Microbial diversity, activity and functional ecology of permafrost and cryptoendolithic microbial life in a hyper-arid Antarctic Dry Valley

By

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Those who contemplate the beauty of the earth find reserves of strength that will endure as long as life lasts. There is something infinitely healing in the repeated refrains of nature -- the assurance that dawn comes after night, and spring after winter.

— Rachel Carson, *Silent Spring*

On first inspection the habitat seems as sterile as the surface of autoclaved glassware... but the trained eye, aided by a microscope, sees otherwise.

— E.O. Wilson, *The Future of Life*

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Abstract

A large fraction of Earth's biosphere is permanently cold, and cold-adapted microorganisms capable of growth at temperatures well below freezing have been found in cryoenvironments globally. It is now well established that permafrost—ground that remains at or below 0°C for at least two consecutive years—can host viable and active communities of microorganisms. The permafrost soils of the high elevation McMurdo Dry Valleys are the most cold, desiccating and oligotrophic on Earth; where the continuous aridity and cold results in the formation of dry permafrost overlaying ice-cemented permafrost, a rare condition that likely only occurs in this region. Little is known about the permafrost microbial communities in the high elevation Dry Valleys other than microorganisms are present.

University Valley is a high elevation Dry Valley (1700 MASL), and is one of the coldest and driest locations in Antarctica (mean annual temperature ~ -25ºC; no degree days above freezing). The objective of this study was to examine the microbial diversity, activity and functional ecology of microbial communities in two distinct habitats in University Valley: permafrost soils, and the sandstone rock which form the valley walls.

We found total microbial biomass in University Valley permafrost was extremely low (10^3 cells/g), and microbial activity under ambient conditions was undetectable. Only 6 isolates were cultured after 2 years of effort using multiple medias and enrichment strategies. Surprisingly, given the low biomass and undetectable activity, University Valley permafrost soil had high microbial diversity, as determined by 454 pyrosequencing of bacteria, archaea, and fungi. Metagenomic sequencing of University Valley permafrost found there was a low diversity of stress response genes, and instead soils were enriched in genes involved with dormancy and sporulation. Our results contrast with reports on lower elevation Dry Valleys and Arctic permafrost, suggesting that

the combination of severe cold, aridity, and oligotrophy is severely constraining microbial survival, and that active microbial life is potentially nonexistent. Intriguingly, genome sequencing of the sole bacterial isolate capable of subzero growth isolated from University Valley soils revealed the presence of genes associated with adaptation to cold and oligotrophy, as well as genome wide amino acid substitutions thought to confer crucial increased protein flexibility at low temperatures.

In comparison to the soils, the sandstone rock in University Valley contained cryptoendolithic ('hidden within rock') microorganisms with a simple but functional community structure which included photoautotrophic algae, and heterotrophic fungi and bacteria. The cryptoendolithic microorganisms were capable of metabolic activity at *in situ* temperatures, and possessed a diverse suite of stress response and nutrient cycling genes to fix carbon under the fluctuating conditions the sandstone rock would experience during the summer months. Pyrosequencing of two cryptoendolithic communities found that these communities share few OTUs in common with the surface soils in University Valley. The source of the diversity seen in University Valley soils is thus likely a mixture of cryptoendoliths and wind deposited cells.

This thesis outlines a natural setting in the high elevation Antarctic Dry Valleys, which is pushing the boundaries of terrestrial life on Earth. The permafrost soils of University Valley are not sterile, but are uninhabitable, and are selecting for dormancy and sporulation rather than for activity and growth. In contrast, the more clement conditions provided by the porous sandstone rock structure has fostered a thriving cryptoendolithic microbial community living within the same valley. These results are relevant to understanding the limits of life on Earth, as well as the possibility of life on other cold, terrestrial planetary bodies such as Mars.

Résumé

Une grande proportion de la biosphère de la Terre est froide en permanence, et des microorganismes adaptés au froid et ayant la capacité de croitre à des températures bien au dessous du point de congélation ont été découverts dans plusieurs milieux catégorisés comme des cryoenvironnements. Il est maintenant bien établi que le pergélisol, sol dont la température reste sous 0 °C pour une période d'au moins deux ans, peut abriter des communautés microbiennes viables et actives. Les pergélisols de régions plus élevées de McMurdo Dry Valleys sont les plus froids, desséchés et oligotrophes sur Terre, où une aridité et un froid continus créent une couche de pergélisol sec surplombant un pergélisol de glace cimenté : une rare structure, qui n'arrive probablement que dans cette région. Les communautés microbiennes du pergélisol des régions élevées des Dry Valleys ne sont que très peu connues, autre le fait qu'elles existent.

University Valley est une vallée sèche de haute altitude (1700 MASL) et est l'un des endroits les plus froids et desséchés en Antarctique (température annuelle moyenne : ~ -25 ºC, avec aucunes journées au dessus du point de congélation). L'objectif de cette étude était d'examiner la diversité microbienne, l'activité, et l'écologie fonctionnelle des communautés microbiennes de deux habitats distincts de University Valley : des pergélisols, et des roches de grès qui forment les murs de la vallée.

Nous avons trouvé que la biomasse microbienne totale du pergélisol de University Valley était extrêmement basse (10^3 cellules/g), et que l'activité microbienne dans les conditions *in situ* était indétectable. Seulement 6 isolats ont réussit à être cultivés après 2 ans d'efforts en utilisant plusieurs médium et stratégies d'enrichissement. Étonnamment, contenu la faible biomasse et absence d'activité détectable, le pergélisol de University Valley avait une grande diversité

microbienne, observée par pyroséquençage 454 de bactéries, archées, et champignons. Le séquençage métagénomique du pergélisol de University Valley a permis de trouver la présence d'une basse biodiversité de gènes impliqués aux réactions de stress. Au lieu, le pergélisol était enrichi de gènes impliqués dans le repos végétatif, et la sporulation. Nos résultats contrastent avec les faits rapportés sur les régions de basse altitude de Dry Valleys et du pergélisol de l'Arctique, insinuant que la combinaison de froid aride, et d'oligotrophie restreint sévèrement la survie de microorganismes, et que la vie microbienne active est potentiellement non existante. Curieusement, le séquençage génétique du seul isolat bactérien capable de croissance sous zéro isolé du sol de University Valley a révélé la présence de gènes associés à l'adaptation au froid et à l'oligotrophie, de même que le substitut d'acides aminés à travers le génome. Ces particularités sont considérées comme pouvant apporter une augmentation cruciale de flexibilité de protéines dans de basses températures.

Contrastant avec le pergélisol, les roches de grès de University Valley abritaient des microorganismes cryptoendolithes (« caché à l'intérieur des roches ») avec de simples, mais fonctionnelles structure des communautés, incluant des algues phototrophes, et des bactéries et champignons hétérotrophes. Les microorganismes cryptoendolithes étaient capables d'activité métabolique aux températures *in situ*, et possédaient une panoplie de gènes de réponse au stress, et liés au cycle des substances nutritives servant à la fixation de carbone dans les conditions fluctuantes des roches de grès durant les mois d'été. Le pyroséquençage de deux communautés cryptoendolithes a trouvé que ces communautés partagent peu de OTUs avec les sols de surface de University Valley. La source de la diversité observée le sol de University Valley est donc probablement un mélange de microorganismes cryptoendolithes et de cellules déposées par le vent.

Cette thèse décrit un environnement naturel dans la haute altitude de Dry Valley en Antarctique, qui repousse les limites de la vie terrestre sur Terre. Le pergélisol de University Valley n'est pas stérile, mais est inhabitable, et démontre une sélection pour la dormance et la sporulation au lieu d'activité et de croissance. En contraste, les conditions plus clémentes fournies par les roches de grès poreuses ont favorisé une communauté microbienne cryptoendolithe prospère, vivant dans la même vallée. Ces résultats sont relevant pour comprendre les limites de la vie sur Terre, ainsi que pour la possibilité de vie sur d'autres planètes froides comme Mars.

Contributions to knowledge

1. This study includes the first next generation sequencing carried out on a permafrost core from the Antarctic, in conjunction with direct and culturable biomass measurements which has added to the scarce data about the microbial ecology of Antarctic permafrost.

2. Identified unique permafrost in University Valley in which microbial activity could not be detected- unlike all other permafrost assayed in this manner on Earth to date.

3. The first polar cryptoendolith metagenome, and the first Antarctic Dry Valley metagenome were sequenced for this study, providing insight into the functional capacity and microbial diversity of two unique cryoenvironments.

4. This study describes the first genome sequenced from a bacterial isolate from Antarctic Dry Valley permafrost (*Rhodococcus* sp. JG3). Giving insights into the cold and low nutrient stress response capacity of this isolate which has allowed it to remain viable in University Valley permafrost.

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List of Abbreviations

BLAST- Basic local alignment search tool

CDS- Coding sequences

CFU- Colony forming units

CPM- Counts per minute

CODH- Carbon monoxide dehydrogenase

COG- Cluster of orthologous groups

MDA- Multiple displacement amplification

MDVs- McMurdo Dry Valleys

OTU- Operational taxonomic unit

PAAT- Polar amino acid uptake transporter

ROS- Reactive oxygen species

SUZ- Stable upland zone

TAG- Triacylglycerol

UV- Ultraviolet

Chapter 1. Introduction and Literature Review: Microbial Life in Cryoenvironments

Portions of this literature review appear in:

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1.1 Introduction: Microbial life in cryoenvironments

Cryoenvironments are generally defined as environments that exist continuously and predominately at subzero temperatures. They exist primarily in polar and alpine regions and consist of large scale geomorphological features such as permafrost, glaciers, ice caps, and sea ice. Cryoenvironments also include relatively rare subzero habitats such as cold lakes and ponds, which can be permanently ice covered, and subzero saline springs, which flow throughout the year, warmed by geothermal gradients, and maintained liquid due to their high salinity (Andersen et al. 2002; Doyle et al. 2012). The primary constraint to life in cryoenvironments is the availability of liquid water; life needs liquid water to survive, mediate biochemical reactions, provide transport of molecules, and act as a solvent. It is not necessarily subzero temperatures that constrain life in cryoenvironments, but rather the conditions that are typically found associated with subzero temperatures, which include freezing, desiccation, or osmotic and oxidative stress. Microorganisms in subzero environments must also be able to cope with the thermodynamic effects of low temperatures including lower reaction rates, increased molecule stability, and conformational changes of proteins (Bakermans 2008). Despite these harsh environmental conditions, there is a recent and growing body of evidence that cryophilic microorganisms (those able to reproduce at <0ºC) exist and are metabolically active in these cryoenvironments at ambient temperatures.

1.2 Permafrost cryoenvironments

Permafrost regions cover 27% of the terrestrial surface of Earth and occur in polar and high altitudes. Permafrost is defined as soil that remains below 0°C for at least 2 consecutive years, and

is typically overlain with an 'active layer' that seasonally rises above 0°C and which can extend several meters down. Any microbial life present in permafrost must cope with long-term exposure to subzero temperatures, background radiation, limited liquid water availability, and often oligotrophic conditions (Steven et al. 2006; Steven et al. 2009). While these extreme conditions are lethal for some cells, there is mounting evidence indicating that permafrost microbial communities are active *in situ*.

Liquid water available for microbial life in permafrost should be present in small amounts despite the subzero temperatures. Concentrated solutes in frozen soils can reduce the freezing point of water causing the presence of briny veins within permafrost (Anderson 1967). The ordering effects of clay minerals are also known to stabilize liquid water into very thin films adsorbed to the mineral grain, and may be the only available water at subzero temperatures (Anderson 1967; Jakosky et al. 2003). Due to surface area differences, particle size greatly influences the fraction of unfrozen water present in permafrost. Whereas Arctic loamy soils can contain up to 2-10% unfrozen water down to -12°C, sandy soils contain virtually no liquid water due to their larger particle size (Gilichinsky et al. 1993; Steven et al. 2006). While these films may be too thin to allow microbial mobility, they are thought to be sufficient to transport wastes and nutrients and to sustain microbial life (Price and Sowers 2004).

Within permafrost, unique geomorphological features may additionally be present such as ice wedges, massive ground ice, cryopegs, pingos, and taliks. For example, polygon patterned ground is common in permafrost affected terrain (Figure 1.1). Depressions forming the polygon boundaries are underlain with V-shaped ice wedges; they are formed over thousands of years by repeated seepage and freezing of water through cracks in the soil created by thermal contraction (Wilhelm et al. 2012). Ice wedges in wet permafrost environments such as those found in the

Canadian high Arctic can range in size from 2-4 meters in width at the top and can extend to depths of 5-10 meters (Wilhelm et al. 2012). In the high elevation McMurdo Dry Valleys of Antarctica, where dry active layer soils predominate, the trough-like depressions may be underlain by sand wedges instead of ice (Singleton et al. 2010). Massive ground ice refers to horizontally extensive bodies (> 2 m thick) of ice also found embedded within permafrost (Steven et al. 2008). Permafrost environments can also contain supercooled (-9 to -11ºC) anaerobic brine lenses (6 to 30% NaCl) known as cryopegs. These relatively rare liquid permafrost habitats are ~1- 2 meters in diameter and can remain isolated on geological timescales. Cryopegs were formed from ancient marine sediments 100–120,000 years ago after the Arctic Ocean regression, when sediments surrounding the remaining pockets of water froze (Gilichinsky, 2003; Gilichinsky et al., 2003, 2005).

1.2.1 Terrestrial Antarctic permafrost and soils

Terrestrial Antarctic surface soils, including McMurdo Dry Valley soils were initially believed to be sterile and inhospitable for life (Horowitz et al. 1969; Horowitz et al. 1972) but are now known to host diverse and significant microbial biomass (Lee et al. 2012; Cowan et al. 2002). In comparison with other Antarctic environments however, relatively little is known about the microbial diversity, activity, and ecology existing within Antarctic permafrost, despite the fact that 37% of all of the world's permafrost exists throughout the ~280,000 km^2 ice-free regions of the Antarctic (Bockheim 1995; Bockheim and Hall 2002).

As of 2015, prior investigations into the prokaryotic component of Antarctic permafrost is limited to five studies: (Cameron and Morelli 1974; Gilichinsky et al. 2007; Blanco et al. 2012; Tamppari et al. 2012; Bakermans et al. 2014). The earliest study investigated the viability of

Antarctic permafrost microorganisms by classical, culture-dependent methodologies. However, the authors were not able to demonstrate that cultured microorganisms were indigenous to the permafrost because of possible contamination from drill fluids used to obtain permafrost cores (Cameron and Morelli 1974). Fluid-less permafrost drilling techniques (Gilichinsky et al. 2007; Blanco et al. 2012) or sampling ice-cemented ground with a hammer and chisel (Tamppari et al. 2012; Bakermans et al. 2014) were more recently employed in microbiological studies in the Antarctic. However knowledge of the habitability of permafrost globally remains primarily informed by investigations from Arctic and Alpine regions. The limited work done on Antarctic permafrost, when compared with data obtained on surface soils, is mainly due to the considerable logistical challenges and very high costs associated with obtaining non-contaminated permafrost samples through permafrost drilling from the very remote regions of Antarctica.

Active layer thickness and depth to ice-cemented permafrost in the Antarctic is influenced by regional climate, proximity to glaciers, age, and presence of vegetation, snow cover and surface albedo. Active layer soils occur in the ice free regions of the Antarctic peninsula, offshore islands, and maritime Antarctica (Bockheim 1995; Bockheim and Hall 2002). The McMurdo Dry Valleys in high elevation and inland Antarctica are a particularly harsh and low water activity permafrost environment, characterized by hyper aridity in addition to the cold. Moisture content is extremely low and dry permafrost (permafrost with <3 % water by weight)(Subcommittee 1988) forms from sublimation of moisture in ice-cemented permafrost over time. The Dry Valleys are the only place on Earth where a layer of dry permafrost overlays ice-cemented ground permafrost (Mckay 2009) and, at high elevations, entirely lack an active layer which rises above 0°C (Marinova et al. 2013). Water exchanges between the dry soil and ice-cemented permafrost via vapour diffusion rather

than liquid water. Despite being in the vapour phase, this water is thought to be available to microbial cells (Stomeo et al. 2012).

Nutrient content in permafrost soils in the Antarctic is heterogeneous. Soils in Antarctica are generally oligotrophic, though soils in close proximity to available water (lakes and streams), ornithographic soils, and coastal Antarctic soils, have higher organic C content. The source of organic carbon and nitrogen in Antarctica may originate from aeolian deposition of organic matter from modern or ancient lacustrine, marine, or lithic sources (Burkins et al. 2000; Hopkins et al. 2009; Cowan et al. 2011). Carbon and nitrogen isotopic signatures that are indicative of primarily ancient aquatic sources of organics, rather than reflective of wind deposition from modern sources, were found in one Dry Valley (Burkins et al. 2000). However, in general, the total contribution of ancient carbon to Antarctic soils and permafrost remains unknown (Hopkins et al. 2009). Antarctic permafrost has been found to contain electron acceptors like nitrates and sulphates, and electron donors such as formate, acetate, and oxalate which could support chemolithoautotrophic growth (Blanco et al. 2012), although the potential for chemolithoautotrophy in Antarctic soils remains largely unexplored.

1.2.2 Microbial Biomass and Diversity of Microorganisms in Permafrost environments

Much of what is known about soil microbiology in the Antarctic is currently derived from investigations of surface active layer soils, and mostly from the Dry Valleys. Similar amounts of microbial biomass are present in Antarctic surface soils (10^6-10^8 cells/g wet soil) as reported for temperate soil habitats, and also high levels of microbial diversity are reported (Cowan et al. 2002;

Lee et al. 2012). Taking into account the very few Antarctic permafrost samples analyzed, it appears that relatively low amounts of microbial biomass are present in Antarctic permafrost samples (10^3-10^4 cells/g wet soil) compared to Arctic regions (10^5 to 10^8 cells / g wet soil) (Table 1.1), and the abundance of microbial cells reported in Dry Valley and coastal permafrost generally decreases with depth (Blanco et al. 2012; Gilichinsky et al. 2007).

Molecular surveys of permafrost from the Arctic and the Antarctic both have found aerobic and anaerobic bacteria, and show a predominance of phylotypes belonging to *Actinobacteria*, *Proteobacteria, Firmicutes* and *Bacteriodetes* (Gilichinsky et al. 2007; Steven et al. 2007a; Steven et al. 2009; Hinsa-Leasure et al. 2010; Yergeau et al. 2010). These bacteria may be selected for in the permafrost environment; *Actinobacteria* are known to be able to metabolize at low temperatures, and may be protected in the permafrost environment by cyst-like resting forms as observed in *Arthrobacter* and *Micrococcus* species isolated from permafrost (Soina et al. 2004), and spore forming Bacteriodetes and Firmicutes may better resist the permanently frozen conditions. Accordingly, *Actinobacteria* were found to dominate active layer samples in a Canadian high Arctic core, while *Actinobacteria, Proteobacteria* and *Bacteriodetes* co-dominated the permafrost samples (Steven et al. 2008; Yergeau et al. 2010). Microbial composition and biomass vary with the environment. The desiccating mineral soils of the McMurdo Dry Valleys have been found to harbor high levels of diversity (Smith et al. 2006), often with a microbial composition of many species occurring in low abundance (Takacs-Vesbach et al. 2010). Maritime influenced Dry Valley soils have been found to contain a surprisingly high biomass on the order of 10^8 cells/g (Cowan et al. 2002), compared to the inland and arid Beacon Valley soils, which were shown to support minimal populations, up to 10^4 cells/g in the dry surface soils, and 10^6 cells/g in the permafrost (Gilichinsky et al., 2007).

Overall, at least 11 phyla have been detected in Antarctic permafrost using molecular methods to date (see Table 1.2). The source of such diversity is likely to be related to the overlying surface soils, atmospheric deposition of bacteria, and nearby colonized habitats such as hypoliths, cryptoendoliths, and microbial mats from colonized streams and lakes (Pearce et al. 2009; Pointing et al. 2009). For example, in Dry Valley permafrost, Gilichinsky et al. (2007) reported that 16S rRNA phylotypes found in surface soils were closely related to those in the underlying ice-cemented permafrost. Nonetheless, the sources and extent of microbial diversity found in Antarctic permafrost remain very poorly characterized.

In addition to prokaryotes, algae, yeast and fungi have been found in permafrost (Gilichinsky et al. 2007; Kochkina et al. 2012; Zucconi et al. 2012)**.** The relative contribution of Archaea in Dry Valley surface soils is not well known. Multiple studies have found no Archaea (Farrell and Pointing 2010; Lee et al. 2012), though the presence of Archaea has been found in Dry Valley hypoliths (Khan et al. 2011). Gilichinsky et al. (2007) was also able to culture methanogens in Dry Valley permafrost. The low abundance, or potential absence of Archaea in Antarctic soils may be indicative that Archaea are generally not adapted to cold and arid environments.

1.2.3 Culturable Microbial Diversity in permafrost

It is widely recognized that culturable microorganisms represent less than 1% of the microbial population in soils, and in permafrost the culturable proportion may be even lower; culturable bacteria from permafrost in the Arctic and Antarctic represent <0.1/ 0.01% of the bacteria identified in samples using molecular methods (Gilichinsky et al. 2007; Steven et al. 2007). In general, culturing permafrost bacteria on low nutrient media such as R2A has yielded

the most isolates. However, increased representation of culturable microorganisms have been obtained by alternative methods such as thawing permafrost samples at high temperatures (52 °C) (Kochkina et al. 2012) and incubating natural samples at the 'warm' temperature of 5 °C prior to plating (Vishnivetskaya et al. 2000). Monitoring permafrost samples over time using this method with DAPI staining revealed that cell numbers increased in the sample, while numbers of CFU did not, suggesting that there is a proportion of viable microorganisms in permafrost that are not amenable to the culturing methodologies being currently employed. The isolated and characterized Antarctic permafrost bacteria have mostly been identified as psychrotrophs rather than true psychrophiles, indicating that these microorganisms may represent a community of 'survivors' rather than organisms which thrive in these environments. Microorganisms isolated to date from Antarctic permafrost are outlined in Table 1.3 and include a small number of representatives from the *Actinobacteria, Proteobacteria, Bacteriodes*, and *Firmicutes*. Many of the genera isolated from Antarctic permafrost have also been identified in Arctic permafrost indicating that such organisms may be particularly adapted to survive and selected for in such harsh cyroenviroments.

1.2.4 Ancient Life in the Permafrost Subsurface

The Antarctic contains some of the Earths oldest sediments, and thus may represent the largest reservoir for ancient microbial life on the planet. Due to the preservative properties of permafrost and ice environments (i.e. stable and permanently cold temperatures) nucleic acids and biomolecules may be able to be maintained over long time scales**.** Lower rates of decay, an order of magnitude for every 10°C drop, should allow extraction of biomolecules from sediments as old as 1 million years (Willerslev et al. 2004). In spite of the constant cold temperatures, with increasing age, damage to nucleic acids will accumulate. Processes that can reduce the integrity of

nucleic acids and biomolecules over time include ionizing background radiation, alkylation, hydrolytic and oxidative damage causing depurination, crosslinking, and single stranded and double stranded breaks of nucleic acids (Hansen et al. 2006; Amato et al. 2010). DNA preserved in Siberian Arctic permafrost ~100,000 to 400,000 years old was found to have an increased frequency of cross-linked DNA with age (Hansen et al. 2006). Crosslinking and single strand breaks inhibit PCR amplification, or cause the generation of short PCR fragments inhibiting molecular surveys of microbiota. Metabolically active cells in permafrost could increase the longevity of their DNA with active DNA repair systems. Data on the metabolic rates associated with the survival of immobilized, starved, and possibly dormant microbes thought to exist in permafrost, suggest that rates of repair of DNA and protein damage in living microbes are similar to rates of incurred damage (Price and Sowers 2004). Lastly, the successful isolation of ancient permafrost bacteria or intact genes or pathways through metagenome analyses, especially from the relatively very old Antarctic permafrost, may serve as novel sources of biomolecules for biotechnology (enzymes, compatible solutes) and health applications (novel antimicrobials) or, for example, elucidating the evolution of resistance mechanisms to antibiotics, as was recently done in high Arctic permafrost (Perron et al. 2013).

1.3 Lithic cryoenvironments

Rock associated microbial communities (lithobionts) are often the only visible forms of life in both hot and cold deserts (Pointing and Belnap 2012). In the Antarctic, three types of rock associated microbial communities can be found. Epilithic communities exist on the top of rocks, hypoliths underneath translucent rocks or along rock margins, and endolithic life is found within rocks either colonizing fissures and cracks (chasmoliths) or hidden in pore spaces underneath the

surface of light coloured or translucent rocks like sandstone, limestone or weathered granite rocks (cryptoendoliths) (Cary et al. 2010). Epilithic communities are the most vulnerable to extremes of wind, UV radiation, desiccation and temperature fluctuation and are largely restricted to milder, coastal influenced Antarctica (Makhalanyane et al. 2014). Hypolithic microbial communities can be found in the inland Dry Valleys, where they primarily occur in the lower and mid elevation Dry Valleys, or in areas effected by water such as snowmelt (Pointing et al. 2009; Cowan et al. 2010). The hypolithic habitat buffers UV radiation, physical disruption from the wind and experiences increased humidity, however the habitat provides little to no buffering of temperature (Cary et al. 2010). Cryptoendoliths can be found even in the extremely cold and arid high elevation Dry Valleys (>1000 m.a.s.l), where they can be found colonizing within porous Beacon supergroup sandstone distributed widely throughout the valleys (Friedmann 1982; Cary et al. 2010). Similar to hypoliths, cryptoendolithic communities are photoautotrophic based, driven by the light which penetrates into the rock (Cowan et al. 2010).

1.3.1 The cryptoendolithic microbial habitat

The hidden life in the cryptoendolithic habitat persists even in the high elevation Dry Valleys because of the more clement conditions which occur within the rock. (Pointing et al. 2009; Goordial 2015a). For example, sandstone boulders can be heated by the sun above ambient temperatures; Beacon sandstone cryptoendoliths at Linneaus Terrace (1600−1650 m.a.s.l) were measured to have within rock temperatures as high as 18ºC above air temperatures (McKay and Friedmann 1985). Rock temperatures are governed by a number of factors, including size and slope of the rock, angle of the sun, cloud cover, or shadowing caused by valley walls. As a result,

cryptoendolithic communities may experience large fluctuations in temperature on a daily or hourly basis, including frequent freezing and thawing (McKay and Friedmann 1985; Vestal 1988). The cryptoendolithic habitat also has a higher moisture content than the surrounding atmospheric air. Water condenses on mineral grain surfaces within porous sandstone rocks, and additionally snowmelt can provide significant amounts of liquid water to the cryptoendolithic community (Friedmann et al. 1987). Within the rock, a steep light gradient occurs due to attenuation of light (Nienow et al. 1988). This light gradient is largely what drives the banding pattern and stratification seen within the cryptoendolithic community (Figure 1.2). They have parallel black and green bands just beneath the rock surface, with a white leached zone in between The black band is composed of lichen, and the green of algae and/or cyanobacteria. Where free living and lichenized fungi and algae exist millimetres below the surface of the rock filtering out harmful UV radiation (black band), and photosynthetic algae and cyanobacteria exist in a narrow zone where sunlight is sufficient to drive photosynthesis and attenuated enough to not cause cellular damage (Friedmann 1982; Selbmann et al. 2005; Henry J. Sun et al. 2010).

1.3.2 Microbial diversity in Antarctic Cryptoendoliths

Cryptoendoliths in the Dry Valleys are trophically simple, consisting of primary producers (algae or cyanobacteria), and consumers/degraders (heterotrophic fungi and bacteria). Cryptoendoliths can be characterized based on the diversity of the dominant photoautotrophs as cyanobacterial dominated or lichen dominated, lichen dominated cryptoendoliths are the most prevalent in the Dry Valleys (Friedmann et al. 1988; de la Torre et al. 2003). In the cyanobacterial dominated cryptoendoliths, *Gloeocapsa, Chroococcidiopsis* and *Hormathonema* were the main

constituents based on culture-based and microscopic identification (Friedmann et al. 1988), and *Phormidium, Plectonema* based on molecular surveys (de la Torre et al. 2003). Molecular techniques identified a single algal species, related to *Trebouxia jamesii* to be the lichen photobiont of the lichen dominated cryptoendoliths (Friedmann et al. 1988). *Stichococcus, Desmococcus, Hemichloris* and *Heterococcus* algal species have been identified through culturable methods in Dry Valley cryptoendoliths (Darling et al. 1987; Meyer et al. 1988 ; Broady and Ingerfeld 1993). Molecular and culturable surveys of both cyanobacterial and lichen dominated cryptoendoliths have shown that there is a high diversity of fungi and bacteria in this trophically simple community (de la Torre et al. 2003). Black melanized 'black yeast' fungi belonging to *Eurotiomycetes* and *Dothideomycete*s are found either free-living or in lichenized associations with photobionts and are known to be desiccation and UV resistant (Selbmann et al. 2005; Selbmann et al. 2013), and psychrophilic *Cryptococcus* yeast have been isolated as well from cryptoendoliths (Vishniac 1985). Molecular survey of cyanobacterial and lichen dominated cryptoendolithic communities found the presence of heterotrophic bacteria belonging primarily to the phyla *Actinobacteria*, *alpha-Proteobacteria*, *Deinococcus-Thermus* and many bacteria which could not be identified through 16s rRNA sequencing (de la Torre et al. 2003). Bacteria identified through culturable methods were genera belonging to *Actinobacteria*, with the exception of *Deinococcus* (Hirsch et al. 1988; Henry J. Sun et al. 2010). Diversity of heterotrophic bacteria does not seem to be strongly correlated with the diversity of photoautotrophs based on culturable and microscopic observation (Hirsch et al. 1988). Microarray based molecular surveys of cryptoendoliths have found the presence of key genes for autotrophy in the phyla *Acidobacteria, Actinobacteria, Chloroflexi, Deinococcus-Thermus*, *alpha-* and *beta-Proteobacteria*, indicating the genetic potential for chemolithoautotrophy taking within the endolithic habitat (Chan et al. 2013), though this has yet

to be established using culturable methods, and no evidence of chemolithoautotrophy using ammonium, nitrite, or sulfate was found by Vestal, 1988. No archaea were detected in cryptoendoliths using molecular methods.

Cultured heterotrophic bacteria, fungi and algae from Dry Valley cryptoendoliths are generally psychrotolerant mesophiles rather than psychrophiles with optimal growth temperatures above 20ºC, temperatures which would not occur *in situ* (Henry J. Sun et al. 2010). Optimal net photosynthesis (CO_2 fixation minus respired CO_2) in Dry Valley cryptoendoliths have been experimentally determined to be 2ºC to -3ºC. Though the individual constituents of the cryptoendolithic community may not grow optimally at *in situ* low temperatures, the community as a whole seems to be adapted. Friedmann and Sun showed in lichen communities that the temperature optima of the community can shift by altering the ratio of producers (algae/cyanobacteria) to consumers (fungal and bacterial) (Friedmann and Sun 2005; Sun and Friedmann 2005). Where a higher proportion of photosynthetic producers are required at warmer temperatures to fix carbon due to a steeper increase in heterotrophic metabolic rates, compared with photosynthetic metabolic rates with warmer temperatures.

1.3.3 Cryptoendolithic fixation and incorporation of carbon

Net fixed carbon by the photoautotrophic component of the cryptoendolithic community is estimated to be 606 mg C m^{-2} y^{-1}, though the net cryptoendolithic ecosystem productivity (amount accreted into community biomass) is estimated to only be 3 mg C m^{-2} y^{-1} (Friedmann et al. 1993). The differences between fixed and accumulated carbon may be the metabolic costs associated with maintaining viability in a fluctuating cold-arid environment, and the remaining is estimated to

leech deeper into the rock, and onto soils to be used by heterotrophic bacteria (Friedmann et al. 1993b; Hopkins et al. 2014a). Colonization of sandstone rocks in the Dry Valleys cause bioweathering of the sandstone, which shows visible patterns of exfoliation based on extent of colonization and age of the community. Oxalic acid produced by lichen dissolves the cement between mineral grains, resulting in exfoliation of biomass from the rock. Once exfoliated colonization continues deeper into the rock, and the cycle resumes until further exfoliation (Friedmann 1982; Blackhurst et al. 2005). The duration of the exfoliation cycle is estimated to take place over 1,000-10,000 years in the high elevation Dry valleys (Friedmann et al. 1993b; Henry J. Sun 1999). Though this contribution of organic matter is small, it is significant in an area with no higher plants. Along with other lithobionts, streams and lakes which are sparsely distributed, cryptoendolithic life in the upper elevation Dry valleys are hot spots of productivity.

1.4 Cryoenvironments, More than Biological Freezers: Subzero Microbial Activity

The preservative properties of cold environments is well established; for example, "freezing" of bacterial isolates in glycerol at -80°C is a routine method for storing bacteria in the laboratory. A fundamental question that remains, however, is whether the microorganisms identified in cryoenvironments are actually active or viable *in situ*. For example, the potential for cryopreservation of nucleic acids in cold, dry environments makes it especially difficult to differentiate between active, dormant, or dead populations based on molecular surveys (Willerslev et al. 2004; Ah Tow and Cowan 2005). The use of treatments such as propidium monoazide (PMA) on environmental samples prior to DNA extraction allows only DNA from cells with intact cell

membranes to be available for downstream enzymatic reactions, permitting identification of viable microorganisms and their functional genes (Yergeau et al., 2010). There is, however, a growing body of evidence that indicates that cryoenvironments are more than natural microbial freezers, sustaining an actively metabolizing population of bacteria. Work on both bulk environmental samples and on microbial isolates in the lab show measureable microbial metabolism at subzero temperatures (Tables 1.4 and 1.5). Additionally, the finding of anomalous CO_2, N_2O and CH_4 gas concentrations and isotopic compositions in ice environments is indicative of active cryophilic life *in situ*.

1.5 Measuring Activity from bulk environmental samples

Activity assays performed on bulk environmental samples have the advantage of targeting whole sample communities without the bias of working on specific isolated strains, and by preserving, at least in some part, the community integrity of the original sample. (Rivkina et al. 2000) assayed activity on Siberian permafrost samples using ^{14}C-acetate to show respiration by native microbial populations down to -20°C. Similar techniques have since been used to asses subzero activity on other Arctic permafrost samples, as well as cold-spring sediments, and reports of active microbial members in cryoenvironments have become numerous (Steven et al. 2006; Steven et al. 2007b; Lay et al. 2012). However, the use of specific substrates (e.g. ^{14}C-acetate, ^{14}C-glucose) to assess activity is limited to the capability of the microbial community to metabolize such compounds and also constitutes a relatively selective method. As such, failed attempts to detect measurable amounts of mineralization in permafrost-associated-ice environments (i.e. ice wedges and massive ground ice) cannot rule out the possibility of active indigenous communities (Wilhelm et al., 2012).

Compared to glacial and permafrost ice environments, increased microbial activity has been observed in sea ice. Subzero activity in summer sea ice has in fact been known since the 1970s (Deming, 2010). The habitability of the more extreme winter-time Arctic sea ice, however, was only recently shown. By combining microscopy with CTC (5-cyano-2,3-ditolyl tetrazolium chloride) respiration experiments, Junge et al. (2004) exposed how sea ice bacteria and archaea not only populate highly concentrated brines in winter sea ice, but are likely metabolically active *in situ* in liquid veins with salinities of 20% at -20°C. Despite many investigations of subzero activity in cryoenvironments, no clear demonstration to date of microbial activity on environmental samples has been reported below -20°C, a temperature that has been proposed by some to constitute a practical limit for cell division and below which metabolism evidences are still scarce (Beaty et al. 2006; Bakermans 2008).

1.6 *In situ* measurements of microbial activity

Independent of laboratory experiments, measurements of putatively microbially produced gases can serve as direct proxies for biological activity *in situ* without the caveats of additional manipulations. Several anomalous CO_2, N_2O, and CH_4 gas concentrations and isotopic signatures have been reported in cryoenvironment studies, hinting at cryophilic life metabolizing *in situ*. For example, the bottom of the Greenland ice sheet has been found to contain elevated concentrations of CH_4 and CO_2 (Tung et al., 2006; Miteva et al., 2009). The findings of high numbers of cells attached on glacial clay minerals at these measured depths (Table 1.1), in concert with F_{420} autofluorescence imaging (as a proxy for methanogenesis), revealed that at least some of the reported cells are active *in situ (Tung et al. 2006)*. Methane concentrations four orders of

magnitude higher in silty glacial ice than in clear ice samples, alongside with CH_4 isotopic compositions, were also indicative of biogenic CH_4 production (Miteva et al. 2009). Similarly, isotopic compositions of the occluded gases O_2 and CO_2 found in ice wedges from the Yukon, suggested microbial respiration by heterotrophic bacteria *in situ* (Lacelle et al. 2011).

To date, the report of anomalous N_2O concentrations and isotopic ratios consistent with biogenic production within Vostok glacial ice (Antarctica) perhaps represents the most extreme case of possible activity in ice at an *in situ* temperature of approximately -40°C (Sowers 2001; Miteva et al. 2007). Nitrification in ice has since been described in both pure culture experiments as low as -32°C (Miteva et al., 2007), and implied in other glacial environments to potentially occur at -40°C (Rohde et al. 2008). While trapped gas measurements are suggestive of *in situ* metabolism, they do not indicate when or on what timescale these gases may have accumulated. Measurements of CO_2 flux have been used to detect putative microbial respiration in polygon and trough surfaces in Canadian high Arctic permafrost; A net CO_2 flux was observed at significantly higher levels above atmospheric values, with ambient surface soil temperatures of -9ºC and underlying permafrost temperatures of -16ºC (Wilhelm et al., 2012). In Arctic permafrost, soil CO_2 flux has been detected in high Arctic permafrost during both summer and late winter ambient conditions (Whyte et al. unpublished) and in permafrost microcosm experiments at temperatures low as -15°C (Steven et al. 2008) to -25°C (Mykytczuk et al. 2013).

1.7 Microbial Activity in Antarctic Permafrost Soils

While nitrate reducers, methanogens, and sulphate reducers have been detected in Dry Valley permafrost, it is still unknown whether they are metabolically active *in situ* (Gilichinsky et al. 2007). It is noteworthy to add that the enzymes associated with these metabolisms have been

detected in permafrost with a protein microarray (Blanco et al. 2012). Whether the detection of these enzymes is reflective of *in situ* metabolisms, or if the stable and cold preservative properties of Antarctic permafrost are preserving these biomolecules, similarly remains to be elucidated.

Aerobic heterotrophic microorganisms are the most commonly cultured from Antarctic permafrost, but their potential metabolic activity in permafrost under *in situ* conditions is generally uncharacterized. In surface soils of the Dry Valleys, soil CO_2 efflux has been detected under summer ambient conditions, indicating microbial respiration may be occurring *in situ* (Burkins et al. 2001; Parsons et al. 2004). After taking isotopic C composition into account, Shanhun et al., 2012 concluded that the CO_2 soil fluxes observed in surface and subsurface Dry Valley soils are partially abiotic in origin and previous measurements likely overestimated the *in situ* activity of heterotrophic microorganisms in Dry Valley soils (Shanhun et al. 2012).

1.8 Pushing the limits for life: Artificial freezing of psychrophiles

Work on isolated strains have rendered it possible to look at cryophilic life under controlled laboratory settings, allowing us to probe the biological limits to cold and gain insights on responses and adaptations to cryo-related stresses. The lowest recorded temperature for microbial division has recently been pushed down to -15°C in *Planococcus halocryophilus* OR1, 3°C below the previously held record of *Psychromonas ingrahamii* (see Table 1.4) (Breezee et al. 2004; Mykytczuk et al. 2013). Experiments on cryophilic isolates have also succeeded in expanding our view of subzero microbial activity. Recent work on ice isolates incubated in frozen M9 medium, for example, showed that viable cells were respiring down to -33°C (Bakermans and Skidmore 2011a). The same research group compared how a liquid brine media of comparable salinity to

that of the liquid veins in frozen M9 media affected microbial growth at -5°C. Diminished activity in ice veins as opposed to brine was observed (Bakermans and Skidmore 2011b), a trend that has also been observed by others at lower temperatures (Amato and Christner 2009). These findings suggest that the osmotic stress caused by the concentration of solutes during freezing is not the sole constraint to microbial activity in ice.

Extreme subfreezing incubations, down to -80°C and -196°C (in liquid nitrogen), have also been performed on permafrost and marine isolates (Junge et al. 2006; Amato and Christner 2009). Though true that such extremely low temperatures are generally not found on Earth, these are relevant to astrobiological research on other planetary bodies and our understanding of how life may cope with such low temperatures. Interestingly, both studies provided evidence of activity, yet only for relatively short incubation periods (less than a day), followed by virtually inactive states, reflecting probable cold-shock responses and entries into dormancy.

1.9 Microbial Adaptations to Cold

Whole genome sequencing, transcriptomic and proteomic work in recent years is revealing several trends into how microorganisms cope with cold environments at the molecular level. To date, at least 47 genomes from cold adapted bacteria have been sequenced (Bakermans, Bergholz et al. 2012), though only a small proportion of these isolates are capable of subzero growth. Extensive proteomic and transcriptomic work has been carried out on a few model organisms, primarily *Psychrobacter cryohalentis*, *Psychrobacter arcticus*, and *Methanococcoides burtonii* (Allen et al. 2009; Bakermans et al. 2009; Ayala-del-Río et al. 2010b; Bakermans et al. 2012). Molecular adaptations to cold environments can be grouped into adaptations which protect the cell

from freezing, increase membrane fluidity, preserve enzymatic function, maintain essential cellular functions such as transcription and translation, and protect against reactive oxygen species (ROS) due to increased oxygen solubility at low temperatures. In addition, cold adapted microorganisms have been found to decrease energy metabolism, or go into a state of dormancy to resist cellular damage, adaptations which promote long term survival in permanently cold environments (Bakermans et al. 2009; Casanueva et al. 2010).

Because the main source of liquid water in subzero environments results primarily from freezing-point depression caused by increased solute and salt concentrations, many of the microbial adaptations to cold include mechanisms to cope with osmotic stress (Chin et al. 2010). As result, a high number of the culturable microorganisms isolated from cryoenvironments are observed to be halotolerant or halophiles. For example, 33% of the culturable organisms isolated from an ice wedge were found to be tolerant to 5% NaCl (Wilhelm et al. 2012). All isolates from a permafrost sample, and 32% of the culturable isolates from the overlaying active layer, were similarly halotolerant (Steven, et al., 2008).

Isolated from an active layer sample from the Canadian high Arctic, *Planococcus halocryophilus* strain OR1 is capable of growth in up to 19% NaCl and in temperatures as low as -15°C, the coldest temperature recorded yet for an isolated microorganism(Mykytczuk et al. 2011b; Mykytczuk et al. 2013). Some of the cold adapted traits of *P. halocryophilus* strain OR1 include a large amount of genes associated with extracellular polysaccharide substances (EPS) production, and a large genetic redundancy in genes involved with osmolyte uptake and synthesis (e.g. glycine betaine) (Mykytczuk et al. 2013). The production of EPS is a commonly observed adaptation to low temperature by microorganisms (Table 1.4). EPS production has been found to counteract the effects of ice-crystal formation, increase brine salinity, and has been found to be a

more effective cryoprotectant for cells frozen at -80°C than glycerol when scaled to equivalent carbon (Marx et al. 2009). Increased compatible solutes uptake (e.g. glycine betaine, choline, glycerol, trehalose, mannitol), antifreeze proteins, and ice-binding proteins (IBPs) can lower the freezing point of the cytoplasm as well as prevent ice crystal formation (Casanueva et al. 2010).

Membrane fluidity can be maintained at cold temperatures by increasing unsaturated lipids, decreasing branched lipids, shortening acyl chain length, and altering polar head groups (Bakermans et al., 2009). Protein function may be preserved in a number of ways that increase flexibility by increasing molecular entropy. This destabilization in protein structure can occur by reducing hydrogen bonds and salt bridges, decreasing acidic and hydrophobic amino acid residues, decreasing proline and arginine content, and increasing solvent-exposed hydrophobic residues (reviewed in Casanueva et al., (2010). The increased production of cold shock proteins and chaperones, which assist in protein folding, as well as maintain transcriptional and translational function, has been observed in several proteomic studies of cold adapted bacteria (Mykytczuk et al. 2011a; Piette et al. 2011).

As proteomic, whole genome, transcriptome and metagenome sequencing efforts on cold adapted strains and cryoenvironments increase, we may begin to answer questions about which adaptations are common to all cold adapted microorganisms, which are common to a specific cryoenvironment, and which are shared by a particular type or family of microorganisms (Bakermans et al., 2009).

1.10 Astrobiology Implications of Terrestrial Cryoenvironments

The primary targets for astrobiology investigations of other solar system bodies are Mars, in the short term, as well as Jupiter's moon Europa and Saturn's moon Enceladus, in the mid to longer term. Extremely cold temperatures characterize these targets, and in this respect, polar cryoenvironments, especially briny subzero habitats, arguably offer the best terrestrial analogue sites that resemble conditions known or suspected to exist on these worlds. With average surface temperatures of ~-190°C on Enceladus, -160°C on Europa, and -60°C on Mars (with lows of -130°C and highs of 20°C), the habitability of extant or extinct life forms on these planetary bodies would be constrained by liquid water availability, similar to earthly cryoenvironments.

Mars possesses ample evidence of past liquid water, as well as the current presence of ice-rich ground beneath dry, extremely cold soils as observed by the Phoenix lander in the North polar region of Mars (Mellon et al. 2009). Intriguing has been the discovery of "contemporary gully activity" on Mars which is found on numerous impact crater slopes, and, which forms and grows in warm seasons (late spring to early fall) and fades or vanishes in cold seasons (Malin et al. 2006; McEwen et al. 2011). Liquid brines near the surface might explain this activity as the presence of salts is found to be widespread on the surface of Mars (Davila et al. 2010; Osterloo et al. 2010); yet, the exact mechanism and source of water are not understood (McEwen et al., 2011). Geomorphological evidence indicate in Mars' past: thermokarst lakes (Soare et al. 2012), spring-like structures (Allen and Oehler 2008; Rossi et al. 2008), hydrated minerals (silicates and sulfates) (Gendrin et al. 2005; Mustard et al. 2008), and deltas and alluvial fans preserved on the surface of Mars- signs that water once flowed on the Martian landscape (Kraal et al. 2008) and thus, could have been a potential abode for past microbial life.

The Phoenix lander also surprisingly detected perchlorate (~1%) in Martian permafrost soils (Hecht et al. 2009). Perchlorates (ClO_4^-) are highly soluble salts with low eutectic temperatures which can act as freezing point depressants, creating subzero salty liquid habitats within frozen permafrost. As an example, a saturated solution of $Mg(ClO_4)_2$ has a freezing point of –67°C, within the range of the diurnal temperature cycle of the Phoenix landing site in the summer (Rennó et al. 2009; Catling et al. 2010; Stoker et al. 2010). Perchlorates can also act as electron acceptors, allowing anaerobic microbial respiration to occur where perchlorate replaces oxygen as the terminal electron acceptor (Coates and Achenbach 2004).

While the cold and dry Martian surface environment is considered inhospitable to microbial life, subsurface permafrost environments are extensive on Mars and are considered to be primary astrobiology targets where life could have survived. The discovery of polygonal terrain on Mars underlain by ice, confirmed at the Phoenix landing site, heightens interest in the possibility that this water-bearing habitat may be, or may have been, a suitable habitat for extant life (Smith et al. 2009). This possibility is supported by the detection of active microbial communities in subsurface permafrost environments, such as ice wedges found beneath tundra polygon features on Earth (Wilhelm et al., 2012) or with depth in permafrost cores (Steven et al. 2008). Antarctic permafrost located in the higher elevation McMurdo Dry Valleys are considered to be the most Mars-like due to their extreme aridity and cold temperatures, and because they are the only place on Earth known to contain both dry-permafrost overlaying ice-cemented permafrost as observed at the Phoenix landing site. As on Mars, the presence of perchlorates is found in the dry permafrost soils of the high elevation Dry Valleys (Kounaves et al. 2010; Hecht et al. 2009). To date however, little is known about the habitability of upper elevation Dry Valley permafrost, other than microorganisms are present (Gilichinsky et al. 2007; Tamppari et al. 2012).

1.11 Objectives of this thesis

The microbiology of high elevation Antarctic Dry Valley permafrost is poorly characterized; little is known about habitability of the unique and extremely old, cold and arid permafrost soils. The overall objective of this thesis was to characterize the microbial communities in permafrost from University Valley (1700 m.a.s.l), a unique high elevation Dry Valley, one of the coldest and driest locations in Antarctica and where water exists primarily as ice or vapor, to determine if these ecosystems harbor an active microbial community. Within the same valley, cryptoendolithic colonization is observed in the Beacon sandstone which makes up the valley walls. The objectives of this thesis were as follows:

1) The first objective was to determine whether University Valley permafrost is habitable for microorganisms and if it harbours an active microbial community. Our goal was to characterize for the first time the microbial diversity, biomass and activity in representative core profiles of dry permafrost and ice-cemented permafrost in a high elevation McMurdo Dry Valley (University Valley) using a combination of culture dependent methods, radiorespiration activity assays and next generation pyrosequencing to identify Bacteria, Archaea and Fungi.

2) The second objective was to examine and compare the ecological functional capacity of microbial communities in permafrost soil and in the sandstone cryptoendolithic microniches in University Valley, by using metagenomic sequencing to identify key nutrient cycling genes, cold adaptive and stress response genes which could enable either community to be active *in situ*.

3) In order to survive the extreme conditions of University Valley, microorganisms would have to be adapted to survive prolonged cold and desiccation. The third objective of this study was to identify the genomic adaptations to cold, stress and oligotrophy in a cold adapted bacterial isolate, *Rhodococcus* sp. JG-3, from University Valley permafrost through comparative genomic analysis with related mesophilic genomes.

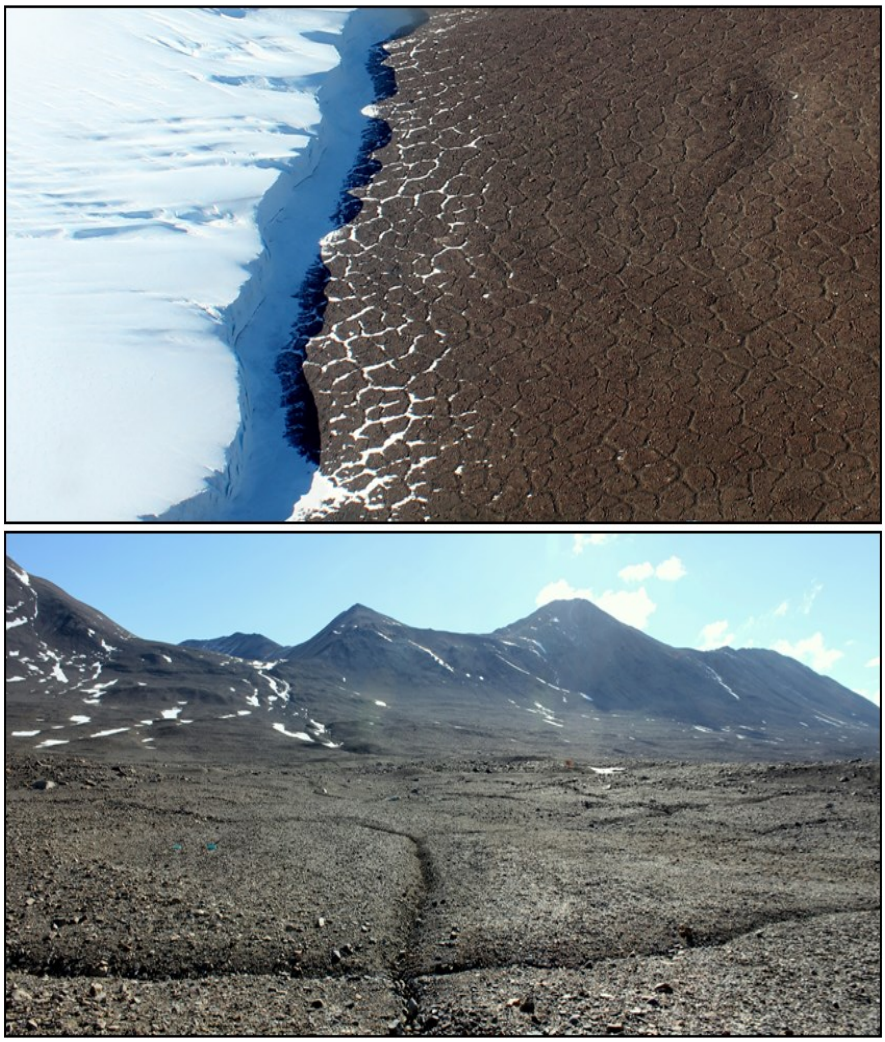

Figure 1.1. Polygon Patterned Permafrost in the Antarctic

Polygon patterned ground in the McMurdo Dry Valleys, aerial view (top), and view from the ground (bottom).

Table 1.1. Microbial biomass in permafrost environments

Location	Age of permafrost (years)	Microbial functional groups reported	Viable cell counts	Direct microscopic cell counts	Reference
Antarctic Dry Valley permafrost Taylor Valley, Miers Valley, Beacon Valley, Mount Feather	30,000 - 8 million	Aerobic heterotrophs Methanogens Sulphte Reducers Denitrifiers	0- 10^4 2- 10^1 0- 10^3 0- 10^1	10^5	Gilichinsky et al. 2007
Antarctic Dry Valley permafrost University Valley	2,500 - 120,000	Aerobic heterotrophs	0- 6	10^3	Goordial, unpublished
Antarctic peninsula permafrost Deception Island	200	Aerobic heterotrophs	0- 10^5	n.d.	Blanco et al. 2012
Canadian high Arctic permafrost Eureka	5,000-6,000	Aerobic heterotrophs	10^1-10^4	10^8	Steven et al.2007; 2008
Canadian high Arctic ice wedge Axel Heiberg	>4,000	Aerobic heterotrophs Ammonia oxidizing Archaea Denitrifiers	10^4-10^5	10^8	(Wilhelm et al. 2012b)

Table 1.2. Bacterial phyla found in permafrost

Phylogenetic Group	Antarctic Dry Valley Permafrost [a]	Antarctic Peninsula Permafrost [b]	Dry Valley Soils [c]	Permafrost Interface in Maritime influenced Dry Valley [d]	Maritime Dry Valley Soils [d]	Maritime Antarctic Soils [e]	Continental Antarctic Soil [e]	Canadian high Arctic permafrost [f]
Acidobacteria	+	+	+	+	+	+	+	+
Actinobacteria	+	+	+	+	+	+	+	+
Armatimonadetes (formally OP10)						+		
Bacteriodetes		+	+	+	+	+	+	+
Chlorobi				+	+			
Chloroflexi	+	+	+			+		
Cyanobacteria		+		+	+	+	+	
Deinococcus-Thermus		+	+		+			
Fibrobacteres	+							
Firmicutes	+	+	+	+	+	+		+
Fusobacteria						+		
Gemmatimonadetes		+	+	+		+		+
Nitrospira			+					
Plantomycetes		+			+	+	+	+
Proteobacteria	+	+	+	+	+	+	+	+
Spirochaetes					+			
TM7		+						
Verrumicrobia				+		+	+	

a. Gilichinsky et al. 2007 b. Blanco et al. 2012 c. Lee et al. 2012 d. Stomeo, et al. 2012 e. Yergeau, et al. 2007 f. Steven et al. 2007

Table 1.3. Phyla and genera of Bacteria cultured from Antarctic permafrost

Phylogenetic group	Antarctic Dry Valley permafrost [a]	Antarctic peninsula permafrost [b]	Dry Valley soil [c]	Antarctic coastal high elevation soil [d]	Canadian high Arctic permafrost [e]
Actinobacteria *			+		
Arthrobacter	+	+	+	+	+
Brachybacterium					+
Cellulomonas	+				
Frigoribacterium				+	
Kocuria		+			+
Micrococcus	+				+
Nocardiaceae (family)**				+	
Rhodococcus	+	+			+
Promicromonospora	+				
Subtercola		+			
Bacteriodetes *			+		
Flavobacterium					+
Hymenobacter			+	+	
Pedobacter					+
			+		
Firmicutes					
Bacillus	+				+
Paenibacillus					+
Sporosarcina		+			+
Staphylococcus					+
Proteobacteria *			+		
Aeromonas	+				
Aztobacter	+				
Brevundimonas				+	
Oxalobaceraceae (family)**				+	
Polaromonas		+			
Pseudomonas	+				+

a. (Gilichinsky et al. 2007); b. (Blanco et al. 2012); c. (Aislabie et al. 2006b); d. (Aislabie et al. 2006a); e. (Steven et al. 2007; Steven et al. 2008; Steven et al. 2009); * could not be classified past the phylum level; ** could not be classified past the family level

Table 1.4. Isolated microorganisms capable of sub-zero growth or metabolism

Isolate (Source)	Salt tolerance (% NaCl)	Temp.	Known adaptations and research interests	Reference
Acetobacterium sp. Ls1 (Lake Fryxell sediment, McMurdo Dry Valleys)	3.6%	-2.5 °C	-Subzero acetogenesis	Sattley and Madigan, 2007
Methanococcoides burtonii * (Ace Lake, Antarctica)		-2.5 °C**	-EPS -Oxidative stress proteins -Subzero methanogenesis -Genome and proteome completed	Allen et al., 2009; Franzmann et al., 1992;Williams et al., 2011
*Planococcus halocryophilus** (Active layer, Canadian high Arctic)	19%	-15°C**	-EPS production -Osmolyte uptake genes -Osmolyte production (e.g. glycine betaine) -Lower acidic residues -Encrustation of cell at cold temperature	Mykytczuk et al., 2011a; Mykytczuk et al., submitted
Psychrobacter arcticus * (Siberian permafrost)	10%	-10°C	-Less proline, arginine residues, and acidic amino acids -Cold shock proteins -First genome sequenced -From a terrestrial psychrophilic bacteria	Bakermans et al., 2006; Ayala-del-Rio et al., 2010
*Psychrobacter cryohalentis** (Siberian permafrost)	10%	-80°C	-Increased ATP levels upon freezing.	Amato and Christner, 2009
Psychromonas ingrahamii * (Arctic sea ice)	10%	-12 °C**	-Less hydrophobic proteins -EPS production -Osmotic pressure regulation -Lowest temperature growth has been observed	Breezee et al., 2004; Riley et al., 2008
Nitrosomonas cryotolerans (Vostok ice core)		-32°C	-Ammonia oxidation of $^{15}NH4$	Miteva et al., 2007
Psychrobacter sp. *Arthrobacter* sp. (Glacial ice isolates)		-15°C	-Incorporation of ^{3}H precursors into DNA and protein	Christner, 2002
Paenisporosarcina sp. *Chryseobacterium* sp. (Antarctic glacial ice isolates)		-33°C	-Microbial respiration of ^{14}C acetate, CTC reduction	Bakermans and Skidmore, 2011a

* Genome sequenced, **Lowest temperature growth observed (Other temperatures indicate temperature observed activity occurred at)

Table 1.5. Microbial activity seen in environmental samples at sub-zero temperatures

Environment	Temp (min)	Activity	Reference
Siberian permafrost	-20°C	Incorporation of ^{14}C acetate into lipids	Rivkina et al., 2000
Arctic permafrost	-15°C	Microbial respiration of ^{14}C acetate	Steven et al., 2008
Siberian permafrost	-16.5°C	Methanogenesis	Rivkina et al., 2004
Arctic ice wedge	-10°C	No microbial respiration of ^{14}C acetate, *in situ* CO_2 flux detected	Wilhelm et al., 2012
Yukon ice wedge		Occluded CO_2 and O_2, isotopic signatures	Lacelle et al., 2011
Massive ground ice	-15°C	No microbial respiration of ^{14}C acetate	Steven et al., 2008
Basal Ice, Greenland ice core	-9°C	Methanogenesis inferred from elevated CH_4 concentrations and isotopic signatures	Tung et al., 2006; Miteva et al., 2009
Arctic wintertime sea ice	-20°C	CTC reduction	Junge et al., 2004
Vostosk ice	-40°C	Occluded N_2O gases, isotopic signatures	Sowers, 2001; Miteva et al., 2007
Lost Hammer Spring (~25% salinity)	-20°C	Mineralization of ^{14}C labeled acetate	Steven et al., 2007b; Lay et al., 2012
Gypsum Hill Springs (7.5-7.9% salinity)	-0.5°C	*in situ* methanogenesis detected	Perreault et al., 2008

CTC: 5-cyano-2, 3-ditolyl tetrazolium chloride

Connecting Text:

Given the scarcity of microbiological data on Antarctic permafrost, and the unique dry and ice-cemented permafrost which occurs only in the upper elevation Dry Valleys, we undertook a comprehensive microbial analysis of the biomass, diversity and activity of University Valley permafrost and surface soils.

Chapter 2. Nearing the cold-arid limits of microbial life in permafrost of an upper dry valley, Antarctica

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2.1 Abstract

Some of the coldest and driest soils found on Earth are located in the high elevation McMurdo Dry Valleys of Antarctica, though little is known about the permafrost microbial communities other than microorganisms are present. Here we describe the microbiology and habitable conditions of highly unique dry and ice-cemented permafrost from University Valley (1700 m.a.s.l), where extreme cold and dry conditions have persisted for over ~150,000 years (mean temperature −23ºC; no degree days above freezing), and where the ice in permafrost originates from vapour deposition rather than liquid water. We found that culturable and total microbial biomass in University Valley was extremely low, and microbial activity under ambient conditions was undetectable. Our results contrast with reports on lower elevation Dry Valleys and Arctic permafrost, suggesting that the combination of severe cold, aridity, oligotrophy, and the age of University Valley permafrost is severely constraining microbial survival, and where active microbial life is potentially nonexistent.

2.2 Introduction

The McMurdo Dry Valleys (MDVs) of Antarctica can be subdivided into three microclimate zones on the basis of seasonal environmental conditions (Figure 1) (Marchant and Head III 2007). The coastal thaw zone (CTZ; <400 m) has the highest mean summer air temperatures (~−5 ºC), where soils have an active layer with temperatures exceeding 0ºC for over 50 days annually, and snowfall (snow water equivalent; SWE) ranges between 25−100 mm yr^{-1}. The stable upland zone (SUZ), located at the highest elevations (~1000 – 2500 m), is the coldest and driest, with mean summer air temperatures around −10ºC and SWE <10 mm yr^{-1}. Both regions are separated by the Intermediate Mixed Zone (IMZ) (Marchant and Head III 2007). The continuous aridity and cold throughout the MDVs result in a layer of dry cryotic soil of variable thickness (cm to m) overlaying ice-cemented permafrost (Lacelle et al. 2013; Marinova et al. 2013), and at elevations greater than ca. 1500 m no seasonal active layer develops.

Previous soil microbial studies in the MDVs have focused on the warmer and wetter CTZ and reported a wide diversity of microbial taxa, forming a simple but functional trophic structure with average soil respiration rates approximately 20 times lower than Arctic tundra (cf. (Cary et al. 2010). On the other hand, practically nothing is known regarding the microbiology of permafrost in the SUZ other than microorganisms are present (Gilichinsky et al. 2007; Tamppari et al. 2012). Based on the capacity of some permafrost microorganisms to grow at temperatures as low as −15ºC and metabolize at −25ºC (Mykytczuk et al. 2013), we hypothesized that soils in University Valley, one of the coldest and driest in the SUZ, would also harbour a viable, active microbial ecosystem similar to other permafrost environments, and that these communities may be some of the most cold-adapted studied to date.

Site Description

We examined the micro-climate, physicochemistry, and microbiology of soils from University Valley, a hanging valley located at high elevation (1650-1800 m.a.s.l) 450 m above Beacon Valley in the Quartermain Range of the Upper Dry Valleys (Figure 2). The valley is northwest facing, 1.5 km long and 500 m wide. Surface soils in University Valley are heterogeneous spatially, with some areas dominated by large sandstone boulders, and other areas dominated by sand-sized particles and where polygon patterned ground and desert pavement have formed (Lacelle et al. 2013). A small glacier is located at the head of the valley, and the depth to the ice table increases with distance away from the glacier, being less than 1 cm close to the glacier, and over 70 cm at the mouth of the valley (Lacelle et al. 2013). Ice-cemented permafrost is widespread beneath a layer of dry permafrost of variable thickness. Permafrost here is defined as ground that remains at or below 0ºC for two years and dry permafrost is defined as permafrost <3% water by weight (Subcommittee 1988). The cores examined in this study were located near the head of the valley close to the glacier, a shadowed region where the soil surface experiences few thaw hours (Table S1), and where the uppermost 50 cm of ice content in the ice-cemented permafrost formed from water vapour diffusion into the cryotic soils rather than from liquid water (Mckay 2009; Lacelle et al. 2013). This area of the valley was selected for sampling because it is thought to be the harshest for microbial life. We studied two soil profiles comprising both dry and ice-cemented permafrost (Figure 2); one profile was a 55 cm long core designated (IT-5cm) since the depth to the ice-table was 5 cm, and the second profile was a 42 cm long core collected further down valley where the depth to the ice-table was 12 cm (designated IT-12cm). The age of soils in both profiles ranges between 10^4–10^5 years, with an average soil accumulation rate ca. 10^{-3} mm yr^{-1} (Lacelle et al. 2013). The soils are largely

derived from the weathering of Ferrar dolerite and Beacon Supergroup sandstone boulders originating from the valley walls (Tamppari et al. 2012; Lacelle et al. 2013).

2.3 Results and Discussion

Air climate and soil environmental data were collected at two sites within the valley between 2009 and 2013 (Table S1). The mean annual air temperature was −23.4±0.9ºC, with the maximum hourly value always below −2.8ºC. The mean annual soil surface temperature ranged between −23.5ºC and −26.5ºC, with the mean daily values remaining below 0ºC, irrespective of ice table depth; as such the soils are perennially cryotic lacking a seasonal thaw layer. Maximum hourly near-surface (top 10 cm) temperatures experienced values above 0ºC for a few hours during clear summer days due to insolation, occurring for <1% of the year (<80 total hrs yr^{-1}). These extremely cold conditions have likely persisted in this region since the Eemian interglacial period, ca. 150,000 years ago (Marchant et al. 2013).

Soil samples were found to be highly oligotrophic (0.01% − 0.05% total carbon, undetectable to 0.09% total nitrogen) and near neutral pH (7.5−8) (Table S2). Total soluble solutes (<5 g L^{-1}) were primarily of a SO_4-NO_3 geochemical composition (Lacelle et al 2013). Very low microbial biomass was found by direct microscopic cell counts ($1.4 - 5.7 \times 10^3$ cells g^{-1}) in both the dry and ice-cemented permafrost using 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) stain as described by Steven et al. 2008. Comparatively, 2 orders of magnitude higher cell counts ($1.2–4.5 \times 10^5$ cells g^{-1}) were detected in active layer and permafrost samples from the relatively warmer Antarctic Peninsula (Marambio). Other fluorescence based staining was used, including DAPI, Live/Dead BacLight viability staining, and catalyzed activated reporter

deposition-fluorescent in situ hybridization (CARD-FISH) analyses, as carried out in (Niederberger et al. 2010); however, cells were not easily or unambiguously discernible using these methods and thus could not be accurately quantified. Close visual inspection of the cores showed no signs of biofilm microbial life, including the ice-cemented ground to dry soil interface, which we had hypothesized to potentially be a habitable niche. Culturing was carried out using a variety of enrichment techniques and media previously used successfully in Arctic and Antarctic permafrost (Gilichinsky et al. 2007; Steven et al. 2007a) and included oligotrophic media as well as a mineral salts media with no carbon added (see methods). Aerobic and anaerobic culturing from 6 soil samples along the two permafrost profiles yielded only 6 heterotrophic isolates on over 1000 agar plates in 2 years (Table 2.1); 2/6 of the soil samples yielded no culturable isolates (IT-5cm, 41 cm depth; IT-12, 28 cm depth). With the exception of one *Chaetothyriales* fungal isolate, the other 5 strains required liquid enrichment steps prior to isolation on media and ~3−5 months incubation at 5°C before colonies appeared on agar plates, indicating that the isolated organisms may have been in dormant or damaged states. Two strains of the 6 isolates (*Rhodotorula* and *Rhodococcus* spp.), isolated from ice-cemented permafrost (IT-12cm, 37 cm depth) with an ambient temperature of ~ −25ºC, and soils estimated to be 10^5 years old (Lacelle et al 2013), were both capable of sub-zero growth at −10°C and −5°C, respectively. Attempts to enrich or isolate photoautotrophs, sulfate reducing bacteria, perchlorate reducing bacteria, methanotrophic or methanogenic bacteria were not successful. Both direct microbial and culturable bacterial counts from University Valley were 4−5 orders of magnitude below those encountered in Arctic permafrost and in the surface soils of mid and low elevation MDVs (Cowan et al. 2002; Gilichinsky et al. 2007; Steven et al. 2008) (Table S3) .

Radiorespiration assays (Steven et al. 2007b) (using ^{14}C acetate as a C source) were carried out on 7 permafrost soil microcosms incubated at 5°C, −5°C, −10°C and −15°C for ~600 days (Figure 3) to determine microbial activity under these conditions. For comparison, radiorespiration assays were also conducted with sandstone samples colonized by cryptoendolithic communities collected from the valley walls, and in permafrost and active layer soils from Marambio (Antarctic Peninsula). CO_2 respiration in University Valley soil microcosms was not detected in any of the soils tested above abiotically produced background levels at sub-zero temperatures ($P<0.05$, paired t-test, 1 tailed) relevant to *in situ* conditions (Figure 3). In 4 of the 7 soil samples tested, radiorespiration activity was not detected at any of the sub-zero temperatures or at +5°C. It is possible that CO_2 respiration at sub-zero temperatures did occur in these low biomass soils but was below the detection limits of this assay, although incubations were carried out for over 600 days in an attempt to detect low signal. In comparison, ^{14}C acetate mineralization assays have detected significant amounts of activity in permafrost soils from the Canadian high Arctic (Steven et al. 2008), Siberia (Rivkina et al. 2000), and in a lower elevation Antarctic Dry Valley (Taylor Valley) (Bakermans et al. 2014) within 50, 160, and 180 days respectively at −5°C. It is also possible that microorganisms in these soils were not able to metabolize acetate although acetate radiorespiration was clearly detected in 3 samples at +5°C, indicating that the capacity for these populations to metabolize acetate can occur under favourable conditions, and that some University Valley permafrost soils have small, viable microbial populations. Low N and P levels were not limiting respiration as NH_4 and PO_4 additions had no effect on mineralization when incubated at −5°C (Figure 4). Addition of water to dry soil samples in a 1:1 ratio also had no effect on mineralization rates. When dry soil samples from University Valley were amended with 10^6 cells g^{-1} soil of *Planococcus*

halocryophilus sp. OR1, an Arctic bacterium that reproduces at −15ºC and metabolizes at −25°C in Arctic permafrost soil (Mykytczuk et al. 2013), high levels of mineralization was observed at −5°C after 30 days, indicating that the lack of detectable microbial activity at sub-zero temperatures was not due to soil toxicity. In contrast, relatively higher respiration rates were measured in the sandstone cryptoendolithic communities as low as −20°C (Figure 5), demonstrating that viable microbial communities exist within the microhabitat provided by the porous sandstone rock in University Valley. Additionally, ~20% mineralization of ^{14}C acetate at −5°C was measured in soils from the Antarctic Peninsula, similar to values observed in Arctic permafrost (Steven et al. 2008)(Figure 3). During a 2013 summer field campaign, *in situ* CO_2 and CH_4 fluxes were not detected (detection limit 0.001 ppm CH_4 and 200 ppm CO_2) in University Valley soils at two sites, where the ice table was located at 42 cm depth (IT-42) and 5 cm depth (IT-5 cm) (data not shown).

The permafrost microbial community structure was determined by pyrosequencing of the 16S rRNA gene for Bacteria and Archaea and the 18S ITS region for Fungi. It was not possible to extract amplifiable RNA, likely due to the extremely low biomass and negligible activity, suggesting that the extracted DNA may be derived from dormant or paleomicrobial communities. As observed in other Dry Valleys (Lee et al. 2012; Stomeo et al. 2012; Crits-Christoph et al. 2013) community composition was cosmopolitan, highly variable (Figure S1) and bacterial diversity (H' =1.1 - 4.3, 1673 unique OTUs) was high considering the extremely low viable and total microbial counts (Table S4). Overall *Proteobacteria* dominated the bacterial community, mainly composed of *Gammaproteobacteria* (25%), and *Betaproteobacteria* (9%). *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were also variably present in samples. OTUs related to bacterial genera commonly associated with soil (*Burkholderia*, *Ralstonia*,

Sphingomonas, *Bradyrhizobium*) and aquatic/marine environments (*Alcanivorax*, *Pelagibacter*, *Gillisia*) were found, although University Valley contains no aquatic habitats. Existing genera represented a diverse suite of potential metabolisms, with aerobic heterotrophy the most predominant; also detected were taxa associated with fermentation (*Clostridia*, *Anaerolineae*), methylotrophy (*Methylobacteria*, *Methylophilus*, *Methylobacillus*), sulfate reduction (*Desulfovibrio*), perchlorate reduction (*Dechloromonas*), sulfur and sulfite oxidation (*Sulfuricella*, *Sulfitobacteria*), nitrite oxidation (*Nitrospira*), and phototrophy; *Cyanobacteria* and *Rhodobacteraceae* were found in the deeper ice-cemented permafrost samples. The genus (or order in the case of *Chaetothyriales*) of all cultured isolates in this study were detected in University Valley soils in our pyrosequencing results. Our molecular survey is consistent with the few diversity surveys reported for Dry Valley permafrost to date (Gilichinsky et al. 2007; Bakermans et al. 2014). Clone libraries in Taylor Valley (Bakermans et al. 2014) (elevation 21 m.a.s.l) and lower Beacon Valley permafrost (1000 m.a.s.l.) (Gilichinsky et al. 2007) reported the presence of *Proteobacteria, Gemmatimonadales, Chloroflexi*, and *Acidobacteria*; ubiquitous soil organisms which we also detected. Beacon Valley permafrost soils were found to be dominated by *Gammaproteobacteria,* results which are similar to one core in University Valley (IT-5cm) but not in any depths of the other core profile (IT-12cm). Previous work on surface soils in other Dry Valleys have reported similarities at the phylum level, but high heterogeneity at the OTU level in soils (Lee et al. 2012), which we also observed in University Valley. The differences in species composition found in surface soils of different Dry Valleys are driven by differing localized physicochemical properties (Lee et al. 2012), although allochthonous sources and dispersal of microorganisms through aeolian processes also likely plays a role in community composition.

Archaeal sequences (H' = n.d. -2.7) (Table S4) were primarily composed of *Halobacteria* and *Methanomicrobia* (only detected in surface soils) and related to sequences previously detected in saline and alpine/cold environments (Figure S2). Recovered fungal sequences (H' = 0.04 - 1.7) were dominated by *Dothideomycetes* (54% of total reads) composed of the orders *Capnodiales* and *Pleosporales*. *Eurotiomycetes* (order *Chaetothyriales*) were only detected in surface soil samples; *Eurotiomycetes* include black yeast fungi known to be both desiccation and UV resistant, and found in hot and cold desert lithic habitats (Selbmann et al. 2005; Ruibal et al. 2009).

OTUs related to halophilic genera in both Archaea (*Halobacterium, Haloarcula, Halorubrum*) and Bacteria (*Halomonas*) could be indicative of selective pressures favourable to microorganisms capable of inhabiting briny films in permafrost. However, the absence of metabolic activity in the radiorespiration assays, and the co-occurrence of halophilic organisms with taxa related to phototrophy and marine environments, as well the presence of obligate anaerobes in aerobic surface soils, is more consistent with mixed allocthonous sources of microorganisms (Hopkins et al. 2006; Nkem et al. 2006). The most obvious allocthonous source of microorganisms is the sandstone (Beacon Supergroup) cryptoendolithic community colonizing part of the University Valley walls. However, pyrosequencing of two cryptoendolith samples showed they shared few bacterial, archaeal or fungal OTUs in common with University Valley permafrost soils (Figure 6), and the most abundant taxonomic groups in the sandstone were poorly represented (<1%) or completely absent. The disparity between the cryptoendolithic and the valley floor microbial communities suggests that airborne bacteria from other regions accumulate through aeolian deposition and become preserved in the dry, cryotic soils, diluting much of the cryptoendolithic signature. An exception was the yeast strain that we isolated from

dry and ice-cemented soils that was most closely related to a previously isolated University Valley cryptoendolith *Chaetothyriales* strain (Table 2.1) (Selbmann et al. 2005), suggesting that some cryptoendolith microorganisms are indeed seeding the soils on the valley floor and maintaining their viability within the permafrost.

2.4 Conclusion

Based on our radiorespiration assays and the extremely low culturable and total microbial biomass observed, and given the continuous and prolonged dryness and subfreezing temperatures (Table S1), it is unlikely that microbial communities in the permafrost soils analyzed here metabolize at any time of the year, even during the austral summer, due to a lack of available water. The presence of salts and solutes in permafrost may reduce the freezing point of water and facilitate the formation of small, but habitable brine veins within the soil matrix. The stable upland zone, due to greater distance from the coast receives limited influx of salt as compared to the coastal thaw zone and intermediate mixed zone. Thus, in addition to experiencing warmer temperatures and receiving more precipitation in the form of snow, the lower and mid elevation dry valley soils are more likely to contain habitable liquid brine veins due to increased concentrations of salts. Thin films are not a relevant source of water in University Valley. Measured average unfrozen water content as a function of soil temperature is shown in Figure 7 for two bulk soil samples from University Valley. We computed the amount of water that would be strongly bound to soil particles (i.e. interfacial water) using the Langmuir's absorption model, and a specific surface area of soil particles of 20 $m^2 g^{-1}$, as measured in Beacon Valley (Sizemore and Mellon 2008), and 1 $m^2 g^{-1}$, as a hypothetical lower value. Unfrozen water content curves

described a rapid transition from bulk (thin films) to interfacial water at −15ºC, with a progressive decrease of the latter as hydrogen-bonds were slowly overcoming van Der Walls bonds at the mineral surfaces under cooling permafrost temperature. A considerable amount of unfrozen bulk water (>10%) was found to exist in the soils only at temperatures above −1°C. Using the climate data (Table S1), we calculated that bulk water (i.e. - T> −15°C, RH>95%) is present in the dry soils for approximately 0.84% of the year (74 h y^{-1}). This is in contrast with conditions on colonized faces of sandstone boulders measured in Linneaus Terrace (1650 m.a.s.l), a dry valley with similar climate conditions as University Valley. In Linneaus Terrace, the colonized area of the boulders was shown to be moist for more than 700 h yr^{-1} (Friedmann et al. 1993a).

These results allow us to place the known limits of life to grow and metabolize in an environmental context. There is unambiguous evidence of active microbial life at similar cold ambient temperatures in other permafrost from the Arctic and in lower elevation Antarctic Dry Valleys (Rivkina et al. 2000; Steven et al. 2008; Bakermans et al. 2014; Tuorto et al. 2014). Soils from the hyper-arid core of the Atacama Desert have cell numbers and culturable counts similar to University Valley permafrost (Table S3), but small, viable microbial communities are activated when Atacama soils were wetted (Navarro-González et al. 2003; Crits-Christoph et al. 2013). It appears that microorganisms in the University Valley permafrost soils analyzed here are not exposed to sufficient transient and periodic clement conditions to allow for cell repair, metabolism or growth. Instead, our results suggest that a fundamental threshold for life can be crossed in some University Valley permafrost soils, where the combination of permanently subfreezing temperatures, low water activity, oligotrophy, and age are constraining the evolution of a functional cold-adapted microbial ecosystem. The microorganisms detected in our study

may represent a transient inoculum rather than a cold-adapted community. The depauperate nature of these soils is in contrast with the sandstone boulders and cliffs that are colonized by cryptoendolithic organisms, and our findings add to a growing body of evidence that extant life in extremely dry, cold or hot deserts is limited to specialized lithic habitats at very small scales (Davila et al. 2008; Pointing et al. 2009). Dry permafrost as observed in University Valley is rare on Earth, likely only occurring in the McMurdo Dry Valleys (Bockheim and Tarnocai 1998; Bockheim et al. 2007), but is commonplace in the northern polar regions of Mars at the Phoenix landing site (Levy et al. 2009). Thus, our results have implications for our understanding of the cold limits of life in terrestrial environments, with potential implications for habitability models of Mars near surface permafrost and other icy worlds.

2.5 Acknowledgments

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2.6 Materials and Methods

Sample collection and processing

University Valley permafrost core samples were collected in the 2009 summer field season with a SIPRE corer, and dry soil above the IT-5 cm core was collected in the 2010 summer field season. The cores were 0.3 km apart from each other, co-ordinates for the IT-5 cm permafrost core is 77d51.970s S, 160d43.943s E and 77d 51.817s S, 160d43.524s E for the IT-12 cm core. Samples were shipped to McGill University in a thermally insulated box and maintained at −20ºC until processing. Initial core processing took place in a walk-in freezer held at −5ºC, in a laminar flow hood where 1 cm of the outside of the core was removed with a sterilized chisel. An additional 1 cm of the outside core was removed in a laminar flow hood at room temperature immediately prior to samples being weighed and aliquoted for analysis.

Nucleic acid extraction

Because of low biomass in soils, 10 grams of soil were used per extraction per sample. Community DNA was extracted from 2 g of permafrost soil using the UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, California, USA), as described in the alternative protocol for maximum yield, and a bead beating step was added to aid lysis. For each sample, 5 extractions were performed and the resulting DNA was pooled and concentrated. DNA from cryptoendoliths was extracted from 100 mg of crushed rock, lysed with cetyltrimethyl ammonium bromide, RNase A, and proteinase K and a bead beating step, followed by phenol/chloroform/isoamyl alcohol extraction at 60°C and ethanol precipitation. Negative controls (H_2O in place of soil) underwent identical handling during the extraction procedure and were used as templates for PCR using 16S rRNA gene primers (27F and 1492R) to ensure no contamination during extraction. Multiple attempts to extract RNA were carried out using the RNA Power Soil kit (MoBio Laboratories Inc., Carlsbad, California, USA). Extractions using 2 g of soil were carried for a total of 20 g per sample and pooled. No RNA was detectable after

extraction, nor was an amplifiable product found after a reverse transcription reaction on the extracted product.

Community Profiling

DNA was sent for SSU rDNA pyrosequencing analyses at the Research and Testing Laboratory (Lubbock, TX, USA) using the Roche 454 GS-FLX platform (Roche 454, Branford, CT, USA). Sample libraries were prepared with the following primers for bacterial 16S rRNA gene (28F-5'-GAGTTTGATCNTGGCTCAG-3', 519R-5'-GTNTTACNGCGGCKGCTG-3'), archaeal 16S rRNA gene (349F- 5'-GYGCASCAGKCGMGAAW-3', 806R-5'-GGACTACVSGGGTATCTAAT-3'), Eukaryal/fungal Internal Transcribed region (ITS) (ITS1F-5'- CTTGGTCATTTAGAGGAAGTAA-3', ITS4R-5'-TCCTCCGCTTATTGATATGC-3') genes. Processing of sequences was performed in Mothur following the 454 SOP that is outlined in (Schloss et al. 2009). Briefly, sequences were quality filtered by removing primer sequences, reads < 150 bp long, sequences with ambiguous base calls, and homopolymer repeats greater than 8bp. Bacteria and archaeal sequences were reduced to only unique sequences and aligned to the Mothur-interpreted Silva bacterial database (contains unique sequences from the SSU Ref database (v.102)) and trimmed to equal size. Chimera removal using chimera.uchime within Mothur was used to further reduce sequencing error prior to clustering. OTUs were clustered using average-neighbour clustering with a 97% cut-off. All sequences were classified with Mothur using the Ribosomal Database Project (RDP) training set (v. 9), and sequences that could not be classified to the kingdom of the target primer set were removed. Pre-alignment steps for fungal ITS sequences were as described above, except that unaligned sequences were pre-clustered at 99% nucleotide similarity, and were then hierarchically clustered into OTUs at 97%, 95%, and 90% similarity with CD-HIT (Li and Godzik 2006), with a word size of 8

nucleotides and default parameters. Output cluster files were reformatted as Mothur.list files, which were used for downstream OTU analysis. Initial classification of sequences was performed in Mothur using the UNITE/QIIME 12_11 ITS reference database. Diversity indices were calculated using Mothur, and were subsampled as appropriate.

Cell enumeration and isolation

Total cell counts on dry and ice cemented permafrost were enumerated with 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) stain as described by Steven et al. 2008. Other fluorescence based staining was used, including 4',6-diamidino-2-phenylindole (DAPI), Live/Dead BacLight viability stain (Molecular Probes Inc., Invitrogen, Eugene, OR, USA), and catalyzed activated reporter deposition-fluorescent in situ hybridization (CARD-FISH) analyses, as carried out in Niederberger et al., 2010 however cells were not easily or unambiguously discernible using these methods and were not chosen for presentation or enumeration. To determine viable heterotrophic cell counts and isolate a diversity of bacteria, a dilution series was used with 11 different solid media. The solid media chosen were R2A agar; 1/2 R2B agar (supplemented with 1.5% agar (*m*/*v*)); mineral salts agar with no carbon source as well as augmented with yeast, tryptone, and soluble starch (MSM+YTS; 0.25 g each per L); tryptone soy agar (TSA); 1/10 TSA supplemented with 1.5% agar (m/v); BG11 media for photoautotrophs (incubated in light), and R2A, 1/2 R2A, TSA and 1/10 TSA supplemented with 5% NaCl (*m/v*). Agar plates were incubated at 5°C and 20°C aerobically for all media, anaerobically (0 ppm [O_2]) at 25 °C in an anaerobic chamber (COY Laboratory Products, Inc., Michigan, USA) and at 0°C for media supplemented with 5% NaCl. All media were adjusted to neutral pH and plate counts were performed in triplicate, except for 0°C incubations and anaerobic incubations which were carried out in duplicate. A total of 4 ice-cemented permafrost and 2 dry soil samples were

used. As only one isolate was obtained using solid media directly, enrichment techniques were attempted. The first enrichment technique consisted of a one month incubation of ice-cemented permafrost at 5°C with no amendment prior to plating on the Medias' described above at 5°C. Enrichments for sulfate reducing bacteria (Postgate medium) and perchlorate reducing bacteria (per L: NH_4Cl, 0.25 g; $NaClO_3$, 1.03 g; CH_3CO_2Na, 1.36 g; NaH_2PO_4,0.6 g; KCl, 0.1 g; $NaHCO_3$, 2.5g, 10 mL trace mineral and vitamin solution) were carried out at 25°C anaerobically. Liquid aerobic heterotroph enrichments were also carried out on dry and ice cemented permafrost at 5°C and 20°C in 0.1% $Na_4P_2O_7$, R2A, TSA, 1/10 TSA, BG11 (incubated in light) and R2A, TSA and 1/10 TSA supplemented with 5% NaCl (*m/v*). Soil was incubated in liquid media and sampled at 2 week intervals for 6 weeks and then plated in duplicate on R2A, TSA and on solid media identical to the liquid enrichments being used at 5°C (MSM+YTS media was used for 0.1% $Na_4P_2O_7$ enrichments.

Radiorespiration assay for heterotrophic activity

Five g of permafrost was added to individual microcosms as Steven et al., 2007. Each microcosm was performed in triplicate, and included triplicate sterilized controls (autoclaved twice for 2 hours at 120°C and 1.0 atm, with a 24 h period between autoclavings). Microcosms were spiked with 0.045 mCi ml^{-1} (~100,000 disintegrations per minute) of $1-^{14}C$ acetic acid. Cold acetic acid was added to a final concentration of 15 mM acetic acid per microcosm in a total volume of 40 μl. The CO_2 trap consisted of 1 M KOH for microcosms incubated at 5°C, −5°C and 1 M KOH + 20% v/v ethylene glycol for microcosms incubated −10°C and −15°C. For nutrient amended microcosms, 3 g of ice cemented permafrost samples (IT-12 surface dry permafrost) was used for each microcosm, and spiked with cold and radioactive acetate as described. Nitrogen and phosphorus was added at a concentration of 1 mg g^{-1} wet soil, in the

form of NH_4Cl and $Ca(H_2PO_4)_2$ respectively. Stationary phase *Planococcus halocryophilus* OR1 biomass incubated at −5°C in Tryptic Soy Broth amended with 5% salt and 2% glycerol was pelleted, and rinsed 3 times with 0.1% $Na_4P_2O_7$ buffer to remove residual media. A total of 10^6 cells in 50 ul buffer was added to individual microcosms. Amended samples were measured after 30 days incubation at −5°C. Measurements of radioactivity were determined by liquid scintillation spectrometry on a Beckman Coulter (CA, USA) LS 6500 Multi-purpose Scintillation Counter.

In-situ **soil gas flux measurements**

Soil CO2 flux was measured in the 2013 summer field season in University Valley with a Los Gatos Ultraportable Greenhouse Gas Analyzer, which is able to detect 0.01 – 100 ppm CH_4 and 200 – 20000 ppm CO_2 (Los Gatos Research, Mountain View, CA, USA).Two PVC soil collars were inserted to a depth of 6 cm at ~IT-5 cm and ~IT-10 cm site, and allowed to equilibrate with the soil for 24 hours prior to measurements being taken. Collars remained in place throughout the experiment, minimizing disturbance while enabling repeated measurements of CO_2 flux from the same soil surface. The closed gas exchange system was customized with a LiCor-8100 chamber, and measurements were taken over 5 and 10 minute intervals to measure small changes in chamber CO_2 concentrations following efflux from soil. Measurements were taken for 2 consecutive days, three times daily in the morning, noon and night.

Soil Analysis

The soils were analyzed for total carbon and total nitrogen by combustion at 900°C with a Carlo Erba Flash EA 1112 NC Soils Analyzer which has an analytical error of ±1%. Gravimetric moisture content was measured as a percentage of dry weight. 20 g of soil was oven

dried at 100°C for 48 hours and weighed using a Mettler AE 163 analytical balance with an accuracy ±0.02 mg. The pH of soils was measured using a 1:2 slurry of soil:deionized water with a Fisher Scientific pH electrode (Fisher Scientific), with an efficiency slope of >95%.

Experimental determination of unfrozen water content

We determined the unfrozen water content of three bulk soil samples from University Valley collected near the head of the valley using Decagon 5TE 3-in-1 soil temperature, moisture and conductivity sensors. Soils were initially dried at 105°C, and approximately 500cc of bulk soils from each site were placed in 1L beakers. The soils were saturated with distilled water (18Ω) and the Decagon 5TE sensor placed in the wet soils. The beakers were then placed in a Burnsco environmental test chamber (Arnprior, ON, Canada) and the ambient temperature in the chamber was programmed to decrease from +2°C to –20°C at steps of 1°C every 1.5 hours. After reaching –20°C, the ambient temperature was increased to +2°C at a rate of 1°C every 1 hour. This allowed determining the unfrozen water content during the cooling and warming of the soils as a slight hysteresis has been shown to occur. The Decagon 5TE sensors recorded temperature and apparent dielectric constant (Ka) at one-minute interval. To ensure the best accuracy in unfrozen water content, the measured apparent dielectric constant was first calibrated with the soils used in the experiments following the method described by Starr et al., 2002 (Starr and Paltineanu 2002). The unfrozen water content (%wt; gH_2O/g soils) is reported using the calibration curve and the accuracy is ±0.5%. The sensor accuracy for soil temperature is ±1°C.

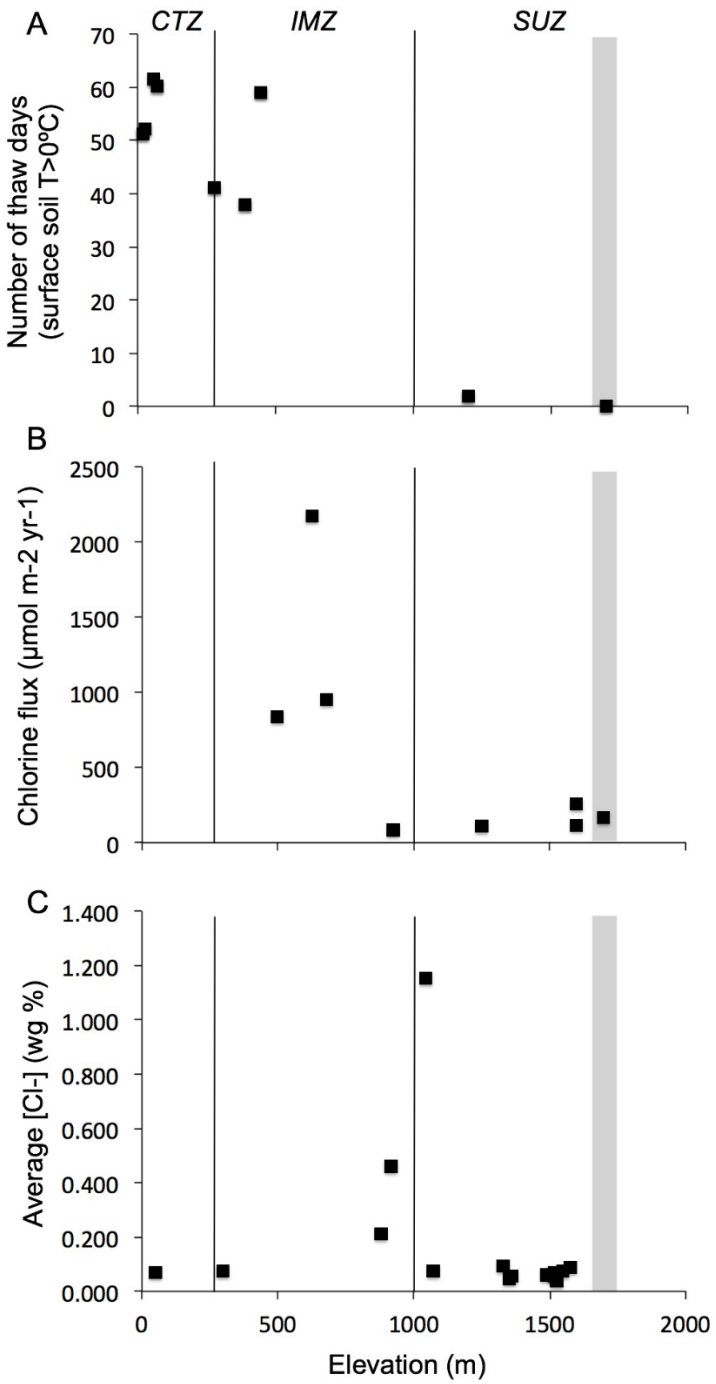

Figure 2.1. Temperature and Chlorine Salt conditions in the McMurdo Dry Valleys

The combination of low temperature (A), low salt influx (B) and low salt concentrations (C) in the stable upland zone (SUZ) of the McMurdo Dry valleys do not permit for liquid water to exist in soils in University Valley (grey band). These conditions are met in the CTZ and IMZ (coastal thaw zone, and intermediate mixed zone). Temperature data compiled from the LTER climate database (http://www.mcmlter.org). Chlorine flux and Chlorine concentration data obtained from Witherow et al., 2006.

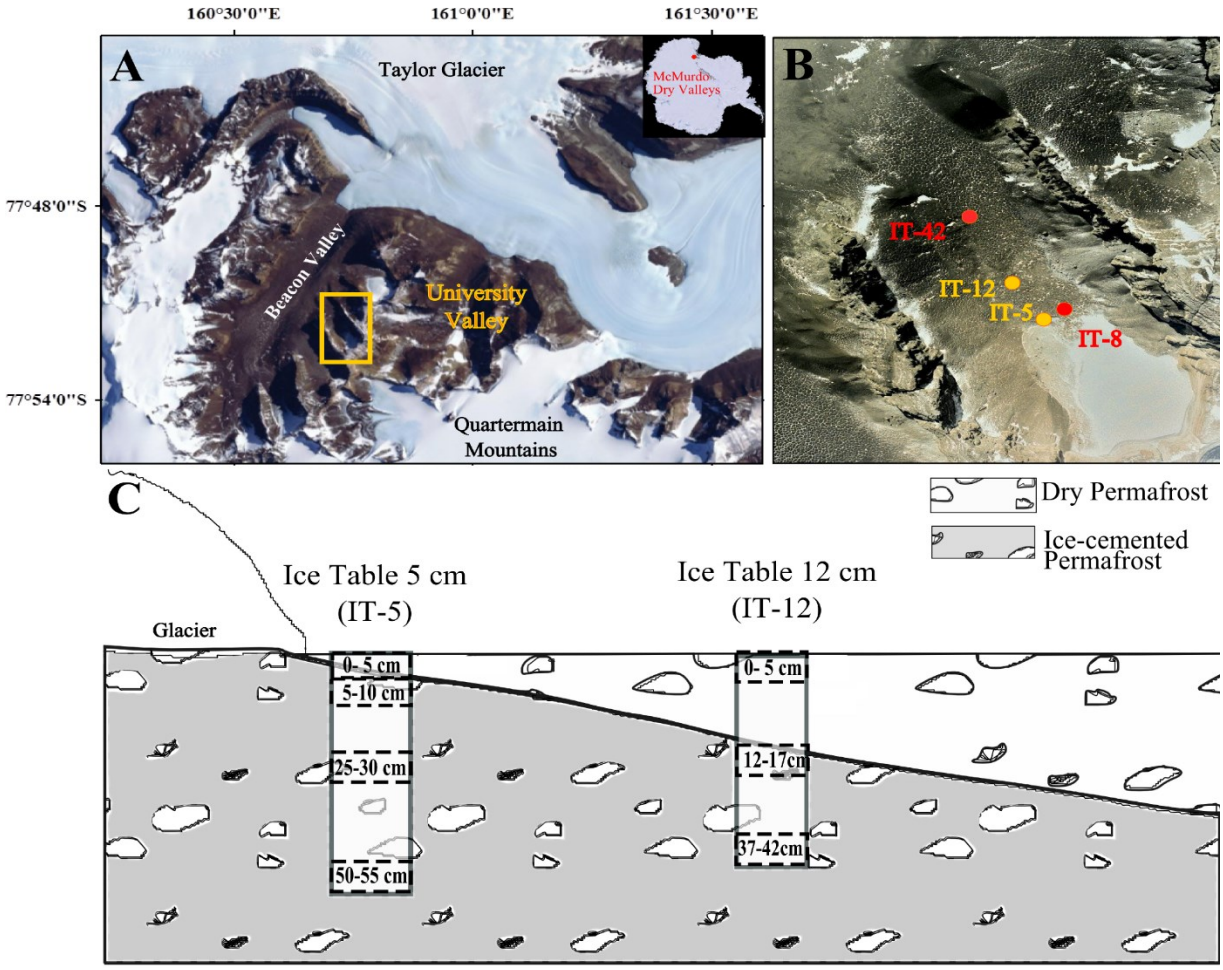

Figure 2.2. University Valley permafrost core locations

(A) University Valleys placement in the Dry Valleys; (B) approximate locations of the two soil profiles used in this study (yellow) and meteorological stations (red) with depth to ice-table indicated; (C) Soil profiles studied are shown in solid rectangles, with sample depths analyzed in dashed boxes.

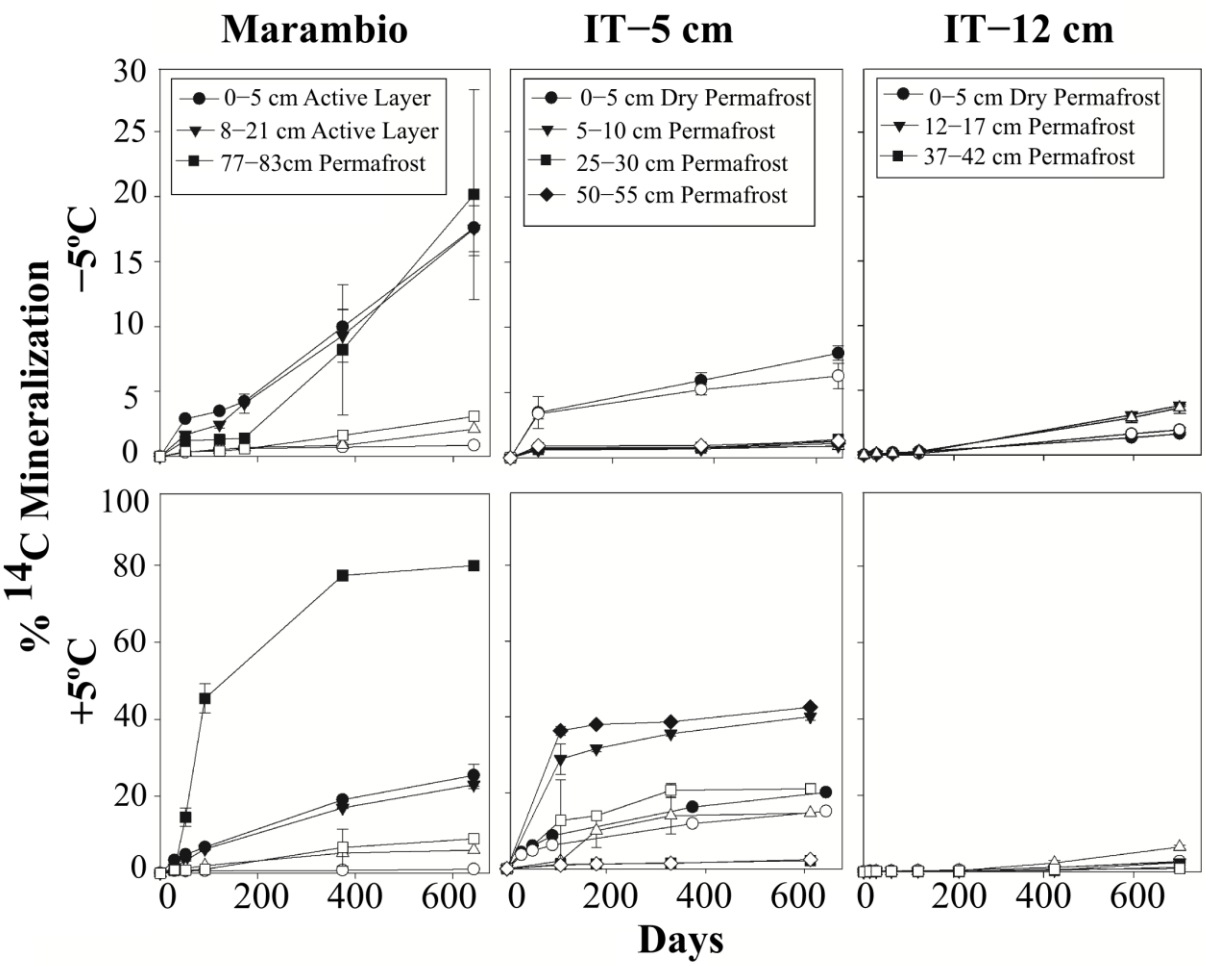

Figure 2.3. Microbial activity assay in dry and ice-cemented permafrost samples

^{14}C labelled acetate radiorespiration assays. Open symbols represent abiotic CO_2 production in killed controls. Standard error is reported here. Not shown are assays carried out at −10°C and −15°C which exhibited no detectable mineralization.

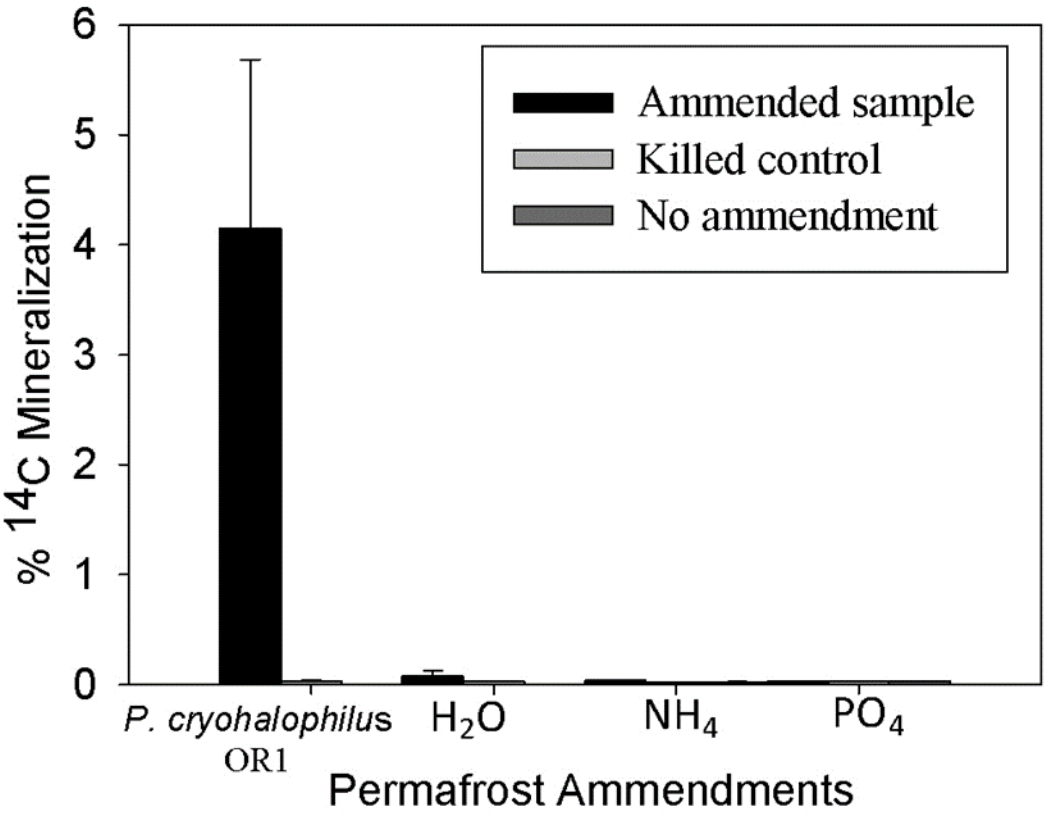

Figure 2.4. Nutrient and biomass amended radiorespiration assays at −5 °C

30 day ^{14}C labelled acetate radiorespiration assays amended with 10^6 cells *P. halocryophilus* OR1, 3 mL H_20, 1 mg g^{-1} soil of NH_4Cl, or 1 mg g^{-1} soil of $Ca(H_2PO_4)_2$. Assays were carried out in triplicate and error bars denote standard error.

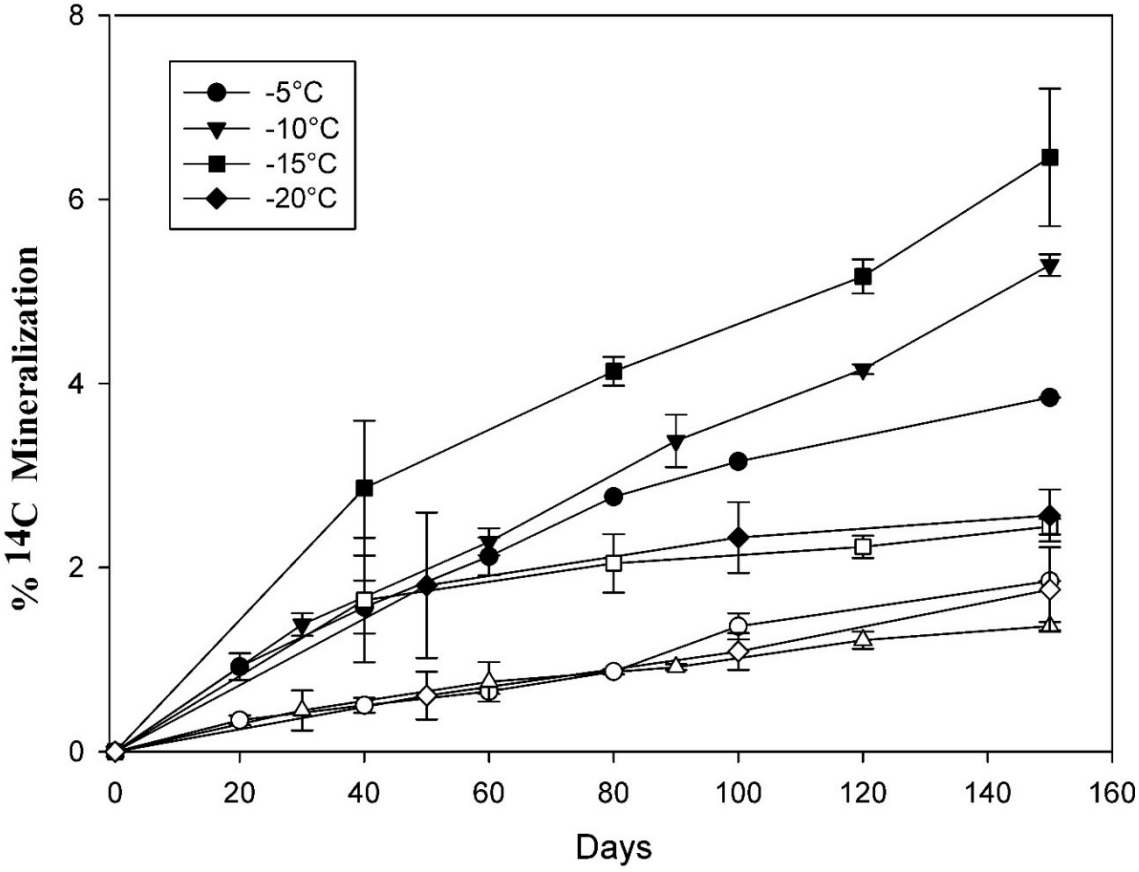

Figure 2.5. Sub-zero respiration of ^{14}C acetate measured in cryptoendoliths.

Open symbols are killed controls and represent abiotic release of CO_2. Bars denote standard error of triplicates.

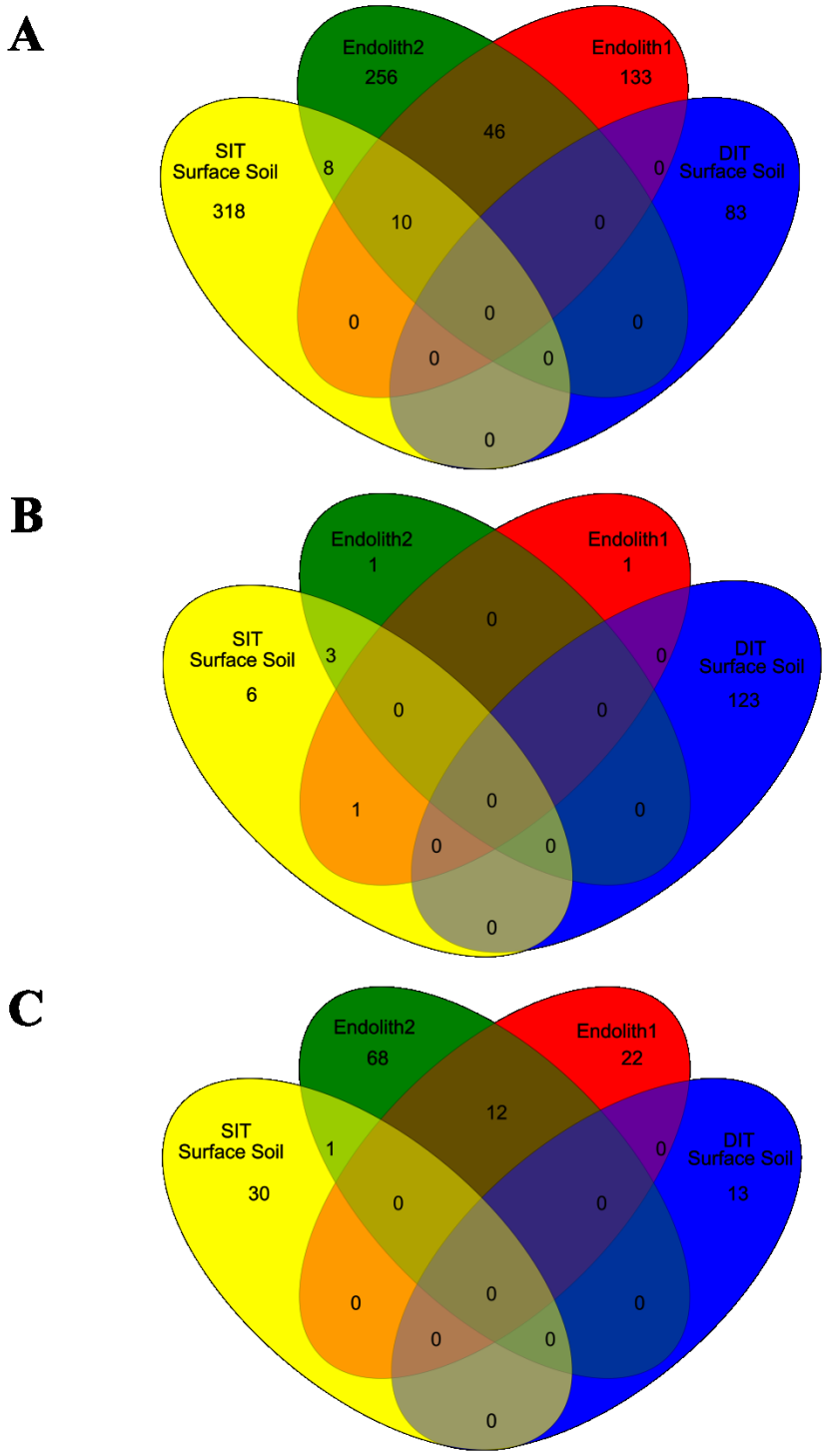

Figure 2.6. Shared OTUs between cryptoendolith and surface soils

Bacterial (A), archaeal (B) and fungal (C) shared OTUs based on 16S rRNA gene sequences. OTUs were defined at a 97% cut-off.

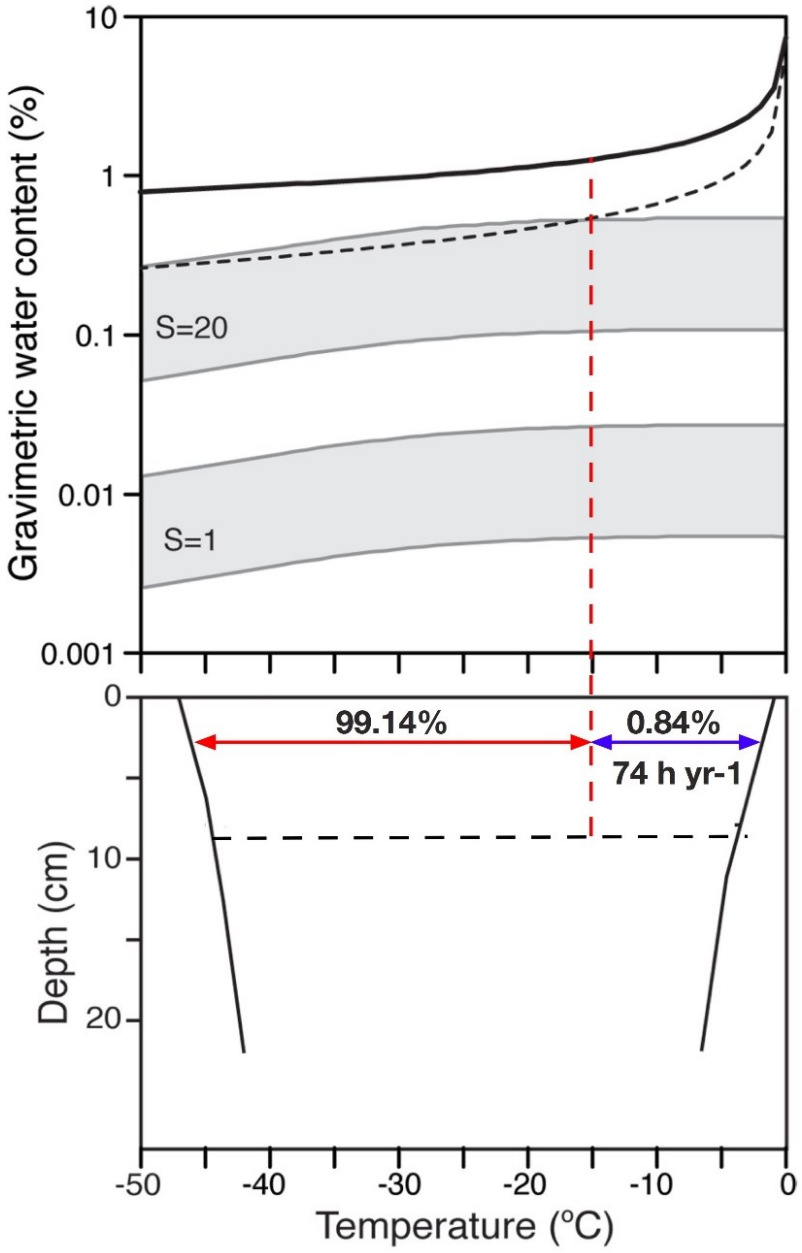

Figure 2.7. Unfrozen water content in University Valley soils

Top. Gravimetric (unfrozen) water content as a function of temperature in University Valley soils. The black dotted line includes the error in the sensors. Grey areas show the region for interfacial water for two values of specific surface area of soil particles. Soil water content transitions from bulk to interfacial at −15ºC (red dotted line, see supplementary text for details). **Bottom.** Yearly envelope of soil temperatures near the head of the valley as a function of depth. The black dotted line shows the ice table at 8cm depth. Bulk water is present in the dry soils above the ice table for up to 74 h yr^{-1}.

Table 2.1. Cultured Isolates from University Valley

Isolate	Environment of closest BLAST match	% similarity	Temp. growth range (°C)[a]	Salinity growth range (% NaCl)	Isolation media	Enrichment step prior to isolation	Sample	Genbank Accession Number
Rhodococcus sp. (Bacteria)	Cloud water (1465 m), France (HQ256820.1)	97%	−5 − 30	0− 7	R2A, 5°C	0.1% $Na_4P_2O_7$, 2 weeks	IT-12, 37− 42 cm	KM279631
Methylobacterium sp. (Bacteria)	Moss phyllosphere (NR_117561.1)	96%	5− 30	0−5	TSA + 5% NaCl	TSA + 5% NaCl, 2 weeks	IT-5, 5−10 cm	KM279632
Rhodotorula sp. (Fungi)	Lake Vostok accretion ice, Antarctica (EU108797.1)	99%	−10 − 30	0−15	R2A, 5°C	0.1% $Na_4P_2O_7$, 2 weeks	IT-12, 37−42 cm	KM279633
Sphingomonas sp. (Bacteria)	Surface Soil, South Korea (NR_043171.1)	95%	5−25	0−5	R2A	TSA + 5% NaCl, 2 weeks	IT-12, 12−15 cm	KM279634
Unidentified *Chaetothyriales*[b] (Fungi)	University Valley sandstone endolith collected 1980/81 (GU250317.1)	99%	0−25	n.d	1/10 TSA, 5°C, 50 days; 1/2 R2A, TSA, 5°C, 40 days	No enrichment	IT-12, surface soil; IT-5, 5−10 cm	KM279635
Bacillus sp. (Bacteria)	Taibai mountain, China (KJ589539)	99%	ND− 25	n.d	R2A, anaerobic conditions	Perchlorate reducing media	IT-5, 5−10 cm	KM279636

[a]-no strains were found to grow at 37°C; [b]-cannot identify to the genus level; n.d – no data

2.7 Supplementary Materials

Supplementary Data Tables 1-4

Supplementary Figures 1-2

Table S2.1. Environmental conditions in University Valley between December 2009-and February 2013.

	Permafrost depth (cm)	Mean annual T (ºC)	Max/Min T (ºC)	Annual thaw-hours*	Mean annual RH (%)	Min/Max RH (%)
	Air	−23.4	−2.8/−45.5	0	40.5	21/82
Ice Table 42 cm below surface	0	−26.5	12.3/−48	77	88	14/100
	10	−23.5	2.8/−44.5	45	n/a	n/a
	42	−23.6	−9/−37	0	96	85/100
	49	−23.5	−10/−37	0	n/a	n/a
Ice Table 8 cm below surface	0	−23.3	15.4/−42.3	36	n/a	n/a
	4	−23.5	2.1/−41.1	1.7	94.7	77/100
	8	−23	−2.2/−40.3	0	n/a	n/a
	15	−23.5	−3.7/−39.9	0	n/a	n/a

Table S2. 2. Geochemical and biomass measurements in permafrost samples analyzed in this study.

Sample	Bacterial cell counts[1]	Total C (%)	Total N (%)	pH	Gravimetric moisture content % mass
University Valley IT-5 Surface soil	2.51×10^3	0.0136	0.0523	7.5	0.56
University Valley IT-5 5-10 cm	5.58×10^3	0.0104	0.0000	7.5	29.17
University Valley IT-5 15-30cm	5.00×10^3	0.0275	0.0258	7.6	22.53
University Valley IT-5 50-55cm	5.70×10^3	0.0236	0.0322	8	27.71
University Valley IT-5 Surface soil	2.51×10^3	0.0196	0.0947	8	-0.32[2]
University Valley IT-12 12-17 cm	3.64×10^3	0.0273	0.0000	7.5	0.33
University Valley IT-12 37-42 cm	1.37×10^3	0.0137	0.0037	7.5	0.28

[1]DTAF- 5-(4,6-dichlorotriazinyl) aminofluorescein counts
[2] Slightly hygroscopic

Table S2. 3. Comparison of Arctic, Antarctic, and Atacama soil and permafrost environments

	Eureka, Arctic Permafrost [13]	Miers Valley, Coastal Influenced Dry Valleys (surface soils and permafrost boundary)[16]	Taylor Valley Permafrost and surface soils [35,25]	University Valley Permafrost (This Study)	Hyperarid core, Atacama desert soils [17,26,36]
Shannon Diversity Index (Bacteria) (97% cut-off)	3.2-4.7[a]	1.7-3.1[b]	2.01[c]	1.1 - 4.3[c]	2.6-3.8[c]
Cell Counts	10^8	10^{7}-10^9	nd	10^3	10^3
Culturable Cell Counts	10^4	n.d.	0-10^3	0-10^1	0-10^6
Laboratory Activity Assay	++++	n.d	+	-	++
***In situ* respiration**	nd	n.d.	0.10 umol CO_2 m^{-2} s^{-1}	< detection limit[d]	n.d.
Carbon	3-6%	0.16-0.69%	<0.1%	0.01-0.02%	0.04-0.16%
Nitrogen	0.01-0.5%	0.009-0.028%	nd	0-0.09%	n.d.
Perchlorate	n.d.	n.d	0-1100 µg/kg	31 to 630µg/kg	6000 ug/Kg
Moisture Content	12-86%	2.1-7.1%	19.6%	<0.5 - 30%	none[e]
Mean annual air temperature	−20ºC	−24.5ºC	−18ºC	−24ºC	16.5ºC

n.d-no data; [a] based on clone library; [b] based on T-RFLP; [c] based on pyrosequencing; [d]detection limit 0.001 ppm CH_4, 200 ppm CO_2; [e] moisture only associated with rare rain event

Table S2.4. Bacterial, archaeal, and fungal sequence reads and diversity indices

		IT-5 cm				IT-12 cm		Cryptoendolith	
	Surface soil	0-5 cm	20-30 cm	45-50 cm	Surface soil	0-5 cm	25-30 cm	1	2
Bacteria									
No. of sequence reads	4847	2726	4096	4175	1672	2521	7714	2182	6975
No. of OTUs (97% cutoff)	335	37	321	127	83	290	596	189	320
Good's coverage	0.94	0.99	0.93	0.97	0.97	0.93	0.91	0.94	0.95
Chao1 richness	412	74	624	165	319	511	512	468	329
Shannon's diversity index	3.6	1.3	2.7	1.7	1.1	4.3	4.1	2.8	2.5
Simpson diversity index	0.08	0.42	0.16	0.37	0.61	0.04	0.05	0.15	0.23
Archaea									
No. of sequence reads	44	2135	[1]	[4]	2839	1743	353	[3]	[4]
No. of OTUs (97% cutoff)	10	80	[1]	[3]	123	76	12	[2]	[4]
Good's coverage	0.93	0.98	nd	nd	0.97	0.97	0.98	nd	nd
Chao1 richness	11	205	nd	nd	273	468	23	nd	nd
Shannon's diversity index	1.9	2.3	nd	nd	2.7	2.5	1.5	nd	nd
Simpson diversity index	0.19	0.13	nd	nd	0.12	0.14	0.26	nd	nd
Fungi									
No. of sequence reads	2290	4171	8202	26021	2362	499	1050	4631	4945
No. of OTUs (97% cutoff)	31	143	69	300	13	9	9	34	81
Good's coverage	0.98	0.94	0.97	0.95	0.99	0.99	0.99	0.99	0.98
Chao1 richness	28	108	95	173	17	30	7	19	33
Shannon's diversity index	0.8	1.9	0.3	1.7	0.1	0.6	0.04	0.4	0.8
Simpson diversity index	0.68	0.25	0.91	0.30	0.97	0.64	0.99	0.83	0.69

Diversity, richness and coverage estimates shown here were calculated by sub-sampling to 1672 sequences for bacterial sequences, 499 for fungal sequences, and not subsampled for archaeal sequences due to low read numbers in some samples. Samples with low archaeal reads are denoted with [], and reads were not used to calculate diversity indices.

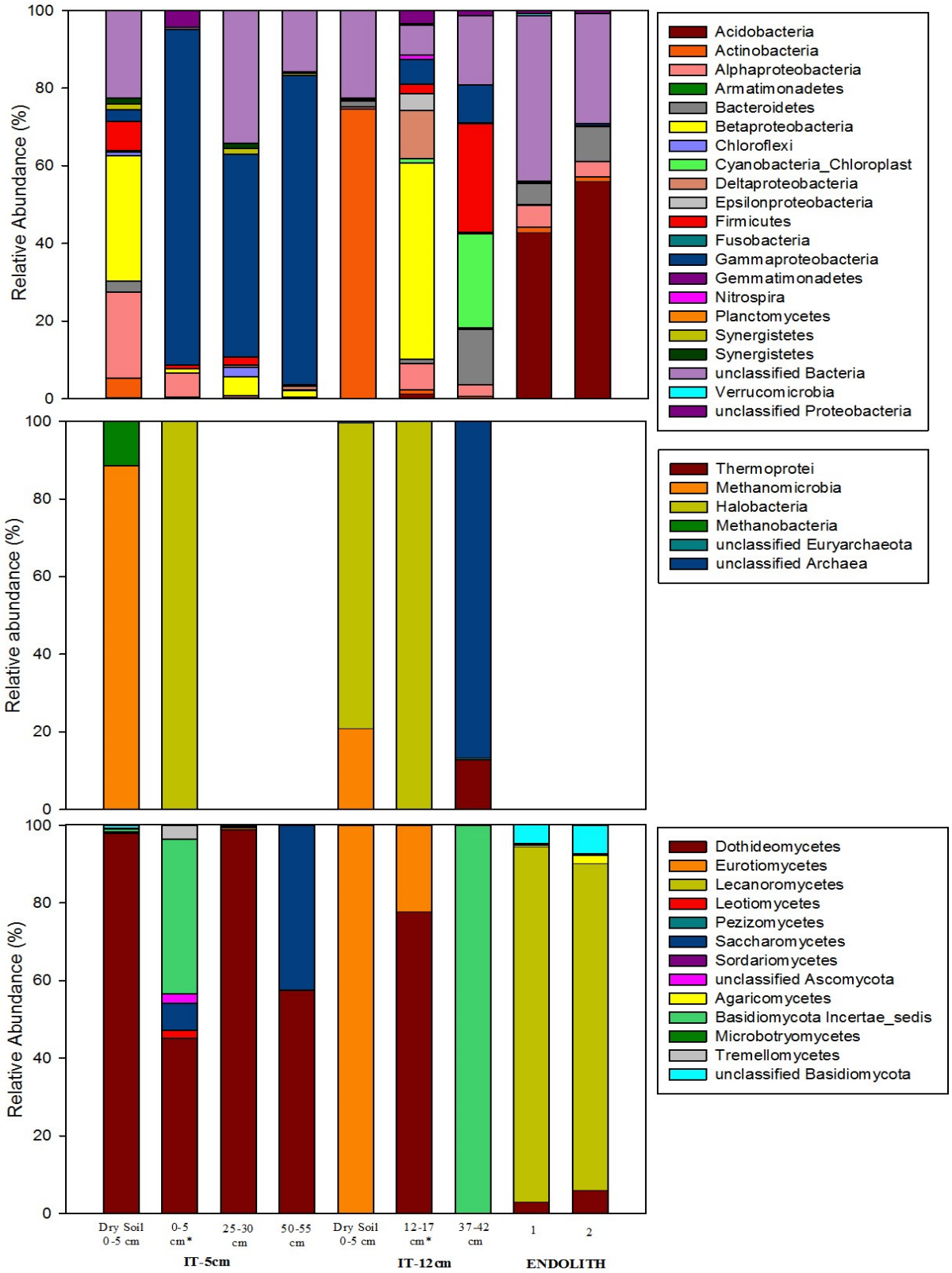

Figure S2.1. Permafrost microbial community composition
Community composition of A) Bacterial phyla (and classes of Proteobacteria), B) classes of Archaea and C) classes of Fungi. *depths that correspond to the ice table


AB663360.1 Halobacterium noricense
UV IIH34TB02IL7Y4
Halorubrum sp. EF660746.1 Salt fermented food
Halorubrum sp. HE802582.1 High altitude lake water-Argentina
UV IIH34TB02JBHLD
UV IIH34TB02H05A2
Uncultured archaeon FJ155599.1 Saline Lake-Tibetan plateau
UV IIH34TB02JTATL
UV IIH34TB02JX1E7
Uncultured haloarchaeon EU869372.1 Saline Lake-Sahara desert
UV IIH34TB02GZZS7
UV IIH34TB02HGJP1
UV IIH34TB02G5QGA
UV IIH34TB02HGFBN
Uncultured haloarchaeon JX668673.1 Saline Lake-Utah
UV HXXTI5Q02DBTC6
UV HXXTI5Q02D9LN4
EF645691.1 Halomicrobium mukohataei strain JCM9738
EU409597.1 Halomicrobium katae strain K-1
Uncultured euryarchaeote KC661836.1 Rhizosphere-Spain
UV IIH34TB02G5H26
AJ277204.1 Haloalcalophilium atacamensis ALT-5
Uncultured archaeon AJ969892.1 saline soil
UV IIH34TB02JZ78Q
Uncultured haloarchaeon FM946152.1 desert salt crust-Israel
HM063952.1 Halomicrobium zhouii strain TBN51
UV IIH34TB02I3NQ1
UV IIH34TB02JNE03
UV IIH34TB02JRS95
Uncultured archaeon HQ400444.1 Petroleum contaminated soil
Haloarchaeon FJ843004.1 Human Intestinal Mucosa
UV IIH34TB02I11F1
JF950943.1 Natronomonas gomsonensis strain SA3
Uncultured euryarchaeote FN650185.1 Hydrothermal vent-Mid Atlantic Ridge
UV IIH34TB02IGBPM
UV IIH34TB02G42YA
NR-044336.1 Haloarcula vallismortis strain CGMCC1.2048
UV HXXTI5Q02DK5SB
Uncultured Halobacterium FN391277.2 Solar Satern-Tunisia
UV IIH34TB02GSZVG
UV IIH34TB02HHP1D
Uncultured archaeon clone GQ861370.1 Water-Dead Sea
UV IIH34TB02HJUM5
Uncultured archaeon FJ696263.1 Great salt plains-Oklahoma
UV IIH34TB02I4TF8
Uncultured haloarchaeon JX668456.1 Saline Lake-Utah
UV IIH34TB02HA9Q0
NR-074200.1 Haloquadratum walsbyi DSM 16790
UV IIH34TB02J3BA0
Uncultured archaeon HQ425156.1 Saline Lake-Iran
UV HXXTI5Q02DCGEM
UV IIH34TB02HP27M
EU931578.2 Haloplanus sp. RO5-8
Uncultured archaeon JQ690685.1 Hypersaline oil resevoir
UV IIH34TB02J3XI7
Uncultured archaeon FJ155607.1 Saline Lake-Tibetan Plateau
UV HXXTI5Q02DN9C9
Halolamina sp. KF650696.1 Cold desert lake-India
UV IIH34TB02H1XA5
UV IIH34TB02H5FZ1
UV IIH34TB02IG5JZ
Uncultured archaeon AB534117.1 Hypersaline sediment-Death Valley
UV IIH34TB02GSWY3
Uncultured euryarchaeote KC465605.1 brine pool water-mud volcano
UV HXXTI5Q02D03WD
Uncultured archaeon HE579843.1 Hydrothermal vent-Mid Atlantic Ridge
UV IIH34TB02HYMSQ
Uncultured haloarchaeon AF505719.1 Hypersaline soda lake- Tibet
UV HXXTI5Q02EYO2X
UV IIH34TB02J153M
Uncultured archaeon AB775723.1 Coastal marine sediment-Japan
UV IH7DPE002I28UE
Uncultured archaeon HQ654815.1 Mud volcano-Northen Appennine mountains
UV HXFL40R01A7QLV
Uncultured archaeon JX857016.1 Oil well-China
Uncultured archaeon GQ127625.1 Meadow soil-Tibetan plateau
Uncultured crenarchaeote AY601298.1 Glacier foreland-Austria
Uncultured archaeon HE796076.1 Freshwater mountain lake-Spain
UV G62FRZZ03HMHC1
UV G62FRZZ03HIX0J
Uncultured archaeon HF951795.1 Freshwater mountain lake-Spain
UV G62FRZZ03GLBDU
UV G62FRZZ03GNYM6
Uncultured archaeon HF922660.1 Hot spring
Uncultured archaeon KF198783.1 Anaerobic sludge digester
UV IH7DPE002I8Q5U
0.05

Figure S2.2. Maximum likelihood phylogenetic tree of the inferred relationship of archaeal 16S rRNA gene sequences (170 bp) showing the position of representative OTUs (97% cut-off, only OTUs with greater than 10 reads) and most similar GenBank taxa sequences with accession numbers, isolate name or environment of sequence shown. Bootstrap values (500 iterations) are shown at branch nodes.

Connecting Text:

The study in Chapter 2 found that the permafrost soils in University Valley harboured lower biomass and undetectable activity, separating these soils from all other permafrost soils studied globally to date. Despite this, University Valley permafrost and surface soils had surprisingly high levels of diversity- the source of which may be cryptoendolithic or aeolian in origin. While the permafrost soil microbiota are nearing the cold-arid limit of life, cryptoendolithic organisms in University Valley, which experience more clement conditions are thriving. We carried out metagenomic sequencing on samples from both ice-cemented permafrost and cryptoendoliths from University Valley with the objective of understanding the functional capacity of both niches which contributes to microbial ecosystem function in University Valley.

Chapter 3. Comparative activity and functional ecology of permafrost soils and lithic niches in a hyper-arid polar desert.

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Contributions of authors: J.G wrote the manuscript, carried out heterotrophic and photosystem activity assays. J.G. and R.C. isolated and characterized the growth of photoautotrophic algae from the cryptoendoliths. J.G isolated DNA, and carried out metagenomic analysis and interpretation. L.G.W, C.W.G, A.D, contributed to interpretation of metagenomic data and helped write the manuscript. J.G., L.G.W, C.W.G., A.D and C.P.M contributed to overall experimental design and planning.

3.1 Abstract

Permafrost soils in the high elevation McMurdo Dry Valleys of Antarctica appear to be largely inhospitable to active microbial life, but sandstone lithic microhabitats contain a trophically simple but functional cryptoendolithic community. We used metagenomic sequencing and activity assays to examine the functional capacity of permafrost soils and cryptoendolithic communities in University Valley, one of the most extreme regions in the Dry Valleys. We found that cryptoendolithic microorganisms are adapted to the harsh environment and capable of metabolic activity at *in situ* temperatures, possessing a suite of stress response and nutrient cycling genes to fix carbon under the fluctuating conditions that the sandstone rock would experience during the summer months. In contrast, permafrost soils have a lower richness of stress response genes, and instead are enriched in genes involved with dormancy and sporulation. The permafrost soils also have a large presence of phage genes and genes involved in the recycling of cellular material. Our results underlie two different habitability conditions under extreme cold and dryness: the permafrost soils that select for traits which emphasize survival and dormancy, rather than growth and activity; and the cryptoendolithic environment that selects for organisms capable of growth under extremely oligotrophic, arid, and cold conditions. This is the first polar cryptoendolith metagenome and the first Antarctic permafrost metagenome completed to date.

3.2 Introduction

A large fraction of Earth's biosphere is permanently cold, and cold adapted microorganisms capable of growth at temperatures well below freezing have been found in Polar and non-Polar Regions (De Maayer et al. 2014). It is now well established that permafrost—ground that remains at or below 0°C for at least two consecutive years—can host viable and active communities of microorganisms (Rivkina et al. 2000; Steven et al. 2008; Bakermans et al. 2014; Tuorto et al. 2014; Goordial 2015a), as well as those capable of resuming metabolic activity upon thawing, even after years of cryobiosis (Legendre et al. 2014). However, permafrost is an absolute extreme environment (Steven et al. 2006; Goordial et al. 2013), and permafrost microorganisms are near the known limits of life (Goordial 2015a).

Some of the coldest permafrost soils occur in the Dry Valleys of Antarctica, where mean annual temperatures can be as low as -25ºC (Lacelle et al. 2013). There, some surface soils are permanently cryotic (Bockheim et al. 2007; D. Lacelle 2015), and the continuous aridity and cold results in the formation of dry permafrost overlaying ice-cemented soils (Marchant and Head III 2007; Lacelle et al. 2013; Marinova et al. 2013), a condition that is rare on Earth. Locally, permafrost soils in the Stable Upland Zone (SUZ) of the Dry Valleys, which is also the driest and coldest (Marchant and Head III 2007), are unlike any other permafrost soils examined to date in that microbial activity can be undetectable *in situ* or in microcosm assays, and can contain negligible microbial biomass (10^3 cells g^{-1}) and culturable organisms ($0–10^1$ CFU g^{-1}) (Goordial 2015a). These soils are potentially devoid of any active microbial life, or alternatively, any existing metabolically active cells are below the detection limits of current methodology. Yet, adjacent to these permafrost soils there is a trophically simple but functional lithobiontic (cryptoendolithic

sensu) microbial community that occupies the pore space of sandstone rocks and is comprised of photoautotrophs, lichenizing and free-living fungi and heterotrophic bacteria (Cary et al. 2010), with demonstrated heterotrophic respiration at temperatures as low as −20ºC (Goordial 2015a). The sharp biological contrast between permafrost soils and sandstone rocks suggests that the physical nature of the microenvironment plays a decisive role in the habitability of extremely cold regions, and the potential absence of *in situ* biological activity in some of the permafrost soils points to a fundamental threshold for life, a very rare case on Earth that can be used to constrain the natural cold limit of biological processes.

The objective of this study was to access the functional differences that underlie the success of the cryptoendolithic communities in comparison to permafrost soils in University Valley, located in the SUZ, and to assess both for the functional capacity of microorganisms to survive in the extremely hyper-arid, cold and oligotrophic environment. Since carbon fixation is thought to be crucial to cryptoendolithic function, we also assessed the activity and diversity of the photoautotrophic community members in such a hostile environment. The data presented here is the first metagenomic sequencing of Antarctic Dry Valley permafrost completed to date, as well as the only polar cryptoendolith metagenome sequenced to date. We used the permafrost soil metagenome to compare to other cold or arid soils globally to gain insight into why permafrost in the high elevation Dry Valleys could be inhospitable to life compared to permafrost elsewhere which experience similarly low temperatures, and in which microbial activity has been unambiguously detected (Goordial et al. 2013).

3.3 Results and Discussion

Metagenome and soil summary

Table 3.1 shows an overview of the metagenome statistics. The permafrost soils used for metagenomics analysis had a gravimetric moisture content of 9.83%, and was very oligotrophic with 0.0189% total carbon, and total nitrogen concentrations below detection limits (<0.001 %). Large amounts of soil were used for DNA extraction (60 g), however due to the low biomass (3 x 10^3 cells g^{-1}), multiple displacement amplification (MDA) was required to generate enough reads. Using MDA was seen as an acceptable compromise to access the functional potential of the permafrost soil, but was not necessary for cryptoendolith samples. Only 63,452 (8.6%) sequences from the permafrost soils and 193,269 (17.4%) sequences from the cryptoendolith metagenomes could be assigned an annotation using the M5 non-redundant protein database (M5nr). With 256,721 annotated reads in this study, we did not identify or capture the entire genetic diversity in these samples.

Microbial community composition

Based on all annotated genes in the metagenomes, the permafrost soil community was Bacteria dominated (85.6%), with a smaller Eukaryotic fraction (12.2%), primarily belonging to the fungal phylum *Ascomycota* (Table 3.2). The cryptoendolith community was mostly Eukaryotic (53.8%), comprised of the fungal phyla *Ascomycota* (45%) and *Basidiomycota* (2.2%), as well as the algal phyla *Chlorophyta* and *Streptophyta* (5.4%), reflecting the lichen dominated community that is prevalent in the Dry Valleys (de la Torre et al. 2003). Algae were nearly absent in the permafrost soils (~0.1%), and similarly, photoautotrophic bacteria belonging to C*hloroflexi,*

Cyanobacteria, and Chlorobi were detected in small amounts in the cryptoendolith but not in the permafrost soil metagenome.

Similar to other Dry Valleys*, Ascomycota* and *Basidiomycota* were the dominant edaphic and lithobiontic fungal phyla, but the phyla *Chytridiomycota,* abundant in Dry Valley soils (Dreesens et al. 2014), was absent in the permafrost soils and was negligible in the cryptoendolith community (<0.001%). The fungi to bacteria ratio was low in the permafrost soil, as has been observed in the Dry Valleys before, likely due to the low water activity, low C:N ratios, and more extreme conditions that restrict fungal growth and dispersal in high elevation inland soils, while still permitting bacterial survival (Dreesens et al. 2014). Though more abundant among the cryptoendolith, similar *Ascomycota* fungi were found in both niches, and consisted mainly of *Eurotiomycetes*, *Sordariomycetes*, and *Dothideomycetes*. Isolates from these classes are known to be polyextremophillic and are found as parasymbionts (symbionts to lichen) in Antarctic lithic niches (Selbmann et al. 2005). *Eurotiomycetes* and *Dothideomycete*s include the 'black yeast' fungi, which are melanized and are known for their desiccation and UV resistance (Selbmann et al. 2005; Ruibal et al. 2009), and may play an important role in community protection from excessive UV radiation, for example by providing an opaque barrier above photobionts in the lithobiontic community (Selbmann et al. 2013).

Actinobacteria were the predominant bacterial phylum identified in both the permafrost soil and cryptoendolith metagenomes (63.7 % and 19.8% respectively). *Firmicutes, Bacteriodetes and Alpha proteobacteria* were also abundant, similar to other regions in the Dry Valleys (Cary et al. 2010; Goordial and Whyte 2014) (Table 3.2). Desiccation and radiation resistant

Rubrobacteridae (cryptoendolith: 0.46%, permafrost: 0.01%) and *Deinococcus-Thermus* group bacteria (cryptoendolith: 0.48%, permafrost: 0.02%) commonly found in hot and cold desert soils were however low in the permafrost soil metagenome. Negligible Archaeal sequences were identified in both metagenomes (0.4% in the cryptoendolith and 0.01% in permafrost soil) consistent with previous reports that Archaea are absent, or difficult to detect in Dry Valley soils and lithobiontic communities (Pointing et al. 2009; Lee et al. 2012). The most abundant archaeal classes were *Methanomicrobia* and *Halobacteria,* which were found to represent 0.1% and 0.09% of reads respectively in the cryptoendolithic community.

Viral reads were a minor component of the cryptoendolith metagenome (0.5%), but comprised a relatively large proportion of the permafrost soil metagenome (2%). Viral families identified were consistent with those detected in other Dry Valley soils and lithic environments (Wei et al. ; Zablocki et al. 2014), and *Microviridae* and *Siphoviridae* were the most abundant in both metagenomes (Table S3.1). *Microviridae* and *Siphoviridae* are known to infect bacteria, and have been found associated with *Arthrobacter, Streptomyces, Staphylococcus* and *Bacillus* species in Antarctic soil (Swanson et al. 2012; Hopkins et al. 2014b), genera also identified in University Valley soils (Goordial 2015a). The comparative paucity of viral reads in the cryptoendolithic community is in contrast to previous comparisons of lithic niches and open soils in the Dry Valleys, which found that lithic habitats harbour a higher abundance and diversity of viruses (Zablocki et al. 2014). The reasons underlying these differences are unknown, as little is yet known about viral roles in community ecology in the Dry Valleys. In Arctic active layer soils, viruses have been demonstrated to exert a top down control on soil communities, decreasing both biomass and activity (Allen et al. 2010).

Dry Valley surface soil communities have been found to be highly localized, suggesting a high degree of endemism within each valley (Lee, 2012), and indicating that aeolian input of microorganisms by strong katabatic winds throughout the Dry Valleys may play a more limited role in community composition compared to local conditions. For example, a recent 16S rRNA gene survey of aerosols in the lower elevation Dry Valleys showed few OTUs in common with the nearby surface soils (Bottos et al. 2014). In University Valley, soils are largely derived from the weathering and erosion of the colonized valley walls (Tamppari et al. 2012; Lacelle et al. 2013), and a previous molecular survey found that the cryptoendoliths and surface soils share few OTU's in common (Goordial 2015a). It is likely that the permafrost soil community in University Valley is derived from a mixture of wind deposited cells and weathered cryptoendoliths, which subsequently undergo negative selection, where only the few cells which can form spores, remain dormant, or have advantageous adaptations to the extremely oligotrophic, arid and cold environment may survive.

Functional diversity in University Valley cryptoendolith and permafrost communities

The most abundant genes in both metagenomes were related to housekeeping functions such as carbohydrate metabolism, amino acids and derivatives, protein metabolism, respiration, and co-factor, vitamin and pigment production. All 28 subsystems detected were significantly different ($P<0.05$) between both metagenomes (Figure 3.3), and for 8 of these subsystems, the differences were determined to be biologically important as inferred from statistical probability modelling using Statistical Analysis of Metagenomic (STAMP) (Parks and Beiko 2010). The biologically relevant subsystems enriched in the permafrost soil metagenome compared to the cryptoendolith included the carbohydrate and amino acid metabolism subsystems, which consisted

primarily of genes related to TCA cycle function, central carbohydrate metabolism, and ubiquitous amino acid metabolisms. Genes responsible for the degradation of cellular material were more abundant in the permafrost soil and included several involved with murein recycling, and N-acetylglucosamine and chitin utilization. These genes could be advantageous for using cellular material as a nutrient source, including potential biomass from eroding cryptoendolithic communities. In the cryptoendolith metagenome, stress response and photosynthesis were enriched biologically relevant subsystems. These subsystems included genes involved with photosystems, CO_2 fixation, and auxin biosynthesis, phytohormones which can stimulate growth and production of antioxidants in algae (Piotrowska-Niczyporuk and Bajguz 2014). Genes reflective of the dense, biofilm like microbial consortia living within a narrow band in the sandstone rock were found in the cryptoendolith metagenome including genes associated with quorum sensing (N-acyl homoserine lactone hydrolase and S-adenosylmethionine synthetase), multidrug efflux pumps, antibiotic resistance genes (penicillin, fluoroquinolones, methicillin, vancomycin) and genes for the production of secondary metabolites known to be antibacterials and antifungals (phenanzine, clavulanic acid). Biosynthesis genes for a number of cofactors, vitamins and prosthetic groups which can support photosynthesis were present in both metagenomes (coenzyme B12, thiamine, biotin). Both metagenomes had the functional potential for catabolism of a diversity of aromatic compounds, poly- and oligosaccharides and carbohydrates (e.g.. catabolism of benzoate, catechol, gentisate, maltose, mannose, xyloglucan, lactose).

Stress Response and cold adaptation

The cryptoendolith metagenome had both a higher relative abundance (Figure 3.3) and higher diversity of stress response genes (measured as number of different stress response genes)

compared to the permafrost soils, with 87 and 34 stress response genes respectively (Figure S3.2). The two metagenomes only shared 25 of the 96 stress response related genes detected. Though the richness of stress response genes differed, known cold-adaptive genes were found in both the soils and cryptoendolith metagenome. General microbial adaptations to cold environments include mechanisms that protect the cell from freezing, preserve enzymatic membrane function, protect against reactive oxygen species (ROS), and protect against osmotic stress caused by the increasing salt and solute concentrations as water freezes (Goordial et al. 2013). The stress response pathways in both niches represented redundant functions, mostly associated with the osmotic and oxidative stresses, which are characteristic of cryoenvironments. For example, shared proteins in both niches included those involved with glycerol uptake, and proline and glycine betaine transport across membranes, these are cryoprotectants and compatible solutes which are commonly used by psychrophilic microorganisms as a strategy to cope with osmotic stress in sub-zero environments (Methé et al. 2005; Mykytczuk et al. 2013). Cold-shock proteins were found in both metagenomes, although bacterial antifreeze protein, which prevents ice-crystal formation, was only found in the cryptoendolith. Other shared stress response genes were related to general stress response functions like chaperones, sigma B stress response, carbon starvation or phage shock protein A (pspA); phage shock protein A is a stress response gene involved in maintaining cell membrane and proton motive force integrity and is induced during filamentous phage infection, extremes of temperature, and osmotic stress. There was also a large proportion of heat shock related genes (Figure S3.2), which mostly grouped with the dnaK gene cluster SEED subsystem (level 3). These proteins unlikely reflect heat shock associated with the perennially cold environment, as dnaK is a general chaperone protein found in other cryoenvironments and in psychrophile isolates (Varin et al. 2012; Lay et al. 2013).

Nutrient Cycling

In highly oligotrophic soils like those encountered in University Valley, carbon and nutrient sequestration is important. Examining the presence and absence of metabolic pathways we found no evidence for functioning autotrophic pathways in the permafrost soil as determined by the absence of key enzymes in the Calvin Benson cycle, the reductive TCA cycle, the reductive acetyl-coA pathway, and the hydroxypropionate cycle carbon fixation pathways (Table 3.3). The paucity of genes associated with autotrophy suggests these soils are dependent on heterotrophic substrates. As expected, the cryptoendolith metagenome contained the genes for CO_2 fixation with the Calvin Benson cycle. Genes associated with metabolism of trace gases and other C1 compounds (methanogenesis, acetogenesis, methanotrophy) were limited or absent in both metagenomes, with the exception of methane monooxygenase (*mmoX*) detected in the cryptoendolith metagenome. Genes required for heterotrophy were abundant in both metagenomes including genes required for acetate metabolism, a compound not mineralized at sub-zero temperatures in University Valley soils (Figure 3.2 and (Goordial 2015a)). Key genes in the glyoxylate pathway were found in both metagenomes, including isocitrate lyase and malate synthase, and it is possible the CO_2 releasing steps of the TCA cycle can be bypassed, although heterotrophic activity as inferred from respired $^{14}CO_2$ was detected in University Valley permafrost soil microcosms at 5ºC so it is unlikely that the glyoxylate pathway is responsible for the lack of microbial activity observed at sub-zero temperatures (Figure 3.2**Error! Reference source not found.**). Previous analysis of University Valley permafrost soils indicated that due to the low salt concentration, the amount of liquid water at below freezing temperatures is limited only to thin films adhering to sand grains (Goordial 2015a). The activity observed only above

freezing temperatures may reflect water newly available for cells, which would be otherwise dormant at *in situ* freezing temperatures.

Nitrogenase reductase (*nifH*) sequences were not detected in either metagenome. Cryptoendolithic communities in the Upper Dry Valleys largely lack the ability to fix nitrogen (as measured by acetylene reduction), likely because of available nitrates which are atmospherically deposited and which have low leaching rates in desert environments (Friedmann and Kibler 1980). Some nitrogen cycling genes (nitrate and nitrite reductases) were recovered from both metagenomes, although other denitrification genes (nitric oxide reductase and nitrous oxide reductase) were absent. The lack of nitrogen and carbon fixation capacity in the permafrost soils separates these soils from Arctic permafrost and lower elevation (<1000 m.a.s.l.) Dry Valleys surface soils where both photoautotrophic and diazotrophic pathways have been identified in functional microarray and PCR surveys (Yergeau et al. 2010; Niederberger et al. 2012; Chan et al. 2013).

Activity and characterization of the photoautotrophic cryptoendolith community

Photoautotrophic microorganisms drive carbon acquisition in the sandstone cryptoendoliths, and may be a source of organic matter to the permafrost soils, and thus could play a key role in ecosystem function. In order to get better resolution of the photoautotrophic diversity we carried out 454 pyrosequencing on two cryptoendolith samples targeting the 23S rRNA plastid gene found in photosynthetic organisms, including cyanobacteria and algae (Sherwood and Presting 2007). The cryptoendolith photoautotroph community was dominated almost entirely by *Trebouxia*, an algae known to form lichenizing associations, which made up over 99% of

sequences. Lichen dominated cryptoendoliths in the high elevation Dry Valleys have been previously shown to be mono-specific (de la Torre et al. 2003), but we found a high diversity of *Trebouxia sp*. in the cryptoendolith samples, with 365 OTU's (97% cut-off) for this genus between both cryptoendolith samples, which only shared 24 OTU's in common (Figure S3.1). *Cyanobacteria* were a minor component made up of 4 OTUS's (representing 33 sequences) in one cryptoendolith sample, and were absent in the other (Figure S3.1). Other photosynthetic organisms were not detected using pyrosequencing, including *Chloroflexi*, and the *Streptophyta* algae annotated in the metagenome.

We were able to isolate green algae from the cryptoendolith samples (Table S3.2) belonging to the genus *Stichococcus* and *Desmococcus*. Photoautotrophs could not be cultured from permafrost soils using the same methodologies. Isolates identified as *Stichococcus* EN2JG and *Desmococcus* EN5JG were adapted for cold temperatures and demonstrated growth (Table S3.2**Error! Reference source not found.**) and chloroplast autofluorescence at −5ºC (Figure 3.2). Notably, the observed growth occurred with no media amendments to prevent cultures from freezing, indicating these isolates are synthesizing freezing point depressants to maintain a liquid culture at sub-freezing temperatures. The isolates were not capable of growth when glycerol (5%) or NaCl (5%) were added as freezing point depressants, and the liquid media tested here froze at the other temperatures tested (< −10ºC), thus potential growth at lower temperatures could not be measured. Two *Stichococcus* isolates differed in their growth characteristics; *Stichococcus* sp. EN2JG was a eurypsychrophile with an optimal temperature of 22ºC and a minimum temperature of −5ºC, while *Stichococcus* sp. UV2BC was a stenopsychrophile incapable of growth at 22ºC, with an optimal temperature of 10ºC and a minimum temperature of 0 ºC. Differing growth optima

may occur in the diverse unculturable algae surveyed here as well, and would result in communities which could fix carbon over the breadth of fluctuating conditions the sandstone cryptoendoliths would experience.

We also carried out Pulse Amplitude Modulated (PAM) fluorometry to determine the activity of the photosystem II [PS(II)] of phototrophic members of the cryptoendoliths. Significant PS(II) activity (measured as variable fluorescence, (Fo - Fm)/Fm) was measured to be 0.618, 0.560, 0.467 at 20ºC, 0ºC and −20ºC respectively, indicating that colder temperatures affected PS(II) efficiency, but photosynthesis could still occur at −20ºC. We found that similar amounts of amounts ^{14}C labelled acetate was mineralized at 5ºC (4.1%), −5ºC (3.8%), and −10ºC (4.4%) over 100 days (**Error! Reference source not found.**Figure 3.2); thus both the heterotrophic and photoautotrophic communities within the cryptoendoliths display thermal plasticity allowing activity over a range of temperatures that overlaps with those observed in the natural environment during the summer months. In contrast, heterotrophic activity in permafrost soils was undetectable at −5ºC and -10ºC, and could only be detected at 5ºC, a temperature which is not encountered *in situ* and likely reflects the activation of dormant but viable cells.

Comparison of University Valley permafrost with other desert and permafrost metagenomes

An ordination (Figure 3.4) was created to examine the functional similarities and differences of University Valley permafrost soil with other permafrost and desert environments. A list of the metagenomes used for comparison in this study is available in Table S3.3. The University Valley permafrost soil metagenome clustered most closely with other permafrost metagenomes from the Arctic, and separately from the more geographically proximate Dry Valley

active layer soils. This may indicate that the permafrost soils in University Valley are more similar to Arctic permafrost than previously assumed based on the low biomass and lack of microbial activity previously detected (7). Compared to the hot and cold desert soils, the permafrost metagenomes were enriched in genes associated with osmotic stress, which would be advantageous in the brine veins thought to exist within permafrost as a potential microbial habitat, where salts, solutes and microorganisms could be concentrated together during freezing in a similar manner to sea ice (Junge et al. 2001). The permafrost soils metagenomes were also enriched in integrases and transposases, and antibiotic and antiseptic resistance genes including beta-lactamases, vancomycin and acriflavin resistance. It is not known what role these genes would have, though it is possible that in permafrost soils microbial biomass becomes concentrated within brine veins, increasing microbial competition for limited nutrients and possibly occurrences of lateral gene transfer. University Valley permafrost soil was an outlier to the permafrost samples in this respect and had the lowest proportion of antibiotic resistance genes, integrases and transposases (Figure S3). The contrast could be due to a combination of unique factors in University Valley soils; the low soil salinity found in SUZ soils is prohibitive to the formation of brine veins where cells could concentrate (Goordial 2015a), biomass in University Valley permafrost soils is extremely low (10^3 cells g^{-1}), and as indicated by the absence of metabolic activity, microorganisms in the permafrost soils are likely not competing, but are dormant. University Valley permafrost soils were less functionally equipped with oxidative stress, general stress response and cold shock genes, though intriguingly had the highest proportion of phage related genes (mostly phage capsid proteins) compared to the other permafrost metagenomes. Rather than a diversity of stress response functions, University Valley had a comparatively high proportion of a number of genes associated with sporulation and spore DNA protection (Figure

S3). While survival on long time scales is important in all permafrost environments (Figure 4), traits which allow cells to persist in permafrost, rather than for growth or activity, are especially emphasized in University Valley permafrost soils where the conditions may be too extreme for the activity of even cold adapted extremophiles.

Comparison of University Valley cryptoendolith metagenome other photoautotroph based metagenomes

To our knowledge, this is the first terrestrial cryptoendolithic metagenome reported to date, as no other sequenced hypoliths or chasmolith metagenomes could be found in public databases. Thus we are limited in our ability to compare the University Valley cryptoendolith to lithic environments in other hot or cold deserts. We chose to compare the biofilm like cryptoendolith community with other communities which have a large photoautotroph component, and included in our ordination metagenomes from an Alpine lichen community, polar microbial mats, and a glacial cryoconite hole microbial community. The cryptoendolith metagenome did not cluster strongly with any of these metagenomes. The cryptoendolith shared with the lichen metagenome a higher proportion of genes involved with quorum sensing, and cofactor, vitamin and pigment production, a reflection of the symbiotic relationship between mycobionts and phycobionts seen in both the cryptoendolith and lichen communities. The genes shared with the Antarctic microbial mat metagenome were important in biofilms, including those involved in adhesion, extracellular polysaccharides, and siderophore production; in the cryptoendolith these traits would be useful in rock colonization, and iron acquisition/mobilization functions which result in the characteristic red banding pattern seen in cryptoendolithic communities (Figure 3.1). Phages, bacterial cytostatic and antibiotic production and resistance was most abundant in the cryptoendolith metagenome,

possibly indicative of a higher level of microbial competition and predation than in the other metagenomes used here for comparison.

3.4 Conclusions

Habitability conditions in University Valley permafrost soils and lithic niches.

It has already been postulated that while microorganisms are present in the permafrost soils (Tamppari et al. 2012; Goordial 2015a), microbial activity is likely non-existent in parts of University Valley where soils are permanently cryotic (Goordial 2015a). We refer to these soils as non-habitable, but not sterile, and the resulting permafrost soil community is likely a mixture of aeolian and cryptoendolithic origin. This interpretation is supported by the metagenomic data presented in this study, which revealed less cold and general stress response functional diversity, critical for life in permafrost soils, whereas sporulation (i.e. dormancy) is an emphasized function. The functional potential for recycling of cellular material, as well as the large presence of phage associated genes suggest that if there is an active component of University Valley permafrost soils, it could survive using scavenged organic matter, possibly of endolithic origin since that is the only relevant source of biomass in the valley.

On the other hand the cryptoendolithic communities that colonize the valley walls appear to be adapted to the harsh conditions within the valley (Figure 3.5), as evidenced by the development of a complete ecological community, including photoautotrophic algae and bacterial/fungal consumers that are viable and active over the range of temperatures the cryptoendoliths experience, and by the diversity of stress response functions and nutrient cycling pathways. We have added to the functional knowledge of lithic communities which are known

'hot spots' of productivity in cold and dry environments, including evidence for the likely presence of community competition in addition to the well-known symbiotic interactions, as indicated by the presence of antifungal and antibacterial production and resistance genes.

The stark biological contrast between permafrost soils and lithobiontic niches is due largely to the physical properties of the lithic substrate. Primarily, the sandstone favours the occurrence of wet events through inducing the melting of snow (Friedmann 1978; Friedmann et al. 1987). Once wet, surface tensions between thin films of water and the rock matrix slow down evaporation, and extends the window for metabolic activity (Friedmann et al. 1987). This, together with the protection from UV radiation while still allowing for photosynthetic activity, represent decisive survival advantages that ultimately control habitability under extreme cold and dry conditions.

Our results evidence that caution should be taken when interpreting function solely from genomic analyses, which cannot differentiate between vegetative, dormant and dead cells, especially in stable and cold permafrost soils which are likely highly preserving for nucleic acids. Metagenomic analysis is best complimented by functional validation and activity assays, though given the difficulties in culturing and isolating organisms from such extreme environments, metagenomics sequencing is a good proxy for the functional potential of environments which may otherwise be inaccessible. Future studies utilizing transcriptomic, proteomic and activity assays targeting some of the functions identified in this study are the next step to understanding how microbial communities are adapted to thrive and survive in one of the coldest and driest terrestrial habitats on Earth.

3.5 Acknowledgments

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3.6 Material and Methods

Sample collection and preparation

The University Valley permafrost core sample used in this study was collected in the 2009 summer field season, located at 77d 51.870s S, 160d43.524s E (elevation 1700 m.a.s.l). Depth from the surface to the ice-cemented ground was 22 cm. An 18 cm ice-cemented permafrost core was collected with a SIPRE corer along with overlaying dry permafrost Samples were shipped to McGill University in a thermally insulated box and maintained at −20ºC until processing. Initial core processing took place in a walk-in freezer held at −5ºC, in a laminar flow hood where 1 cm of the outside of the core was removed with a sterilized chisel. An additional 1 cm of the outside core was removed in a laminar flow hood at room temperature immediately prior to samples being weighed and aliquoted for analysis. Cryptoendolith samples used in this study were collected in the 2013 summer field season, from Beacon supergroup sandstone boulders located on the South-

East facing valley walls. Samples were aseptically collected and maintained at −20ºC until processing.

Soil Analysis

The soils were analyzed for total carbon and total nitrogen by combustion at 900°C with a Carlo Erba Flash EA 1112 NC Soils Analyzer which has an analytical error of ±1%. Gravimetric moisture content was measured as a percentage of dry weight. 20 g of soil was oven dried at 100°C for 48 hours and weighed using a Mettler AE 163 analytical balance with an accuracy ±0.02 mg. The pH of soils was measured using a 1:2 slurry of soil:deionized water with a Fisher Scientific pH electrode (Fisher Scientific), with an efficiency slope of >95%.

DNA extraction

The top ten 10 of the ice-cemented permafrost core (22-32 cm depth from the surface) was used for metagenomics analysis. Community DNA was extracted from 2 g of permafrost soil using the UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, California, USA), as described in the alternative protocol for maximum yield, and a bead beating step was added to aid lysis. 30 extractions (60 g total permafrost soil) were performed and the resulting DNA was pooled and concentrated. DNA from cryptoendoliths was extracted from 6 grams total of crushed rock using the same DNA extraction protocol as for permafrost. Negative controls (H_2O in place of sample) underwent identical handling during the extraction procedure and were used as templates for PCR using 16S rRNA gene primers (27F and 1492R) to ensure no contamination during extraction.

Metagenomic Sequencing and Analysis

DNA was sent to Molecular Research LP (Shallowater, Texas, USA) for sequencing. There, the library was prepared using Nextera DNA Sample preparation kit (Illumina) following the manufacturer's user guide. Both the samples were first purified using PowerClean DNA Clean-up Kit (MoBio) and concentration of purified gDNA was evaluated using the Qubit dsDNA HS Assay Kit (Life Technologies). Because of low DNA concentration for the ice-cemented permafrost sample Multiple Displacement Amplification (MDA) was performed at 30°C for 16h using the REPLI-g Midi Kit (Qiagen) according to the manufacturer's instructions for 2.5uL of input DNA. Once amplification was complete, the concentration of the sample was again determined and each sample was diluted accordingly to achieve the recommended DNA input of 50ng at a concentration of 2.5ng/μL. Subsequently, the sample underwent the simultaneous fragmentation and addition of adapter sequences. These adapters are utilized during a limited-cycle (5 cycles) PCR in which unique index was added to the sample. Following the library preparation, the final concentration of the library was measured using the Qubit dsDNA HS Assay Kit (Life Technologies), and the library size was determined using the Experion Automated Electrophoresis Station (Bio-Rad). The libraries (12.5 pM) were pooled together and was sequenced by using 600 Cycles v3 Reagent Kit (Illumina) in MiSeq (Illumina). Sequences were processed using MG-RAST (Meyer et al. 2008), artificial replicate sequences produced by sequencing artifacts were removed (Gomez-Alvarez et al. 2009), and sequences were quality trimmed using the default settings for dynamic trimming (sequences contain <5 bp below a phred score of 15). Any human or chordata contaminants were removed from the dataset. To determine the presence or absence of functional genes, we used GenBank annotated proteins in MG-RAST (e-value $\leq 10^{-5}$, alignment length >15). We used the statistical probability model in Statistical Analysis of Metagenomic

Profiles (STAMP) (Parks and Beiko 2010) (version 2.08; Faculty of Computer Science, Dalhousie University) to identify the biologically relevant differences between the permafrost and cryptoendolith metagenomes. A pairwise statistical comparison of the two metagenomes analyses was carried out using clustering based SEED subsystem annotations ($E \leq 10^{-5}$, similarity > 60 %, alignment length >15), using a two-sided Chi-square test (with Yates) statistic with the DP: asymptotic-CC confidence interval method and the Bonferroni multiple test correction. A *P*-value of <0.05 was considered significant, and an effect size filter for ratio of proportions (RP) effect size <2.00 and a difference of proportions of <2.5. Comparisons of the University Valley metagenomes were made to other publically available metagenomes from similar hot and cold deserts, or microbial mat communities (Table S3.3), all metagenomics data was processed through MG-RAST to make analyses comparable. Relative abundance was used to calculate Bray-Curtis distances between sample pairs using the "vegdist" function of the "vegan" package (http://vegan.r-forge.r-project.org/) in Rstudio (version 0.98.1091). Principle coordinate analyses (PCoA) analyses were performed using the 'cmdscale' function. Relative abundance of level 1 of the SEED hierarchy were superimposed on the ordination using the "envfit" function.

Pyrosequencing of plastid gene amplification and high-throughput sequencing

DNA was sent for pyrosequencing analyses at the Research and Testing Laboratory (Lubbock, TX, USA) using the Roche 454 GS-FLX platform (Roche 454, Branford, CT, USA). Sample libraries of partial bacterial/ algal 23S rRNA amplicons were produced using the forward primer (5'GGACAGAAAGACCCTATGAA-3') and reverse primer (5'-TCAGCCTGTTATCCCTAGAG- 3') that flank the V domain of the 23S plastid rRNA gene. Data was processed using Mothur (Schloss et al., 2009), Briefly, sequences were quality filtered by

removing primer sequences, reads < 150 bp long, sequences with ambiguous base calls, and homopolymer repeats greater than 8bp. Chimera removal using chimera.uchime within Mothur was used to further reduce sequencing error prior to alignment and clustering. A total of 13,557 sequences were analyzed after quality control. Sequences were aligned to the Silva LSU bacterial database (Accessed March 2015) and OTUs were clustered using average-neighbour clustering with a 97% cut-off. The 'get.oturep' command of Mothur was used to retrieve a representative sequence for each OTU. Representative sequences were classified using the MEGAN5 software (v. 5.3.0) (Huson et al. 2007) after BLASTn searches against the GenBank nt database (http://www.ncbi.nlm.nih.gov/GenBank/) (accessed June 2014) with default settings, and by excluding noncultured/environmental sequences from the target database. For MEGAN5 classification, LCA parameters were changed from default as to favour the taxonomic information of the best BLASTn hits to be assigned to a given read; LCA parameters were set to 'Min Support: 2', 'Min Score: 100', 'Top percent: 2', and 'Min complexity:0'. The primers used in this study were found to amplify some non-phototrophic bacteria (belonging to acidiphilum, and caulobacter) representing <0.01% of reads and which were manually removed from the dataset.

Heterotrophic Radiorespiration Assay

5 g of permafrost was added to individual microcosms as Steven et al. 2007. Each microcosm was performed in triplicate, and included triplicate sterilized controls (autoclaved twice for 2 hours at 120°C and 1.0 atm, with a 24 h period between autoclavings). Microcosms were spiked with 0.045 mCi ml^{-1} (~100,000 disintegrations per minute) of 1-^{14}C acetic acid. Cold acetic acid was added to a final concentration of 15 mM acetic acid per microcosm in a total volume of 40 µl. The CO_2 trap consisted of 1 M KOH for microcosms incubated at 5°C, −5°C and 1 M KOH

+ 20% v/v ethylene glycol for microcosms incubated −10°C and −15°C. For cryptoendolithic microcosms 3 g of crushed rock from the visibly colonized area of the sandstone was used for each microcosm, and spiked with cold and radioactive acetate as described. Measurements of radioactivity were determined by liquid scintillation spectrometry on a Beckman Coulter (CA, USA) LS 6500 Multi-purpose Scintillation Counter.

Pulse Amplitude Modification PAM methodology

Chlorophyll *a* fluorescence was measured with a PAM fluorometer (WATER-PAM, Heinz Walz GmbH). After 30 minutes dark adaptation, initial fluorescence ($F_{O)}$ was measured and represents the point where all PSII reaction centres are open and the most light energy can be used for photochemistry rather than being emitted as fluorescence. The sample was then given a saturation pulse until all reaction centres were closed and electron acceptors saturated, all light energy is given off as maximal fluorescence in this state (F_M). Photosynthetic efficiency of photosystem II (PSII) was measured as F_V/F_M, where variable fluorescence (F_V) is calculated as the difference between initial fluorescence (F_O) and maximal fluorescence (F_M).

Isolation and characterization of photoautotrophs

1g of permafrost soil, or of the colonized band of the cryptoendolith was sampled, homogenized and added to a sterile tube containing 3mL of 0.1% sterile sodium pyrophosphate and 0.5g of glass beads. Following 1 min of vortexing, 100µL of the suspended cell solution was used to inoculate liquid media and agar plates of BG11, CHU-10 and SNAX media. Plates and liquid enrichment cultures were incubated at 5°C and 20°C, in the presence of 24 hours 6400K full spectrum light (T5HO bulb, Sunblaster) until growth was observed. Isolates were then

characterized for growth at −5°C, 0°C, 5°C and 20°C, as well as with 5% NaCl and 5% glycerol added to media.

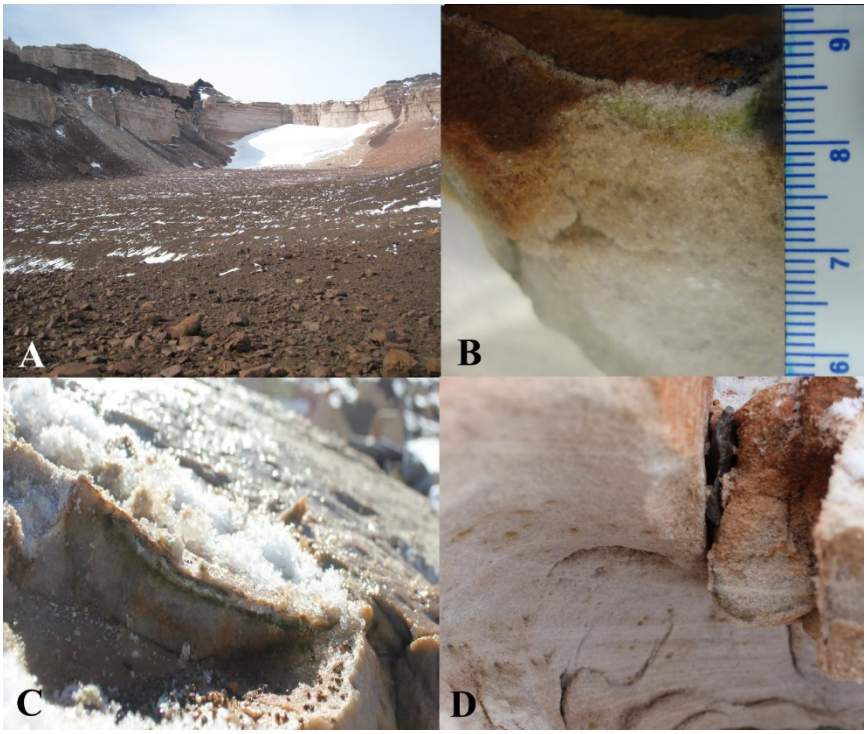

Figure 3.1. University Valley permafrost and cryptoendolith niches

A. University Valley; B. University Valley Cryptoendolith; C-D. Field cryptoendolith photos C. An exposed cryptoendolith in University Valley after a snowfall event D. Cryptoendolith community underneath the rocks surface utilizing low sunlight reflected by sandstone wall

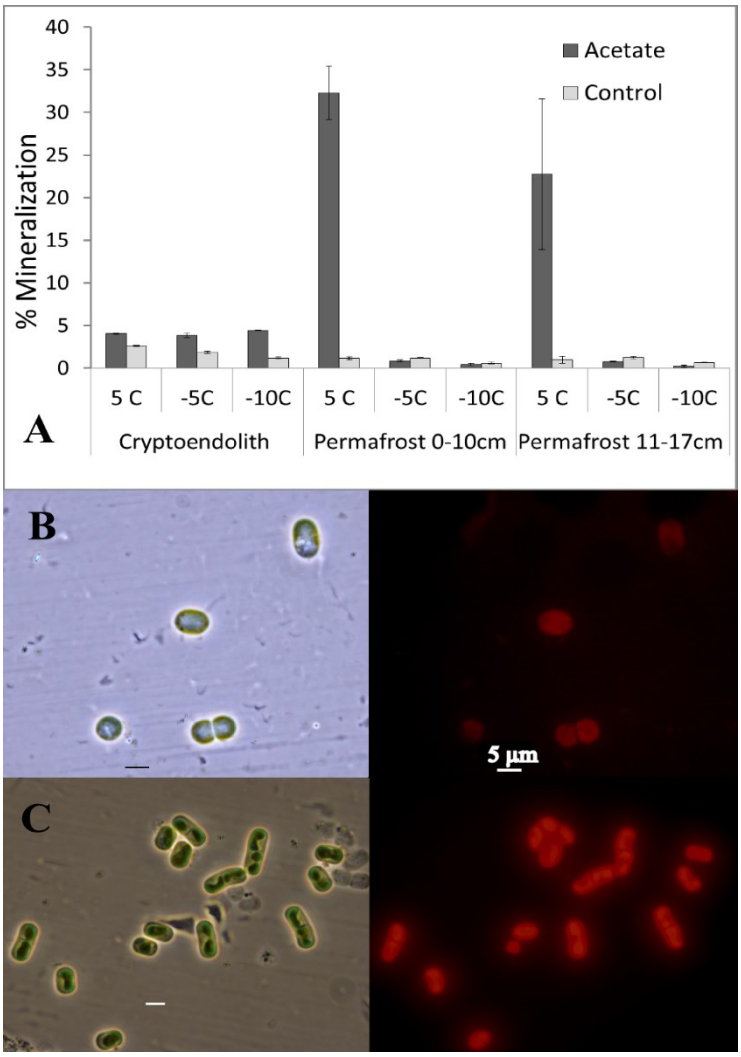

Figure 3.2. Heterotrophic and photosystem activity at sub-zero temperatures

A. ^{14}C acetate mineralization detectable in cryptoendolithic at all temperatures tested, and in permafrost samples only above 5ºC. B-C. *Diplosphaera* (B) and *Stichococcus* (C) isolates demonstrating chloroplast autofluorescence activity after 200 days culturing at −5ºC.

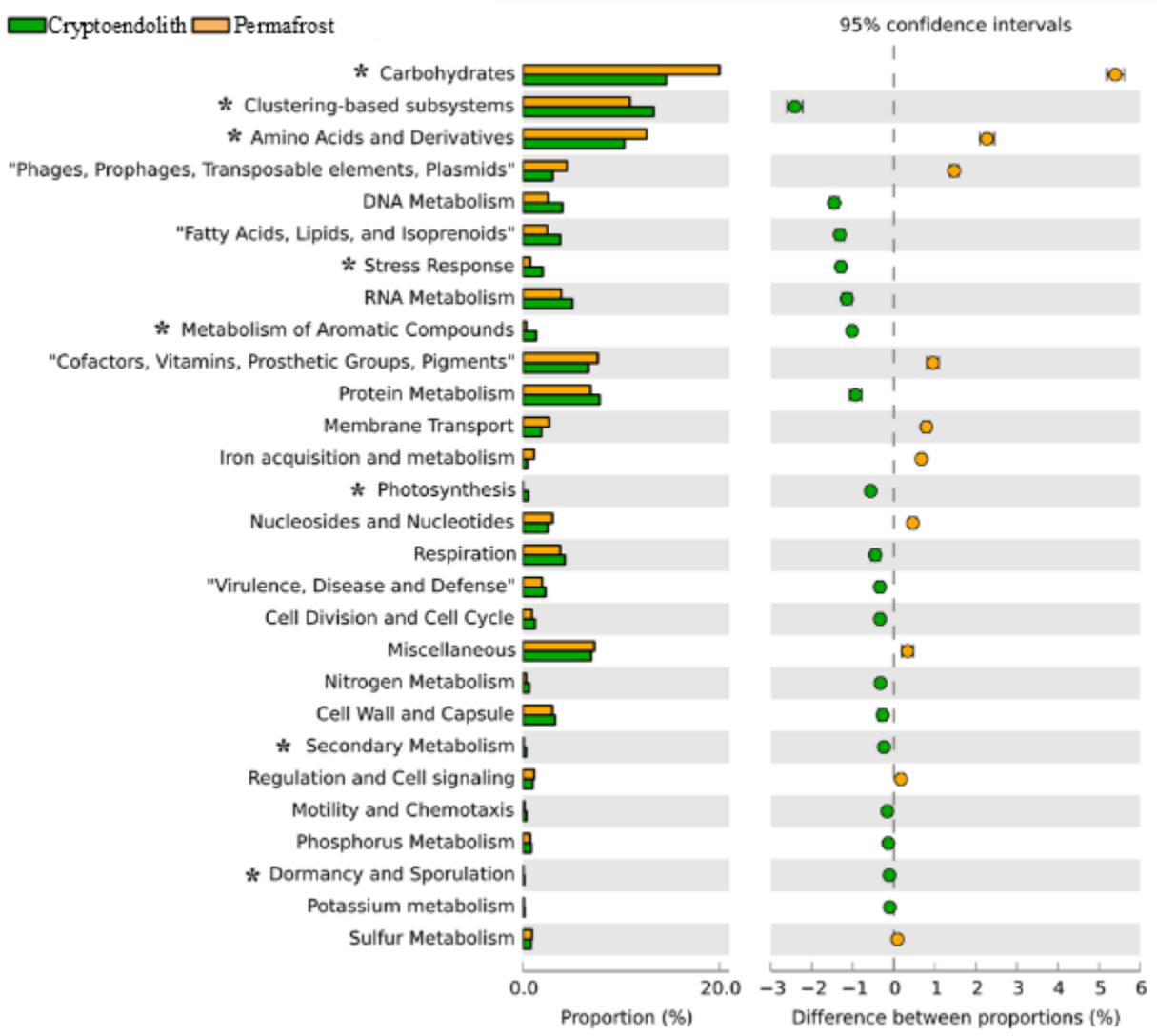

Figure 3.3. Functional profiles of University Valley cryptoendoliths and permafrost

Level 1 SEED subsystems found in the cryptoendolith and permafrost metagenomes. Indicated by * are subsystems which were found to be biologically important as inferred from statistical probability modelling using Statistical Analysis of Metagenomic (STAMP) using *P*-value <0.05, for ratio of proportions (RP) effect size <2.00 and difference of proportions effect size <2.5.

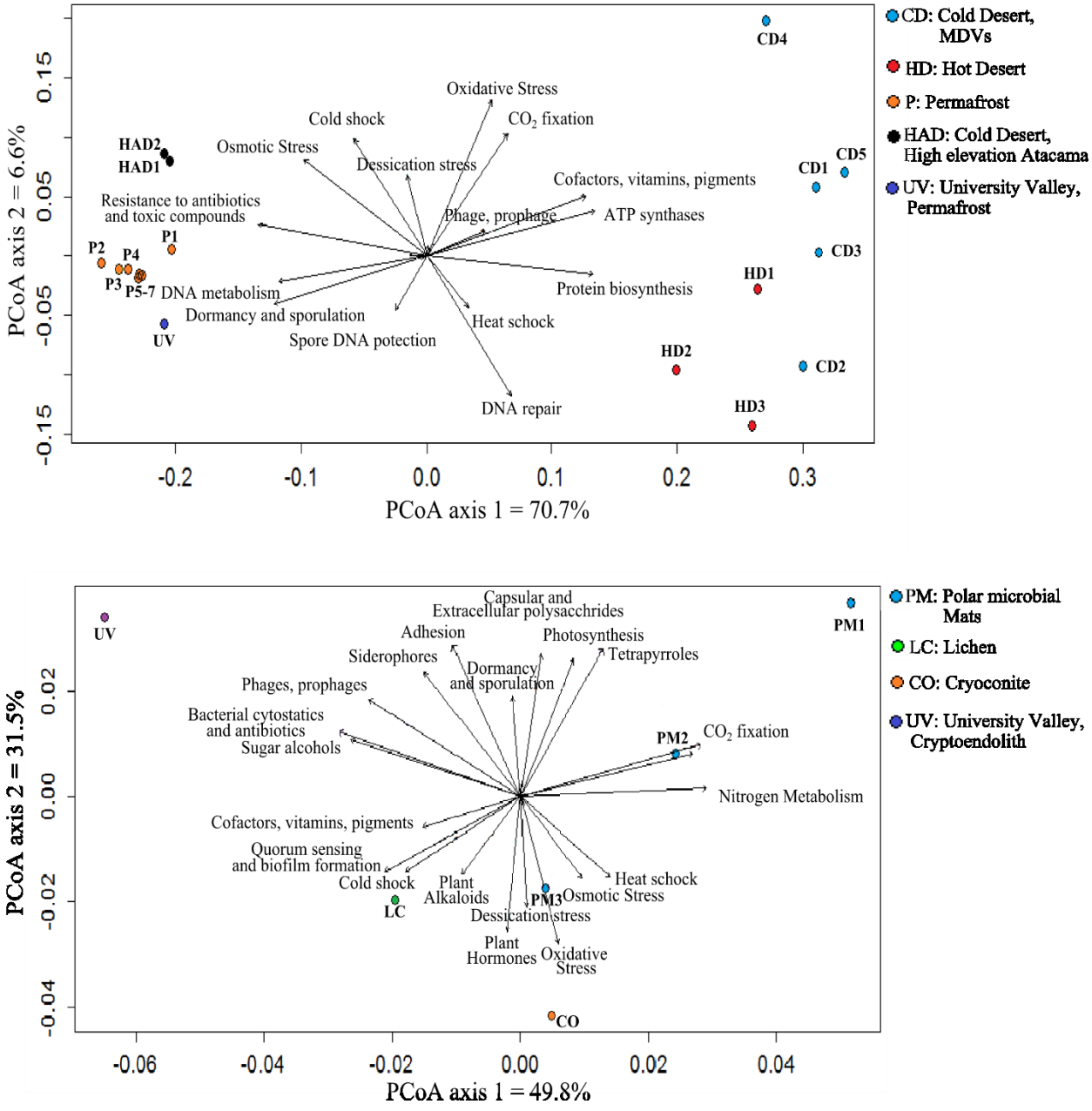

Figure 3.4. Ordination of functional community composition in University Valley

Principle coordinate analyses using Bray-Curtis distances of the relative abundance of level 2 SEED subsystems in the University Valley permafrost (Upper) and cryptoendolith (Lower) compared with other publically available metagenomes. Metagenomes used for comparison are outlines in Table S3. Arrows represent the relative abundance of level 2 subsystems.

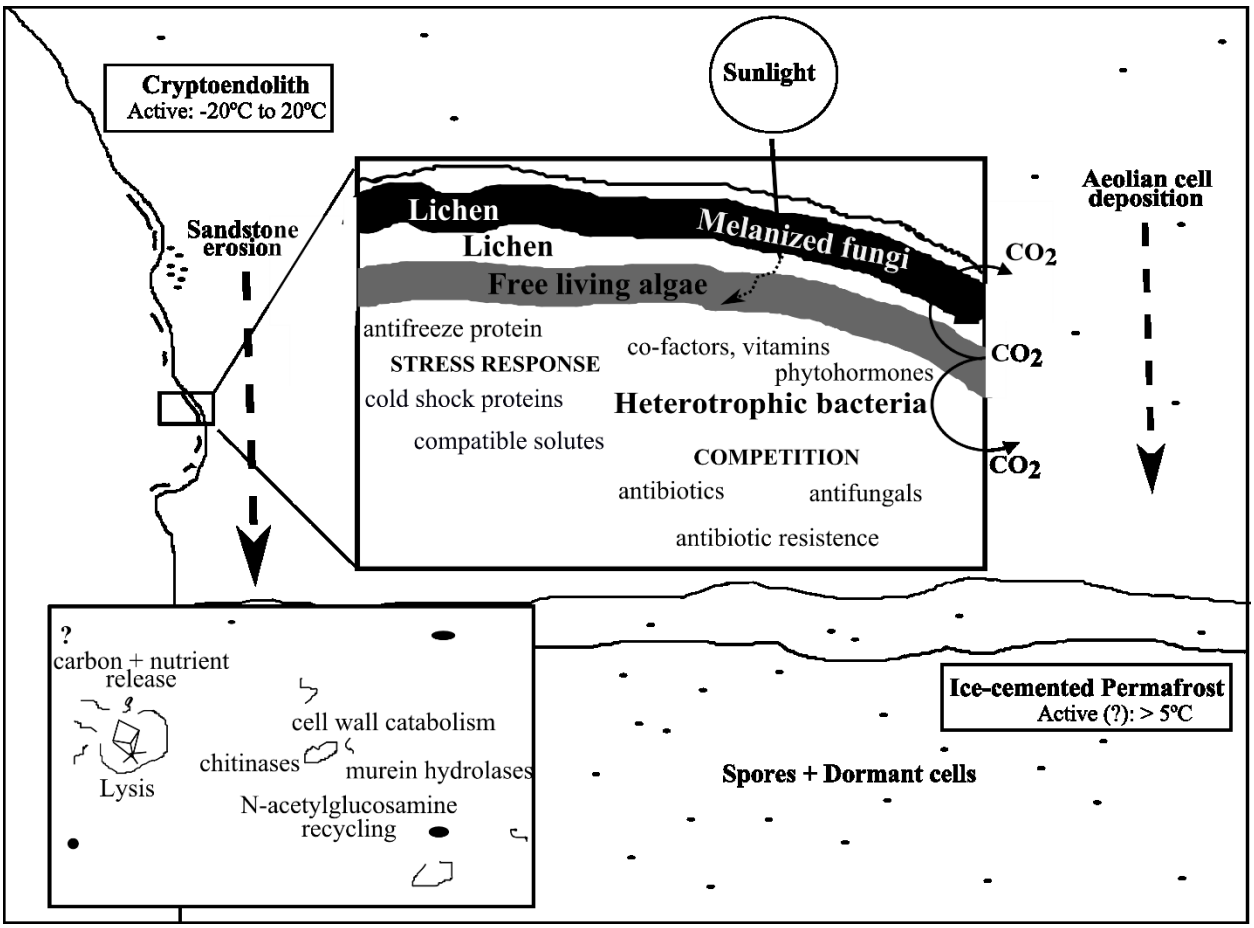

Figure 3.5. Predicted functions in University Valley permafrost and cryptoendolithic systems

Table 3.1. University Valley permafrost and cryptoendolith metagenome statistics

Parameter	Cryptoendolith Metagenome	Permafrost Metagenome
Total no. of sequences before QC	1,293,156	3,124,825
No. of sequences that passed QC	1,112,128	737,531
Total sequence size (bp) after QC	309,810,374 bp	211,084,258 bp
Av. sequence length (bp) after QC	278 bp	286 bp
No. of predicted/identified protein features	770,392/ 193,269	146,715/ 63,452
No. of predicted/identified rRNA features	7,444/ 441	41,691/ 242
No. of identified functional categories	128,195	42,837
GC content (%)	50 %	54 %

Table 3.2. Abundant phyla and classes in University Valley metagenomes

Domain	Phylum [class]	Percent (%) abundance cryptoendolith metagenome	Percent (%) abundance permafrost metagenome
Eukaryota		**53.8**	**12.2**
	Ascomycota	45.0	9.0
	[Eurotiomycetes]	24.1	2.8
	[Sordariomycetes]	10.0	1.7
	[Dothideomycetes]	5.2	3.9
	[Leotiomycetes]	4.1	0.3
	Chlorophyta	3.4	<0.1
	[Trebouxiophyceae]	1.4	0
	[Chlorophyceae]	1.2	<0.1
	Basidiomycota	2.2	0.5
	[Tremellomycetes]	1.7	<0.1
	Streptophyta	2.0	0.1
Bacteria		**45.0**	**85.6**
	Actinobacteria	19.8	63.7
	(order) *Actinomycetales*	17.4	63.2
	Proteobacteria	10.3	13.6
	[Alphaproteobacteria]	4.6	5.2
	[Gammaproteobacteria]	2.0	2.8
	[Deltaproteobacteria]	1.9	0.4
	[Betaproteobacteria]	1.6	5.0
	Bacteroidetes	3.9	1.6
	[Sphingobacteria]	1.3	0.4
	[Cytophagia]	1.2	0.2
	Firmicutes	2.2	5.9
	[Clostridia]	1.2	0.8
	[Bacilli]	1.0	5.0
	Chloroflexi	2.0	0.0
	Acidobacteria	1.9	0.1
	Cyanobacteria	1.7	0.3
	(order) *Chroococcales*	0.8	0.2
	(order) *Nostocales*	0.4	<0.1
	(order) *Oscillatoriales*	0.2	<0.1
	(order) *Gloeobacterales*	0.2	<0.1
Viruses		**0.5**	**2.0**
Archaea		**0.4**	**<0.1**

Only Phyla which represent >1% of total reads are presented here.

Table 3.3. Key Nutrient Cycling gene(s)

	Pathway	Key gene(s)	Cryptoendolith No. of reads	Permafrost No. of reads
Carbon-Autotrophy	**Calvin-Benson Cycle**	ribulose-1,5-bisphosphate carboxylase/oxygenase phosphoribulokinase	127 14	0 0
	Reductive TCA Cycle	2-oxoglutarate:ferredoxin oxidoreductase ATP citrate lyase (aclB)	0 7	0 0
	Reductive acetyl-coA pathway	CO dehydrogenase/acetyl-CoA synthase (CO-DH)	0	0
	Hydroxypropionate cycle	acetyl-CoA/propionyl-CoA carboxylase (pcc) malonyl coA reductase	0 0	0 0
Carbon	**Methane Oxidation**	Methane monooxygenase (mmoX) Particulate methane monoxygenase (pmoA)	13 0	0 0
	Methanogenesis	Methyl coenzyme M reductase (mcrA)	0	0
	Acetogenesis	Formyltetrahydrofolate synthetase (FTHFS)	0	1
	Carbon monoxide	CO dehydrogenase/acetyl-CoA synthase CO-DH	0	0
	Glyoxalate pathway	isocitrate lyase malate synthase	29 63	326 78
Nitrogen	**Nitrogen Fixation**	Nitrogenase Reductase (nifH)	*0*	0
	Nitrification	Ammonia monooxygenase (amoA)	3	0
	Denitrification	Nitrate Reductase (narG, nasA, napA) Nitrite reductase (nirK, nirS, nirA, nirB, nrfA) Nitric Oxide reductase (norB, norVW) Nitrous Oxide reductase (NosZ)	66 62 0 0	62 56 0 0
	Mineralization	Glutamate dehydrogenase (gdh) Urea amidohydrolase (ureC)	120 1	89 0
Phosphorus	**Phosphate metabolism**	Alkaline Phosphatases (phoA and PhoX) Phosphate-specific transport (Pst operon)	81 103	3 861
	Phosphonate metabolism	Phosphonoacetaldehyde hydrolase (phnX)	0	1
	Polyphosphonate metabolism	(polyphosphatase kinase (ppK) Exopolyphosphatase (ppX)	0 27	0 7

No. of reads of key genes based on 60% protein identity, an e-value cut-off of e-5 and a minimum alignment length of 15 aas against the GenBank database

3.7 Supplementary Materials:

Supplementary Figures 1-3

Supplementary Tables 1-3

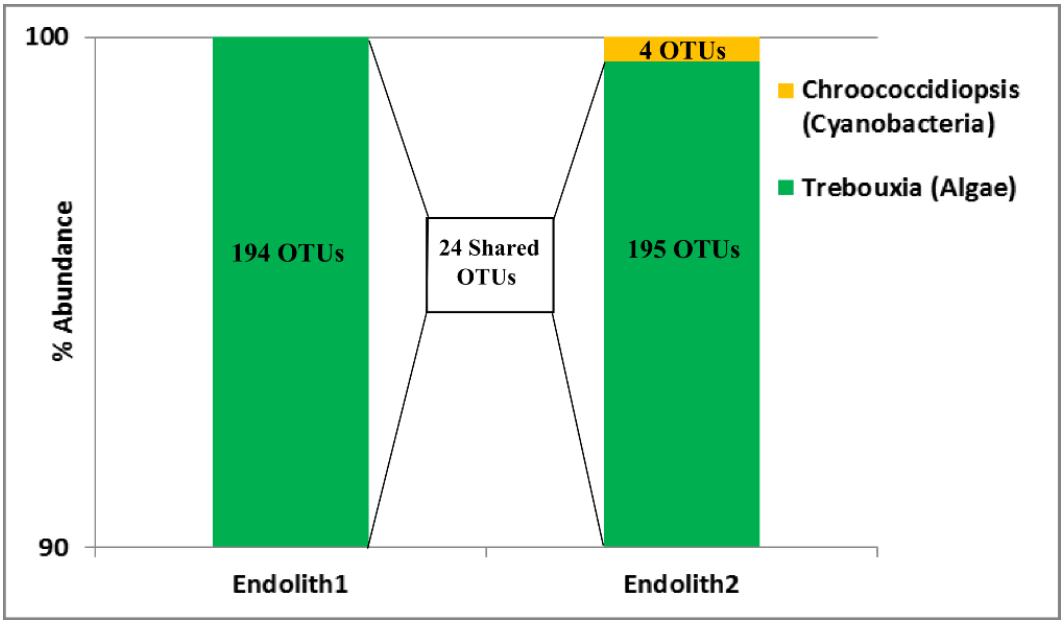

Figure S 3.1. Plastid 23s microbial community composition. OTUS defined t a 97% cut-off.

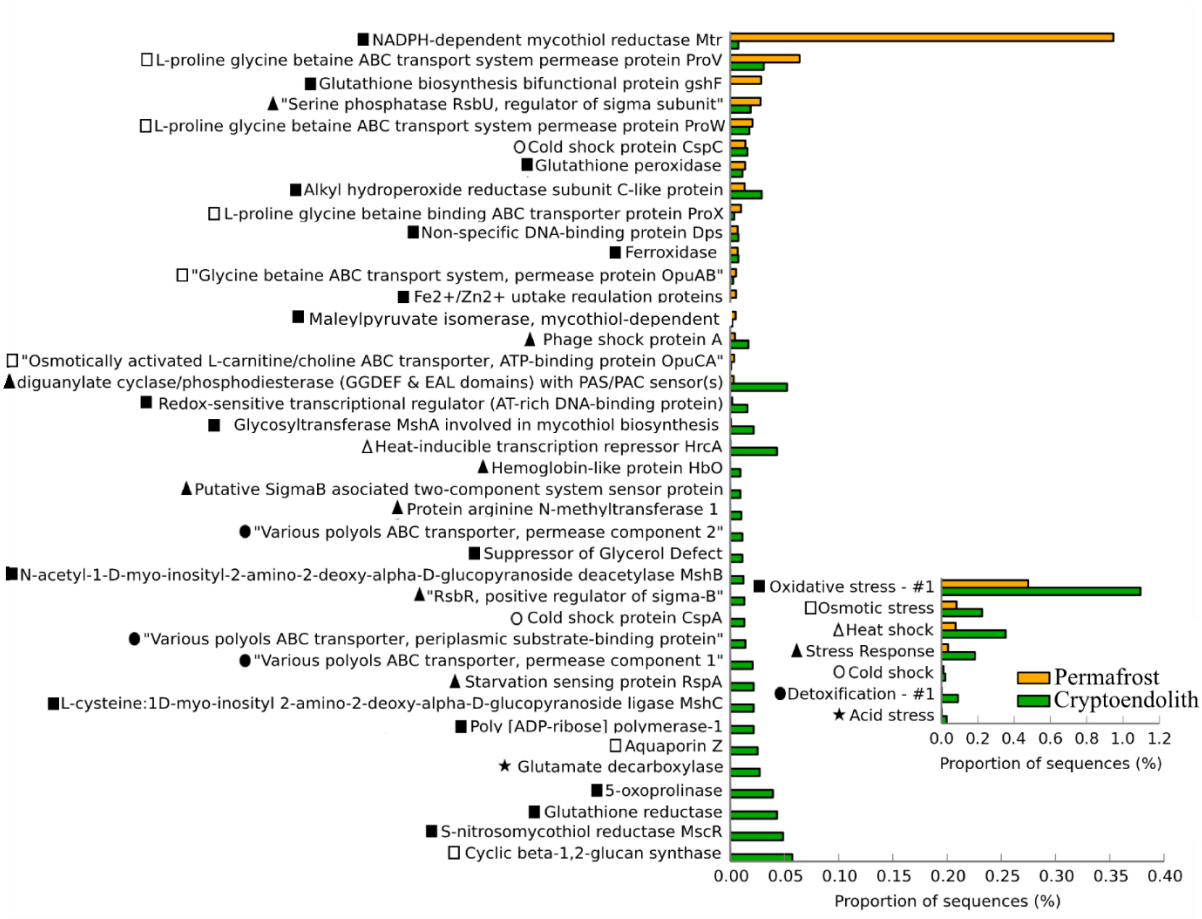

Figure S 3.2. Stress response genes in University Valley cryptoendolith and permafrost

Symbols indicate level 2 SEED subsystem stress response category. For ease of presentation, only functions which have a minimum of 10 reads in either sample are shown here.

Figure S 3.3. Heatmap of selected functions in permafrost metagenomes
1. Sporulation genes; 2. Genes involved in recombination and HGT; 3. Phage genes; 4. DNA repair genes; 5. Antibiotic production and resistance; 6. Osmotic stress response; 7. General and oxidative stress response; 8. Cold shock genes. P1-P7. Arctic permafrost metagenomes as outlined in Table S3, UV. University Valley permafrost metagenome.

Table S 3.1.Virus composition in Cryptoendolith and ice-cemented permafrost metagenome

Virus Order [Family]	Host(s)	Cryptoendolith Relative viral abundance % (number of reads)	Permafrost Relative viral abundance % (number of reads)
Caudovirales			
[Siphoviridae]	Bacteria, archaea	0.83 (23)	32.9 (4780)
[Podoviridae]	Bacteria	0.29 (8)	0.45 (66)
[Myoviridae]	Bacteria, archaea	0.61(17)	0.01(2)
[unclassified	n.a.	0.1 (3)	0.007 (1)
Caudovirales]			
Herpesvirales			
[Herpesviridae]	Vertebrates	0.1 (3)	0.03 (5)
[Alloherpesviridae]	Vertebrates	0.03 (1)	0 (0)
Unclassified order			
[Microviridae]	Bacteria	97.1 (2705)	66(9603)
[Retroviridae]	Vertebrates	0 (0)	0.46 (68)
[Poxviridae]	Arthropods, vertebrates	0.14 (4)	0(0)
[Mimiviridae]	Amoebae	0.14 (4)	0(0)
[Phycodnaviridae]	Algae	0.40 (11)	0.03 (5)
[Baculoviridae]	Invertebrates	0.07 (2)	0.07 (11)
[Ascoviridae]	Invertebrates	0.04(1)	0 (0)
[Potyviridae]	Plants	0 (0)	0.006 (1)
[unclassified family]	n.a.	0.07(2)	0(0)

Relative abundance as a total of viral sequences which total 0.5% in cryptoendolith and 2.0% in the permafrost metagenome. n.a- not applicable.

Table S 3.2. Algal Isolates from University Valley Cryptoendoliths

Strain ID	Isolation Media	Growth								Identification based on 18S rRNA sequence	Percent similarity to closest BLAST match	Environment of closest culturable BLAST Match (GenBank Accession)	GenBank accession no.
		35 ºC	22 ºC	10 ºC	5 ºC	0 ºC	-5 ºC	2% Glycerol	5% NaCl				
EN2JG	BG-11 CHU-10 SNAX	-	+*	+	+	+	+	-	-	*Stichococcus* sp.	100%	Siberian Permafrost, 4.65 meters (EU282451.1) Antarctic Peninsula surface soil (AY380557.1)	KR054371
UV2BC	CHU-10	-	-	+*	+	+	-	-	-	*Stichococcus* sp.	100%	Siberian Permafrost, 4.65 meters (EU282451.1) Antarctic Peninsula surface soil (AY380557.1)	KR054373
EN5JG	BG-11	-	-	+*	+	+	+	-	-	*Diplosphaera* sp.	99%	Chasmoendolith, Marie Bird Land, Antarctica (EU434026.1) Photobiont in lichen, Switzerland (JN573888.1)	KR054372

*Optimal growth of temperatures tested

Table S 3.3. Metagenomes used for comparison to University Valley metagenomes

Metagenome Accession number (database)	ID	Sample Environment	Location	Reference
4594282.3 (MG-RAST)	UV	Dry Valley ice-cemented permafrost	University Valley	This study
4477901.3 (MG-RAST)	CD1	Dry Valley surface soil	Lake Bonney, Antarctica	(Fierer et al. 2012)
4477904.3 (MG-RAST)	CD2	Dry Valley surface soil	Wright Valley, Antarctica	(Fierer et al. 2012)
4477902.3 (MG-RAST)	CD3	Dry Valley surface soil	Lake Frxyell, Antarctica	(Fierer et al. 2012)
4477803.3 (MG-RAST)	CD4	Dry Valley surface soil	Lake Bonney, Antarctica	(Fierer et al. 2012)
4477900.3 (MG-RAST)	CD5	Dry Valley surface soil	Garwood Valley, Antarctica	(Fierer et al. 2012)
4477805.3 (MG-RAST)	HD1	Hot Desert soil	Mojave Desert	(Fierer et al. 2012)
4477872.3 (MG-RAST)	HD2	Hot Desert soil	New Mexico desert	(Fierer et al. 2012)
4477873.3 (MG-RAST)	HD3	Hot Desert soil	New Mexico desert	(Fierer et al. 2012)
4522025.3 (MG-RAST)	HAD1	Cold Desert soil	High altitude Atacama soil	(Lynch et al. 2014)
4522026.3 (MG-RAST)	HAD2	Cold Desert soil	High altitude Atacama soil	(Lynch et al. 2014)
1618 (IMG)	P1	Arctic Permafrost	Alaska Permafrost	(Mackelprang et al. 2011)
4443232.3 (MG-RAST)	P2	Arctic Permafrost	Ellesmere Island permafrost	(Yergeau et al. 2010)
4443231.3 (MG-RAST)	P3	Arctic Permafrost	Ellesmere Island active layer	(Yergeau et al. 2010)
4481800.3 (MG-RAST)	P4	Arctic Permafrost	Axel Heiberg permafrost	(Chauhan et al. 2014)
4481801.3 (MG-RAST)	P5	Arctic Permafrost	Axel Heiberg permafrost	(Chauhan et al. 2014)
4481804.3 (MG-RAST)	P6	Arctic Permafrost	Axel Heiberg permafrost	(Chauhan et al. 2014)
4481805.3 (MG-RAST)	P7	Arctic Permafrost	Axel Heiberg permafrost	(Chauhan et al. 2014)
4594281.3 (MG-RAST)	UV_C	Dry Valley Cryptoendolith	University Valley	This study
4445845.3 (MG-RAST)	PM1	Polar Microbial Mat	McMurdo ice shelf, Antarctica	(Varin et al. 2012)
4445129.3 (MG-RAST)	PM2	Polar Microbial Mat	Ellesmere Island ice shelf	(Varin et al. 2012)
4445126.3 (MG-RAST)	PM3	Polar Microbial Mat	Ellesmere Island ice shelf	(Varin et al. 2012)
4491734.3 (MG-RAST)	CO	Cryoconite	Glacial Cryoconite	(Edwards et al. 2013)
4530091.3 (MG-RAST)	LC	Lichen	Lichen, Alps, Austria	(Grube et al. 2015)

Connecting Text for chapter 4 and 5:

Only one bacterium isolate from University Valley permafrost was found to grow at the subzero temperatures which would be relevant to what the bacterium would experience *in situ* (Chapter 2). In order to better understand the molecular traits which allowed for survival in the cold, arid and oligotrophic University Valley soils on long timescales, we sequenced the genome of *Rhodococcus* sp. JG3. Chapter 4 outlines the genome statistics of *Rhodococcus* sp. JG3. Chapter 5 is a comparative genomic analysis of the functional genes and amino acid substitutions which confer cold adaptation in *Rhodococcus* JG3 as compared with publically available mesophilic *Rhodococci* genomes.

Chapter 4. Improved-high-quality draft genome sequence of *Rhodococcus sp*. JG-3, a eurypsychrophilic *Actinobacteria* from Antarctic Dry Valley permafrost

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Contributions of authors: The overall development and writing of this manuscript was performed by J.G. Genomic analysis was carried out by J.G. Extractions of DNA for DNA sequencing was carried out by J.R. N.S., T.W., carried out sequencing of the strain. L.W, I.R., J.R and C.B provided critical editting. L.W and C.B are principle investigators for the genomic adaptations to cold project through which this strain was able to be sequenced.

This manuscript has been accepted for publication (accepted March 31st, 2015) in the journal Standards in Genomic Sciences and is currently in press: MS 1322273826142816

4.1 Abstract

The actinobacterium *Rhodococcus sp*. JG-3 is an aerobic, eurypsychrophilic, soil bacterium isolated from permafrost in the hyper arid Upper Dry Valleys of Antarctica. It is yellow pigmented, gram positive, moderately halotolerant and capable of growth from 30ºC down to at least -5ºC. The 5.28 Mb high-quality-draft genome is arranged into 6 scaffolds, containing 9 contigs and 4998 protein coding genes, with 64% GC content. Increasing the availability of genome sequences from cold-adapted species is crucial to gaining a better understanding of the molecular traits of cold adaptation in microbes.

4.2 Introduction

Actinobacteria is a ubiquitous phylum in the biosphere, including many environments that exist predominantly and perennially at sub-zero temperatures (cryoenvironments) such as massive ground ice, polar and alpine saline springs and lakes, cryopegs, and permafrost, where it is often a dominant phylum (Goordial et al. 2013). The molecular traits which allow *Actinobacteria* to predominate in cryoenvironments remains largely unknown. *Actinobacteria* may be protected in the permafrost environment by cyst-like resting forms or arthrospores, as observed in *Arthrobacter* and *Micrococcus* species isolated from permafrost (Soina et al. 2004). It is also possible that dominance of *Actinobacteria* are due to increased viability and activity in this phylum, as *Actinobacteria* that can metabolize at sub-zero temperatures have been found (Bottos et al. 2008; Tuorto et al. 2014). Though Antarctic permafrost has generally been found to harbor orders of magnitude lower culturable microorganisms (0-10^5 cells/g) than Arctic permafrost, *Rhodococcus* spp. have been readily isolated from both Antarctic and Arctic permafrost (Goordial and Whyte 2014). The genome sequence of *Rhodococcus* sp. JG-3 is also of interest since species within the genus *Rhodococcus* are known to have versatile degradative metabolisms for recalcitrant xenobiotics (Larkin et al. 2005), including the capability to degrade halogenated organics (Haeggblom and Salkinoja-Salonen 1991), short and long chain alkanes (Whyte et al. 1998), and petroleum hydrocarbons (Margesin et al. 2012). Several reports have investigated the catabolic potential of *Rhodococcus* spp. for contaminant removal at cold temperatures (Whyte et al. 1998; Whyte et al. 1999; Ruberto et al. 2005). The public availability of other mesophillic *Rhodococcus* genomes, in addition to other cryophilic bacterial isolates will enable identification of genes and molecular traits which enable cryophilic organisms like *Rhodococcus* sp. JG-3 to thrive in cold and extreme environments.

4.3 Organism information

Rhodococcus sp. JG-3 is a yellow pigmented strain capable of growth from 30ºC down to at least -5ºC. It does not require salt, but is moderately halotolerant up to 7% NaCl. It is a Gram positive short rod (Figure 1) that forms hyphae, and grows well on TSB and R2A media. *Rhodococcus* sp. JG-3 was isolated from University Valley, a small hanging valley (1650-1800 m.a.s.l) above Beacon Valley in the upper elevation McMurdo Dry Valleys, Antarctica. This bacterium was isolated from ice-cemented permafrost soils aged ca. 150,000 years old (Lacelle et al. 2013) which experience permanent darkness, hyper oligotrophy (0.013% total carbon), low water activity (<1% gravimetric soil moisture content) and constant cold temperature (mean annual soil temperature -24ºC). The classification and general features of *Rhodococcus* sp. JG-3 are summarized in Table 4.1.

The 16S rRNA gene sequence of *Rhodococcus* sp. JG-3 was compared using NCBI nucleotide BLAST (Altschul et al. 1990) against the nucleotide collection database (nr/nt) under default parameters, and excluding uncultured microorganisms. *Rhodococcus* sp. JG-3 showed 99 % similarity to that of *R. cercidiphylli* str. BZ22 (Li et al. 2008) (GenBank accession: HQ588861.1), a cold adapted isolate from an industrial site contaminated with heavy oil and heavy metals, and which has demonstrated low temperature degradation of petroleum hydrocarbons (Margesin et al. 2012), and 99% similarity to *Rhodococcus* sp. K4-07B (GenBank accession: EF612291) isolated from a semiarid lead-zinc mine tailing site (Mendez et al. 2008). Phylogenetic analysis based on the 16S rRNA gene of taxonomically classified type strains of the family *Nocardiaceae* placed *Rhodococcus fascians* DSM 20669 (Goodfellow 1984) as the closest validly named species to

Rhodococcus sp. JG-3 (Figure 2). *R. fascians* DSM 20669 was originally isolated from sweet peas and has an optimum growth temperature of 24 to 27 °C (Goodfellow 1984) .

4.4 Genome sequencing information

4.4.1 Genome project history

Rhodococcus sp. JG-3 was selected for sequencing in 2012 as part of a DOE Joint Genome Institute (JGI) Community Sequencing Program (Quarterly) project to sequence 12 cryophilic isolates from permafrost and cryoenvironments. The Improved Quality Draft assembly and annotation were completed on May 30, 2013. The complete genome sequence of strain JG-3 is available for public access in DDBJ/EMBL/GenBank under accession numbers AXVF01000001- AXVF01000009. The date of Release was December 12, 2013. Table 4.2 presents the main project information and its association with MIGS version 2.0 compliance (Field et al. 2008).

4.4.2 Growth conditions and DNA isolation

Rhodococcus JG-3 was grown to stationary phase on TSB medium at room temperature. Genomic DNA was isolated using the Epicentre MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, Wisconsin) as per the manufacturer's instructions. Purified DNA was evaluated with the NanoDrop 1000 (Thermoscientific, Wilmington, Delaware), according to the standards of the DOE Joint Genome Institute (Ling et al. 2015).

4.4.3 Genome sequencing and assembly

The draft genome of *Rhodococcus sp*. JG–3 was generated at the DOE Joint Genome Institute (JGI) using the Illumina technology. An Illumina std shotgun library and long insert mate pair library was constructed and sequenced using the Illumina HiSeq 2000 platform (Bennett 2004). 20,820,738 reads totaling 3,123.1 Mb were generated from the std shotgun sequence and 41,292,560 reads totaling 3,757.6 Mb were generated from the long insert mate pair library. All general aspects of library construction and sequencing performed at the JGI. All raw Illumina sequence data was passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artifacts. Filtered Illumina reads were assembled using AllpathsLG (PrepareAllpathsInputs: PHRED 64=1 PLOIDY=1 FRAG COVERAGE=75 JUMP COVERAGE=25; RunAllpathsLG: THREADS=8 RUN=std pairs TARGETS=standard VAPI WARN ONLY=True OVERWRITE=True)(Gnerre et al. 2011). The final draft assembly contained 9 contigs in 6 scaffolds. The total size of the genome is 5.3 Mb. The final assembly is based on 3,122.6 Mb of Illumina Std PE, 3,757.6 Mb of Illumina CLIP PE post filtered data, which provides an average 1298.1X Illumina coverage of the genome.

4.4.4 Genome annotation

Genes were identified using Prodigal (Hyatt et al. 2010), followed by a round of manual curation using GenePRIMP (Pati et al. 2010) for finished genomes and Draft genomes in fewer than 10 scaffolds. The predicted CDSs were translated and used to search the National Center for Biotechnology Information (NCBI) nonredundant database, UniProt, TIGRFam, Pfam, KEGG,

COG, and InterPro databases. The tRNAScanSE tool (Lowe and Eddy 1997) was used to find tRNA genes, whereas ribosomal RNA genes were found by searches against models of the ribosomal RNA genes built from SILVA (Pruesse et al. 2007). Other non–coding RNAs such as the RNA components of the protein secretion complex and the RNase P were identified by searching the genome for the corresponding Rfam profiles using INFERNAL. Additional gene prediction analysis and manual functional annotation was performed within the Integrated Microbial Genomes (IMG) platform developed by the Joint Genome Institute, Walnut Creek, CA, USA (Markowitz et al. 2009).

4.4.5 Genome properties

The improved high quality draft genome includes 9 contigs in 6 scaffolds, for a total size of 5286918 bp, 64.41% GC content. Most of the genome (96%, 5092715 bp) assembled into one scaffold. For the genome, 5067 genes were predicted, 4998 of which are protein-coding genes; 3977 protein coding genes were assigned to a putative function with the remaining annotated as hypothetical proteins. The properties and statistics of the genome are summarized in Tables 3 and 4.

4.5 Conclusion

The genome sequence of *Rhodococcus* sp. JG-3 will be used for examination of the molecular traits of cold adaptation and to aid understanding of carbon metabolism in cryoenvironments. This is the first reported genome of a bacterium isolated from the Upper Dry Valley permafrost and will provide insight into how microbes survive such extreme conditions. As the availability

of genomes from cryophilic strains increases, it may be possible to infer if there is a phylogenetic basis for some cold adaptive traits, as well as identify novel molecular mechanisms for cold adaptation.

4.6 Acknowledgements

This work was supported by NASA's ASTEP program and with field support via NSF/OPP (project B-302-M). Support was provided by the Natural Sciences and Engineering Research Council (NSERC) Discovery Grant Program, NSERC Northern Supplements Program, and NSERC CREATE Canadian Astrobiology Training Program (CATP). The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Additional thanks to Marcel Huntemann, James Han, Amy Chen, Nikos Kyrpides, Victor Markowitz, Krishna Palaniappan, Natalia Ivanova, Natalia Mikhailova, Galina Ovchinnikova, Andrew Schaumberg, Amrita Pati, Dimitrios Stamatis, Tatiparthi Reddy, Henrik P. Nordberg, Michael N. Cantor, and Susan X. Hua of JGI.

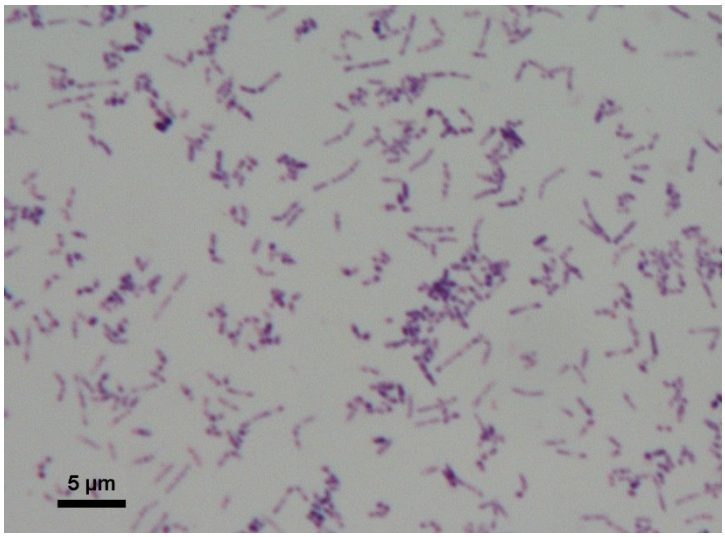

Figure 4.1. Gram stain Rhodococcus sp. JG-3

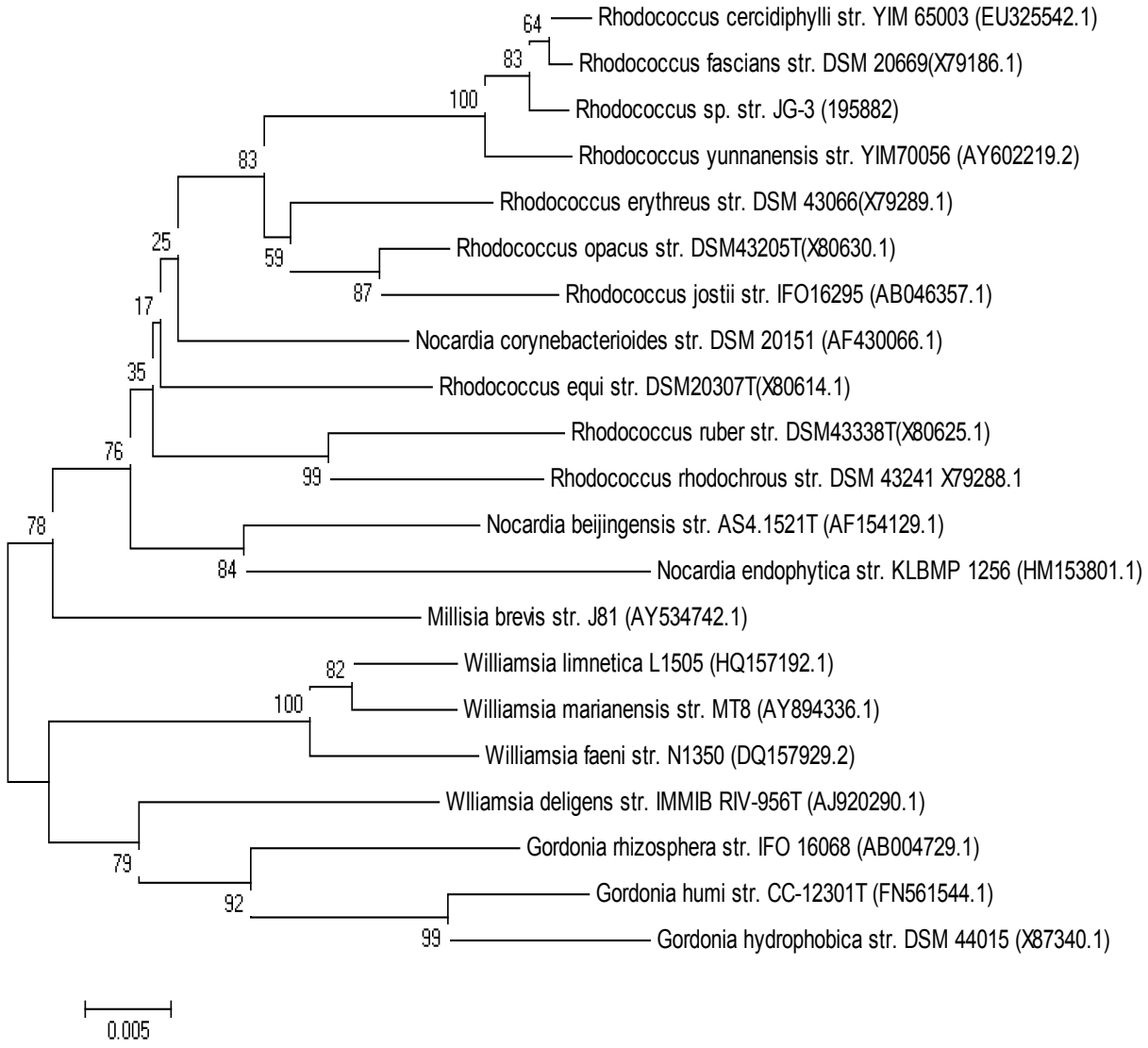

Figure 4.2. Phylogenetic tree highlighting the position of *Rhodococcus* sp. JG-3

Relative to selected taxonomically classified strains within the genus *Rhodococcus* and within the family *Nocardiaceae*. Phylogenetic inferences were obtained using the neighbor-joining method within MEGA6.05 (Tamura et al. 2013). Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1,000 times to generate a tree using the maximum composite likelihood model. The GenBank accession numbers for the 16S rRNA gene are in parentheses.

Table 4.1. Classification and general features of *Rhodococcus sp. JG-3*

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain Bacteria	TAS (Skerman et al. 1980)
		Phylum Actinobacteria	TAS (Stackebrandt et al. 1997)
		Class Actinobacteria	TAS (Stackebrandt et al. 1997)
		Order Actinomycetales	TAS (Skerman et al. 1980)
		Family Nocardiaceae	TAS (Castellani and Chalmers 1919)
		Genus Rhodococcus	TAS (Skerman et al. 1980)
	Gram stain	positive	IDA
	Cell shape	Rod	IDA
	Motility	Not reported	IDA
	Sporulation	Not reported	NAS
	Temperature range	<-5ºC to 30ºC	NAS
	Optimum temperature	~20ºC	IDA
	pH range; Optimum	7	IDA
	Carbon source	R2A, TSA complex media	IDA
MIGS-6	Habitat	Terrestrial, permafrost soil	IDA
MIGS-6.3	Salinity	0-7% NaCl	IDA
MIGS-22	Oxygen requirement	aerobic	IDA
MIGS-15	Biotic relationship	free-living	IDA
MIGS-14	Pathogenicity	Non-pathogen	NAS
MIGS-4	Geographic location	University Valley, Dry Valleys, Antarctica	IDA
MIGS-5	Sample collection	December, 2009	IDA
MIGS-4.1	Latitude	77d 51.817s S	IDA
MIGS-4.2	Longitude	160d43.524s E	IDA
MIGS-4.4	Altitude	37-42 cm below soil surface, in ice-cemented permafrost	IDA

a) Evidence codes - IDA: Inferred from Direct Assay, TAS: traceable author statement (i.e., a direct report exists in the literature), NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are derived from the Gene Ontology project (Ashburner et al. 2000).

Table 4.2. Project information

MIGS ID	Property	Term
MIGS 31	Finishing quality	Improved-high-quality draft
MIGS-28	Libraries used	Illumina Std. PE, Illumina Clip PE
MIGS 29	Sequencing platforms	Illumina HiSeq 2000
MIGS 31.2	Fold coverage	1298.1X Ilumina coverage
MIGS 30	Assemblers	AllpathsLG
MIGS 32	Gene calling method	Prodigal, GenePrimp
	Locus Tag	K414
	Genbank ID	AXVF01000000
	GenBank Date of Release	December 12, 2013
	GOLD ID	Gi22490
	BIOPROJECT	PRJNA195882
MIGS 13	Source Material Identifier	ARS culture collection, NRRL: B-65292
	Project relevance	Permafrost, adaptation to cold, carbon metabolism

Table 4.3. Nucleotide content and gene count levels of the genome

Attribute	Value	% of Total
Genome size (bp)	5286918	100.00
DNA coding (bp)	4884848	92.40
DNA G+C (bp)	3405333	64.41
DNA scaffolds	6	100
Total genes	5067	100.00
Protein coding genes	4998	98.64
RNA genes	69	1.36
Pseudo genes	60	1.18
Genes in internal clusters	NA	
Genes with function prediction	3977	24.18
Genes assigned to COGs	3805	75.09
Genes with Pfam domains	4134	81.59
Genes with signal peptides	370	7.30
Genes with transmembrane helices	1192	23.52
CRISPR repeats	1	-

Table 4.4. Number of genes associated with general COG functional categories.

Code	Value	%age	Description
J	176	4.17	Translation, ribosomal structure and biogenesis
A	1	0.02	RNA processing and modification
K	443	10.50	Transcription
L	173	4.10	Replication, recombination and repair
B	1	0.02	Chromatin structure and dynamics
D	31	0.76	Cell cycle control, Cell division, chromosome partitioning
V	53	1.26	Defense mechanisms
T	213	5.05	Signal transduction mechanisms
M	179	4.24	Cell wall/membrane biogenesis
N	6	0.14	Cell motility
U	43	1.02	Intracellular trafficking and secretion
O	133	3.15	Posttranslational modification, protein turnover, chaperones
C	275	6.52	Energy production and conversion
G	288	6.83	Carbohydrate transport and metabolism
E	380	9.01	Amino acid transport and metabolism
F	97	2.3	Nucleotide transport and metabolism
H	184	4.36	Coenzyme transport and metabolism
I	233	5.52	Lipid transport and metabolism
P	244	5.78	Inorganic ion transport and metabolism
Q	166	3.93	Secondary metabolites biosynthesis, transport and catabolism
R	566	13.42	General function prediction only
S	333	7.89	Function unknown
-	1262	24.91	Not in COGs

The total is based on the total number of protein coding genes in the genome.

Chapter 5. Cold adaptive traits revealed by comparative genomic analysis of eurypsychrophile Rhodococcus sp. JG3 isolated from high elevation McMurdo Dry Valley permafrost, Antarctica

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Contributions of authors: The overall development and writing of this manuscript was performed by J.G. Genomic comparisons was primarily carried out by J.G, with significant contributions by I.R.B. Extractions of DNA for DNA sequencing was carried out by J.R. N.S., T.W., carried out sequencing of the strain. Y.Z, L.D.B. and M. S constructed python scripts for cold adaptation analysis. L.W, I.R., J.R and C.B provided critical editting. L.W and C.B are principle investigators for the genomic adaptations to cold project through which this strain was able to be sequenced.

5.2 Abstract

The permafrost soils of the high elevation McMurdo Dry Valleys are coldest, most desiccating and oligotrophic on Earth. *Rhodococcus* sp. JG3 is one of very few bacterial isolates from Antarctic Dry Valley permafrost, and displays subzero growth down to −5°C. To understand how *Rhodococcus* sp. JG3 was able to survive in extreme permafrost conditions for ~150,000 years we sequenced its genome and compared it to the genomes of 14 mesophilic *Rhodococci*. We discuss the genome wide amino acid substitutions and genomic content which enables *Rhodococcus* sp. JG3 to be metabolically active at subzero temperatures, and possibly be active within some of the most inhospitable permafrost soils on Earth.

5.3 Introduction

As much as 80% of the Earths biosphere is permanently cold, including polar and alpine regions, the world's oceans, and the atmosphere (De Maayer et al. 2014). Within these cryoenvironments, cold adapted microorganisms from diverse genera can be found living at the low temperature limits of life. Though these extreme conditions are lethal for most cells, microorganisms have been found which can grow at temperatures as low as −15°C (Mykytczuk et al. 2013), metabolize down to −32°C (Bakermans and Skidmore 2011b), and there is an increasing body of evidence indicating cold-adapted microorganism are active *in situ* (Goordial et al. 2013).

Relatively few sequenced genomes from cold-adapted microorganisms are available publically, and even fewer from organisms which are capable of subzero growth (cryophiles) (Bakermans et al. 2012). Though permafrost regions cover 27 % of the Earth (Goordial et al. 2013), to date few publications analyzing the genomes of subzero growing permafrost bacteria are available, including *Planococcus halocryophilus* OR1 (Mykytczuk et al. 2013), *Psychrobacter arcticus* 273-4 (Bergholz et al. 2009; Ayala-del-Río et al. 2010a), *and Exiguobacterium sibiricum* (Rodrigues et al. 2008). The genomic mechanisms for cold-adapted metabolism below subzero temperatures remain poorly understood, though the environmental pressures associated with freezing are significantly harsher. In addition to decreased enzymatic reaction rates due to kinetics, cryophiles must be adapted to dessication, and increased osmotic and oxidative stress. Increasing availability of whole genome sequences of isolates capable of subzero growth will provide insight into how cryophiles mitigate the multiple stressors of cryoenvironments, allow metabolism at subzero temperatures, and give insight into the ecological roles of these isolates.

General adaptations in cold-adapted microorganisms include possession of proteins required to maintain DNA replication, transcription, translation and membrane fluidity at low temperature, as well as proteins which mitigate osmotic, oxidative and cold stress (Goordial et al. 2013). Cold adapted organisms often increase copy number of these proteins in order to counteract the lowered enzymatic reaction rates which occur at cold temperatures by increasing the number of active sites (Bakermans et al. 2012). Additionally, molecular adaptations at the amino acid level can allow proteins to maintain flexibility to preserve function at low temperatures. Some of the general features found in psychrophilic proteins believed to impart increased flexibility include fewer salt bridges, a lower content of proline residues, fewer hydrogen bonds, a reduced Arg/(Arg+Lys) ratio, and extended and highly charged surface loops (Aghajari et al. 1998; Russell et al. 1998; Georlette et al. 2000; Huston et al. 2004; Bakermans et al. 2012).

The coldest and driest permafrost on Earth occur in the stable upland zone (SUZ) of the Antarctic McMurdo Dry Valleys. This permafrost is unlike any other permafrost examined to date in that microbial activity is undetectable *in situ* or in microcosm assays, and permafrost soils can contain negligible microbial biomass (10^3 cells g^{-1}) and culturable organisms ($0–10^1$ CFU g^{-1}) (Goordial 2015a). University Valley is a small hanging Dry Valley (1650-1800 m.a.s.l.) located in the SUZ, and is among the most extreme environments studied to date with a mean temperature of −23°C and no days above freezing annually. Isotopic data indicates that water-ice present in permafrost in University Valley is vapour deposited, and has not been deposited by liquid water for at least the past ~100,000 years (Lacelle et al. 2013). The last period of liquid water deposition of ice corresponds with the Eemian interglacial period which is the warmest the area has seen in the past 200,000 years (Lacelle et al. 2013) . Instead, ice in the ice-table exchanges with the atmosphere via water vapour diffusion, rather than liquid water (Mckay 2009). After considerable

efforts to culture microorganisms from University Valley soils, only six isolates were obtained; two isolates, a *Rhodotorula sp.* fungus and a *Rhodococcus* sp. bacterium demonstrated subzero growth (Goordial 2015a). *Rhodococcus* sp. JG3 was isolated from University Valley permafrost soils aged approximately 150,000 years old (Lacelle et al. 2013) which experience permanent darkness, hyper oligotrophy (0.013% total carbon), low water activity (<1% gravimetric soil moisture content) and constant cold temperature (mean annual soil temperature -24ºC) (Goordial 2015a).

Given the extreme conditions in University Valley permafrost soils, and the presumed age of deposition of *R. sp.* JG3, the objective of this study was to identify the cold adaptive traits which confer *R. sp.* JG3 with the ability to survive the extremely arid, cold, and oligotrophic conditions of University Valley permafrost for ~150,000 years. We characterized subzero growth, respiration, and genomic content associated with cold and stress adaptation. *R.* sp. JG3 is the first Dry Valley permafrost isolate to have its genome sequenced; we used a comparative genomic approach with the *R. sp.* JG3 genome and 14 mesophillic *Rhodococcus* genomes to reveal the genomic adaptations at the amino acid level which could allow subzero growth and metabolism within the permafrost environment in some of the most inhospitable permafrost soils on Earth.

5.4 Results and Discussion

General growth characteristics and activity of Rhodococcus sp. JG3

*R. sp.*JG3 grew well on TSA and R2A media, was moderately halotolerant up to 7% NaCl, and grew optimally in the absence of additional NaCl. It is Gram positive, and rod shaped. *R.* sp.

JG3 can be characterized as a eurypsychrophile, capable of growth from 30ºC down to -5ºC, with an optimal growth temperature of 20 ºC determined by optical density measurements (Figure 5.1); no growth was observed at -10 ºC after 200 days. At -5ºC, generation time was 14 days in *R. sp.* JG3, consistent with other characterized psychrophiles such as *Psychromonas ingrahamii* which has a generation time of 10 days at -12ºC (Breezee et al. 2004), and *Psychrobacter arcticus* and *Planococcus halocryophilus* which have a doubling times of ~40 days at -10ºC (Bakermans et al. 2003; Mykytczuk et al. 2013). Although growth below -5ºC was not observed, microbial mineralization of ^{14}C-labelled acetate in permafrost microcosms was significantly higher than background levels at -10ºC and -15ºC, the coldest temperatures tested here (Figure 5.2). The activity observed well below -5ºC likely reflects metabolism used for maintenance of cells rather than growth (Price and Sowers 2004). Similar mineralization rates were seen at temperatures of 20ºC and 5ºC, demonstrating that R. sp. JG3 is adapted to a broad temperature range (Figure 5.2).

Comparative Genomic Analyses with mesophilic Rhodococci

General overview

The *R. sp.* JG3 isolate was sequenced, annotated and compared with other publically available *Rhodococci* genomes available from the Integrated Microbial Genomes (IMG) system and which were annotated as mesophilic isolates. The high-quality-draft genome of R. sp. JG3 was 5.28 Mb, with 5067 predicted genes, of which 3977 were protein coding genes that were assigned a putative function, and the remaining were annotated as hypothetical proteins. The basic genome statistics for the 14 mesophillic strains used for comparison to *R. sp.* JG3 are found in Table 5.1; all strains varied in genome size (5.0-9.7 Mb) and number of predicted genes (4570-9242). *R. sp.*

JG3 did not have a smaller genome, or fewer rRNA genes than the compared mesophilic strains, a possible adaptation to cold which is observed in *Paenibacillus darwinius* strains from the Antarctic when compared to temperate *Paenibacillus* strains (Dsouza et al. 2014). To determine inter- and intra- genomic similarity we carried out an all-against-all BLAST comparison with the coding sequences (CDSs) from all the *Rhodococcus* genomes (Figure S5.1) using CMG biotools (Vesth et al. 2013). Few CDSs were in paralogous groups in *R. sp.* JG3 (3.7%) when compared to the related mesophilic strains, possibly reflective of streamlined genomic content as an adaptation to extreme environments, as observed in other Antarctic isolates (Dsouza et al. 2014). The pan genome (all gene families) and core genome (only gene families shared by all 15 *Rhodococcus* genomes) consisted of 23122 and 1819 gene families respectively. For all *Rhodococcus* genomes approximately 60-70% genes could be assigned to a clusters of orthologous group (COG) category. When all genomes were normalized for number of genes, *R. sp.* JG3 had more genes involved in amino acid transport and metabolism, and cell wall/membrane envelope biogenesis (Figure S5.2). Genes in these COG categories were found to have increased expression during growth at -15ºC in the eurypsychrophile *Planoccocus halocryophilus,* likely due to the relative importance of cell wall/membrane alteration in response to subzero temperatures.

Cold adaptive and stress response genes in Rhodococcus sp. JG3

The *R. sp.* JG3 genome contains CDSs for proteins known to be associated with adaptation and response to low temperatures including genes for osmotic stress response, cold shock proteins, oxidative stress, and cell wall alteration (Table 5.2). While the presence of many such CDSs were found in all *Rhodococcus* strains, when we compared copy number of these CDSs with the 5 mesophillic *Rhodococcus* strains with similar genome size to *R. sp.* JG3 (Table 5.1), we found that *R. sp.* JG3 had a higher copy number for many of these genes compared to the mesophilic strains

(Table 5.3). For example, the ability to cope with osmotic stress, mediated through compatible solutes like glycine betaine, choline and sarcosine, was relatively important in *R. sp.* JG3. This is similar to what is observed in *P. halocryophillus*, which also has a large number of glycine betaine related genes, although this is not a general trait shared with all cryophilic bacteria (Mykytczuk et al. 2013). Water soluble organic compounds, such as glycine betaine, choline, and trehalose are important compatible solutes used by cryophiles to resist ice formation and osmotic pressure caused by increases in salinity in subzero conditions (Mader et al. 2006; Chin et al. 2010). In addition to their role as osmolytes, compatible solutes play a role in increasing enzyme activity and stability (Thomas et al. 2001). Cold shock proteins, which can bind to DNA/RNA, and are involved with regulation of transcription and translation at low temperatures are found in the *R. sp.* JG3 genome, as well as numerous protein chaperones including *dnaJ, dnaK, clpB,* and *grpE*, important for proper folding and maintaining protein functionality. The number of cold shock proteins was similar among the *Rhodococci* analysed here. Maintaining membrane fluidity through increasing disorder is essential for cell viability at low temperatures; to overcome decreased membrane fluidity, the *R. sp.* JG3 genome encodes for proteins which regulate the proportion of unsaturated fatty acids (fatty acid desaturase), and for fatty acid synthesis of straight and branched chain fatty acids (KAS-II, KAS-III). *R. sp.* JG3 also encoded for the carotenoid phytoene synthase and desaturase; carotenoid production has been linked with fine-tuning membrane fluidity at low temperatures (Chattopadhyay and Jagannadham 2001) and has been associated with increased resistance to freeze-thaw cycles in bacteria isolated from the Antarctic (Dieser et al. 2010). To cope with reactive oxygen species *R. sp.* JG3 is equipped with superoxide dismutase and several catalases, as well as peroxiredoxins which *R. sp.* JG3 was relatively enriched in. Interestingly, *R. sp.* JG3 had a higher copy number of proteins related to photolyase and photorepair protein *PhrA*,

proteins involved in UV radiation DNA repair. It is unknown what role these proteins have in *R. sp.* JG3 since it is assumed that the isolate has been in the dark subsurface permafrost environment since the soil was deposited ~150,000 years ago, and the photolyase enzyme requires visible light to function (Sancar 1994). Resistance to radiation has been closely linked to desiccation resistance (Mattimore and Battista 1996), and could be useful in the low water activity soils of University Valley. It is also possible that this strain was originally cryptoendolithic in origin, where UV resistance would have had an important ecological role. *Rhodoccoci* were detected in University Valley cryptoendoliths colonizing the sandstone walls which erode over time and form the valley floor (Goordial 2015a).

Carbon cycling in Rhodococcus sp. JG3

In order to survive in the hyper-oligotrophic conditions of University Valley permafrost, *R. sp.* JG3 must have the metabolic capacity to acquire or store carbon. *R. sp.* JG3 possesses the genetic capacity to store energy in storage molecules like triacylglycerols (TAGs) to aid survival. The CDSs associated with TAG biosynthesis included fatty acid synthase, glycerol-3-phosphate O-acyltransferase, 1-acylglycerol-3-phosphate O-acyltransferase, and diacylglycerol acyltransferase, an enzyme unique to TAG biosynthesis (Alvarez et al. 2013). TAGs are the main storage molecule accumulated by *Rhodococci* and are known to serve as carbon and energy reserves in *Rhodococcus* species under starvation conditions (Alvarez et al. 2000; Alvarez et al. 2013), though other functions for TAG metabolism may include regulation of membrane fluidity (Alvarez and Steinbüchel 2002). In addition to lipids, another storage reserve compound which can accumulate in *Rhodococci* is glycogen (Hernández et al. 2008); *R. sp.* JG3 possessed CDSs for glycogen metabolism, including glycogen debranching enzyme (Table 5.2).

R. sp. JG3 displays aerobic heterotrophic growth, and the genome encodes complete glycolysis, TCA, and pentose phosphate cycle pathways. Additionally, the *R. sp.* JG3 genome also had 3 operons encoding the subunits of carbon monoxide dehydrogenase (CODH). CODH interconverts CO and CO_2, and allows assimilation of CO or CO_2 into biomass. In *R. jostii* RHA1, a strain highly resistant to nutrient starvation, CODH expression and enzymatic activity was induced in response to carbon starvation (Patrauchan et al. 2012). Aerobic carbon monoxide utilization as a sole carbon and energy source has been reported in carboxydotrophic, and extremely oligotrophic *R. erythropolis* N9T-4, an isolate from crude oil (Yano et al. 2012). As an additional oligotrophic adaptation, *R. erythropolis* N9T-4 utilizes a glyoxylate shunt to avoid the CO_2 releasing steps of the TCA cycle (Yano et al. 2015). *R. sp. JG3* also contains the proteins required for the glyoxylate pathway (isocitrate lyase, malate synthase), however measurable respired CO_2 from the ^{14}C mineralization assays (Figure 5.2) indicate that this strain does not utilize this pathway when using acetate as a sole carbon source. The ability to store carbon and energy reserves, as well as assimilate CO/CO_2 into biomass could explain the resilience of *R. sp.* JG3 in the oligotrophic permafrost environment.

Genome wide comparisons: Adaptations at the amino acid level

Cold adaptation can be the result of genome-wide changes, which may only be revealed at the genome level rather than at the level of the individual gene (Bakermans et al. 2012). Genome wide adaptation through amino acid substitutions was identified by comparing the genome of *R. sp.* JG3 against the 14 mesophillic *Rhodococcus* genomes for substitution patterns known to be associated with cold adaptation. Significantly more ‘cold adapted’ proteins compared to ‘hot

adapted' proteins were found in *R. sp.* JG3 for 4 of the 6 indices examined (Bonferonni corrected P< 0.0001), as identified by lower amounts of proline, acidic and aliphatic residues, and a lower arginine to lysine ratio (Figure 5.3). These changes increase flexibility in proteins by lowering the number of potential salt bridges and hydrogen bonds as well as reducing rigid kinks caused by proline residues (D'Amico et al. 2006; Doyle et al. 2012). The grand average of hydropathicity (GRAVY) index, a measure of hydrophobicity, was found not to reflect cold adaptation in *R.* sp. *JG3*, and instead was significantly 'hot adapted'. Overall, for most indices the majority of proteins across the *R.* sp. *JG3* genome were found to be 'neutral' (not significantly different than the average of the mesophilic homologs) (Figure 5.3). The presence of 'hot', 'cold' and 'neutral' genes in *R.* sp. JG3 is reflective of the broad growth temperature range of *R.* sp. JG3 and is also observed in *P. halocryophilus*, a permafrost bacterium which can grow at subzero and warm temperatures (−5°C to 37°C) (Mykytczuk et al. 2013).

To identify CDSs which may be cold adapted, we assigned each protein in *R. sp.* JG3 a cold adaptation score based on the sum of the 6 indices (see methods). The majority of proteins in *R. sp.* JG3 were not found to be significantly different than its mesophilic relatives when all 6 indices were taken into account (3502 'neutral' proteins); 192 proteins were found to be 'cold' adapted and 135 proteins were found to be 'hot' adapted. Cold adapted proteins were higher in COG categories for cell division, transcription, lipid metabolism, and energy production and conversion (Figure S5.3), functions that would be important for cell growth and energy acquisition in sub-zero conditions. Genes associated with cell wall and membrane biogenesis surprisingly had more hot adapted genes.

Specific protein-protein comparisons: Adaptation at the Amino Acid level

Using the cold adaptation scores revealed that many proteins that are functionally associated with cold adaptation had amino acid substitutions which could allow for better functionality at low temperatures (Table 5.4). A subset of proteins involved with altering membrane fluidity or in TAG synthesis were found to have high cold adaptation scores, including 3-oxoacyl-[acyl-carrier-protein] synthase II/III, acyl-coA dehydrogenase, Acyl-CoA synthetase and 1-acylglycerol-3-phosphate O-acyltransferase. Glycogen debranching enzyme, important in glycogen biosynthesis was also found to be cold adapted. Cold adapted proteins with important functions in modulating transcription and translation were also found, including the anti-termination protein *nusG*, a DNA and RNA helicase, and the chaperone *DnaJ*. Numerous proteins involved in the cold/osmotic stress response, including compatible solute synthesis and uptake, ion transport and the ROS response were also found to be cold adapted. Considering the importance of these genes for conferring resistance to cold and salt, it is not surprising that they would exhibit characteristics of cold adaptation at the amino acid level. A Polar Amino Acid Uptake Transporter (PAAT) family amino acid transporter was found to be strongly cold adapted, and was cold adapted for all adaptation indices tested. It is uncertain what role this protein would have at cold temperatures, though increased expression of amino acid metabolic genes have been previously observed during subzero growth (−15°C) in *P. halocryophilus* (Mykytczuk et al. 2013). Glucose-6-phosphate dehydrogenase, which catalyzes the first step of the pentose phosphate pathway, was also found to be highly cold adapted, this step of the pentose phosphate pathway generates NADPH required for reductive biosynthesis.

Cold adapted genomic redundancy

One adaptation to cold found in psychrophilic organisms is the presence of proteins that have the same function, but have differing amino acid composition. We analysed the cold-adaptation scores of CDSs with multiple copies in the *R. sp.* JG3 genome, and identified several that varied in predicted cold, neutral and hot adaptation (Table 5.5). These included CDSs related to osmotic stress such as choline/betaine transporters, PAAT family amino acid transporters, and choline dehydrogenases. It is interesting to note that several of these CDSs were also found to have the highest cold adaptation scores in the genome, as discussed above. *R. sp.* JG3 has 7 copies of choline dehydrogenase, one copy was found to be cold adapted, one hot adapted, and 5 neutral. Though functionally annotated as choline dehydrogenases, percent identity among the isozymes varied (Table S5.1-Table S5.7) and the low percent similarity suggests that these isozymes were not due to recent intragenomic duplication and subsequent mutation. Multiple CDSs for subunits of carbon monoxide dehydrogenase (CODH) were also identified. The CODH middle subunit (4 copies) and the small subunit (3 copies), were found in separate operons along with a CODH large subunit or the structurally similar xanthine dehydrogenase. Analysis of the CODH operons did not reveal any operon to be solely hot or cold adapted for all genes in n operon. Adaptation of single subunits of enzymes may allow cells to only alter small, but critical catalytic portions of proteins, which are otherwise flexible.

Several *Rhodococci* are well-known alkane degraders and carry multiple copies of alkane 1-monooxygenases, though this is not a universal trait in this genus. *Rhodococcus* sp. JG3 has 3 alkane 1-monooxygenase genes, varying in cold adaptation score. Long-chain n-alkanes and n-alkanoic acids have been found in cryptoendoliths inhabiting Beacon sandstone in the Dry Valleys (Matsumoto et al. 1992), and stable isotopic signatures indicate that erosion of colonized sandstone

is the source of alkanes in some Dry Valley soils (Matsumoto et al. 2010); thus *R. sp.* JG3 may be able to utilize alkanes as a carbon source from the weathered cryptoendoliths known to colonize the sandstone valley walls in University Valley.

5.5 Conclusion

Rhodococcus sp. JG3 is clearly adapted to cold as demonstrated by growth at -5°C and measurable respiration down to -15°C. Based on genomic comparisons with mesophilic *Rhodococcus* strains, *R. sp.* JG3 employs several complementary strategies for adaptation to the cold and oligotrophic conditions of University Valley permafrost. *R. sp.* JG3 is equipped with the necessary genes for cell wall/membrane alteration, osmotic and oxidative stress protection, accumulation of energy and carbon storage molecules, and possibly carbon fixation through the presence of carbon monoxide dehydrogenase. Many of these CDSs are present in increased copy number when compared to mesophilic *Rhodococci* of similar genome size, and these CDSs also preferentially possess amino acid compositions favourable for maintained enzymatic activity at low temperature. The majority of proteins in *R. sp.* JG3 are neither cold nor hot adapted, an attribute which is consistent with the broad temperature range that *R. sp.* JG3 is active within. Taken together, these data present insights into the crucial role of fatty acid and lipid alteration, osmotic stress response and carbon acquisition for cell viability at cold temperature.

Genomic analysis of the *R. sp.* JG3 strain has given us some insight into the potential ecological role this strain has, or may have had in University Valley. The relative high abundance of photolyase and photo repair proteins, only functional in the presence of visible light, point to

the possibility that *R. sp.* JG3 may have originally been endolithic in origin. To overcome extremely low nutrient availability, *R. sp.* JG3 is equipped to utilize and store reserve molecules such as TAGs and glycogen for use under starvation conditions. Additionally, *R. sp.* JG3 possesses the full glyoxylate pathway to avoid carbon loss in the form of respired CO_2, and can possibly utilize CODH to fix carbon. The reservoir of cold adapted and stress response genes in *R. sp.* JG3 has allowed it to survive on long time scales in the dark and oligotrophic subsurface permafrost.

Genomic analyses only indicate the functional potential of microorganisms, and future inquiries in *R. sp.* JG3 will investigate whether the cold adapted proteins identified in this genomic analysis are increased in expression, or activity, under conditions similar to those encountered *in situ* in the upper elevation Dry Valleys.

The genomic comparisons competed in this study were aided by the abundance of available *Rhodococcus* genome sequences in public databases, a consequence of their diverse degradative capabilities. As sequencing costs continue to decrease, and more genomes from diverse genera are available, future studies will allow insight into the convergent evolution of cold adaptive traits, as well as identify any taxa-specific cold adaptations.

5.6 Acknowledgements

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5.7 Materials and Methods

Isolation and characterization

*Rhodococcu*s sp. JG3 was isolated from ice-cemented permafrost from a depth of 37 cm in University Valley, Antarctica. Soil was incubated with 0.1% $Na_4P_2O_7$ at 5°C for 2 weeks prior to the slurry being plated on R2A media incubated at 5°C. Growth characterization was carried out on R2A agar plates and in TSB liquid media (amended with 1%, 2%, 5%, 7% and 10% NaCl), at at 30, 22, 5, 0 -5,-10, and -15 °C in thermostat incubators (±0.5 °C). Growth was measured by optical density at 600 nm.

Acetate mineralization radiorespiration assay

Five g of permafrost was added to individual microcosms as Steven et al., 2007. Each microcosm was performed in triplicate, and included triplicate sterilized controls (autoclaved twice for 2 hours at 120°C and 1.0 atm, with a 24 h period between autoclavings). Microcosms were spiked with 0.045 mCi ml^{-1} (~100,000 disintegrations per minute) of 1-^{14}C acetic acid. Cold acetic acid was added to a final concentration of 15 mM acetic acid per microcosm in a total volume of

40 µl. The CO_2 trap consisted of 1 M KOH for microcosms incubated at 5°C, -5°C and 1 M KOH + 20% v/v ethylene glycol for microcosms incubated -10°C and -15°C.

DNA extraction

Rhodococcus sp. JG-3 was grown to stationary phase on Tryptic Soy Broth medium at room temperature. Genomic DNA was isolated using the Epicentre MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, Wisconsin) as per the manufacturer's instructions. Purified DNA was evaluated with the NanoDrop 1000 (Thermoscientific, Wilmington, Delaware), according to the DOE Joint Genome Institutes recommendations.

Genome Sequencing and annotation

The draft genome of Rhodococcus sp. JG-3 was carried out at the DOE Joint Genome Institute (JGI) using the Illumina HiSeq 2000 platform platform (Bennett 2004) as outlined in (Goordial 2015b). All general aspects of library construction and sequencing were performed at the JGI. Genes were identified using Prodigal (Hyatt et al. 2010), followed by a round of manual curation using GenePRIMP (Pati et al. 2010) for finished genomes and Draft genomes in fewer than 10 scaffolds. The predicted CDSs were translated and used to search the National Center for Biotechnology Information (NCBI) non-redundant database, UniProt, TIGRFam, Pfam, KEGG, COG, and InterPro databases. The tRNAScanSE tool (Lowe and Eddy 1997) was used to find tRNA genes, whereas ribosomal RNA genes were found by searches against models of the ribosomal RNA genes built from SILVA (Pruesse et al. 2007). Other non–coding RNAs such as the RNA components of the protein secretion complex and the RNase P were identified by searching the genome for the corresponding Rfam profiles using INFERNAL (Gilichinsky et al. 1993). Additional gene prediction analysis and manual functional annotation was performed

within the Integrated Microbial Genomes (IMG) platform (Goordial et al. 2013) developed by the Joint Genome Institute, Walnut Creek, CA, USA (Markowitz et al. 2009). The complete genome sequence of strain JG-3 is available for public access in DDBJ/EMBL/GenBank under accession numbers AXVF01000001- AXVF01000009.

Molecular analysis of cold adaptation

The genomes of 14 *Rhodococcus* strains annotated as mesophiles in publically available databases were used for comparison to the cryophilic *Rhodococcus* JG3 genome. No thermophillic *Rhodococcus* genome was available for comparison. A custom blast target database was created using the 14 mesophillic *Rhodococcus* genomes, and the *Rhodococcus* sp. JG3 genome was compared to this database using stand-alone Blastp with a cut-off e-value of $1e\text{-}^{15}$ (Camacho et al. 2009). The top blast match from each mesophilic genome for each *Rhodoccocus* sp. JG3 protein was then used for downstream analysis. Cold adaptation analysis was carried out similarly to (Ayala-del-Río et al. 2010a; Mykytczuk et al. 2013) with the exception that a custom database of genomes of the same genus was created instead of using the Swiss Prot database. Non-hypothetical proteins and their mesophilic matches were assessed for cold adaptation at the amino acid level for the following indices: arginine to lysine ratio, frequency of acidic residues, proline residues, aromaticity, aliphacity, and Grand Average of Hydropathicity (GRAVY). The results were averaged for mesophilic proteins and compared to the cryophilic R. sp. JG3 protein using a one-sample t-test. Proteins from *Rhodococcus* sp. JG3 that were found to be significantly different from the mesophilic proteins ($P < 0.05$) were then assigned as "cold-adapted" or "hot-adapted" depending on the direction of change. The frequency of cold and hot adapted genes were compared using a chi-square ratio test and a bonferoni corrected P-value < 0.00001305483 to determine if the differences seen were significant. All analyses were carried out using in-house python scripts

publically available at https://github.com/ColdAdaptationScripts. Cold adaptation scores were given to each protein based on the 6 indices. A point of 1 was given for each index that was determined to be cold adapted, and a point of -1 for each index found to be hot adapted. No points were given for indices that were not significantly different between R.JG3 and the 14 mesophillic strains. An individual protein was considered cold adapted if it had a score of 3 or greater, hot adapted if it had a score of -3 or lower, and neutral if between 2 and -2. Proteins with a score greater than 5, or less than -5 were considered to be strongly cold or hot adapted respectively.

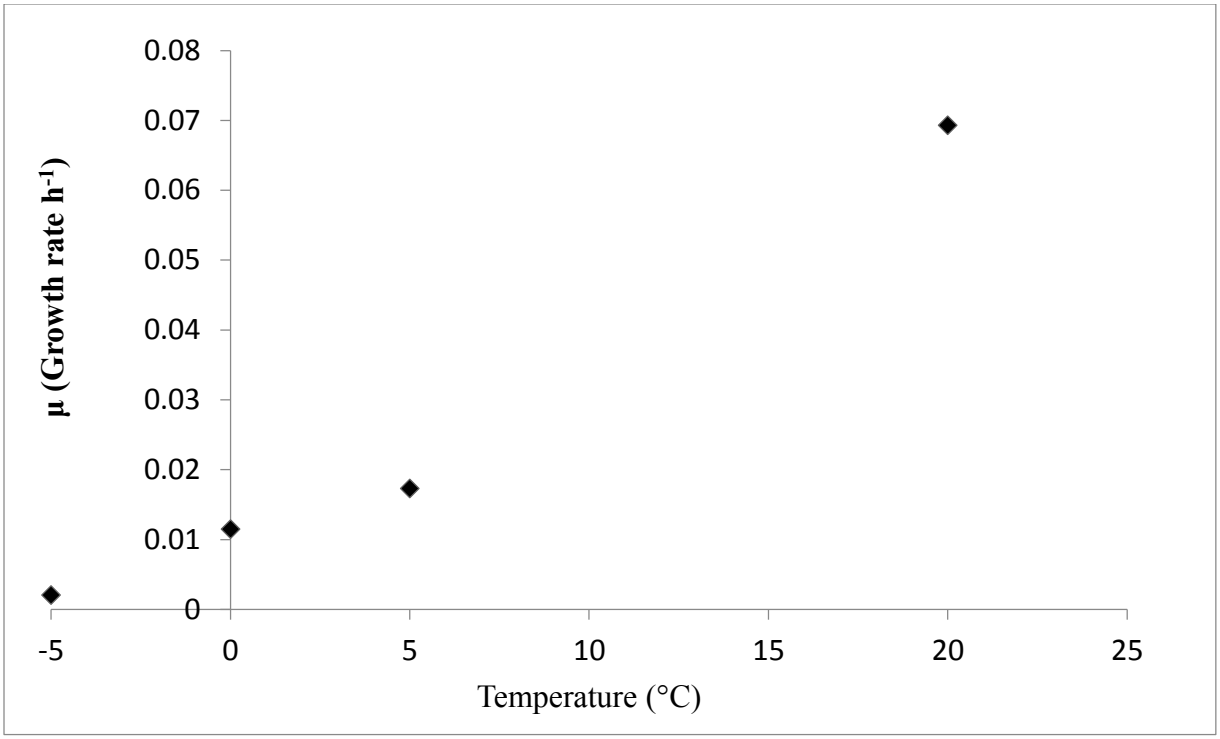

Figure 5.1. Growth rate (h-1) in Rhodococcus JG3 at 20°C, 5°C, 0°C, and -5°C.

Optimal growth temperature was found to be 20 °C, with a generation time of 10 hours. Generation times for 5°C, 0°C, and -5 °C were 40, 60 and 335 hours respectively.

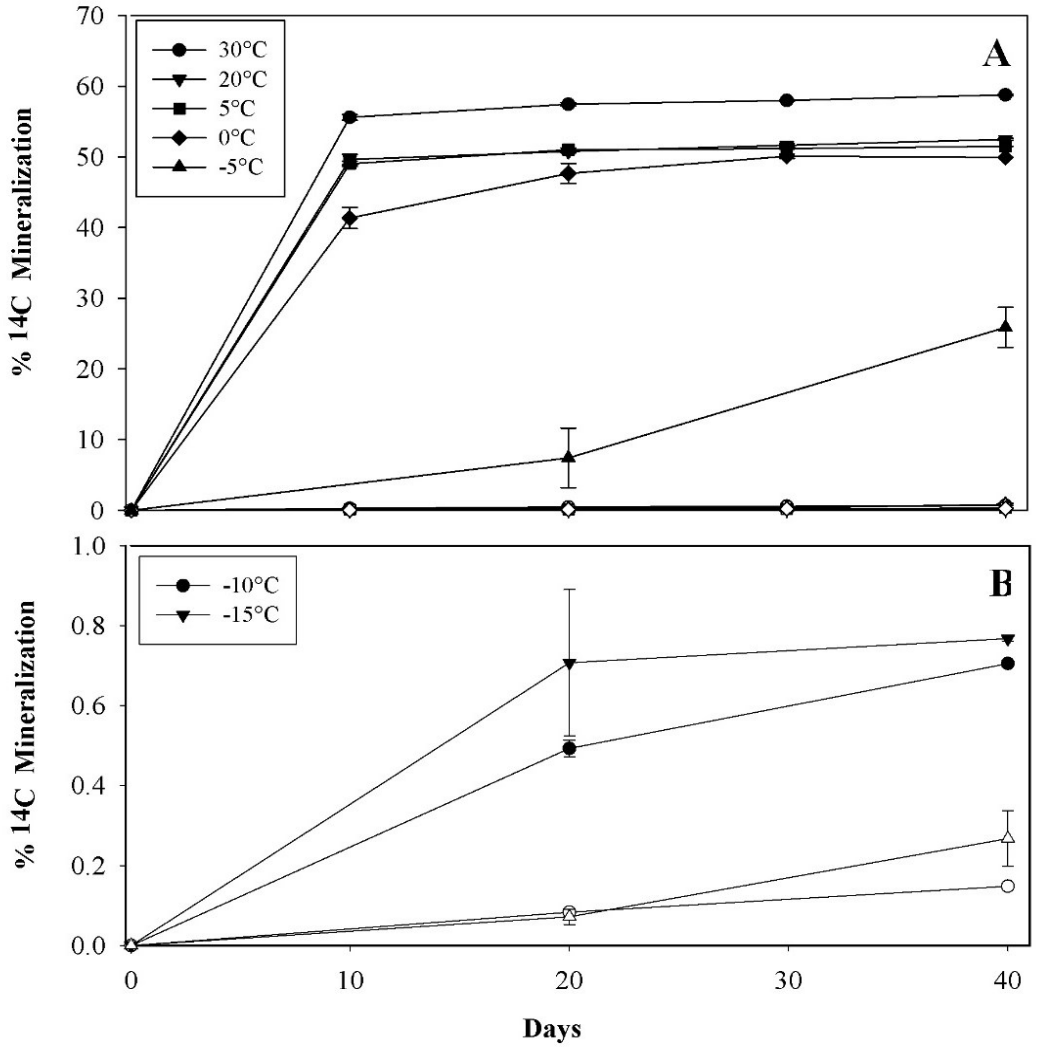

Figure 5.2. Mineralization assay of ^{14}C labelled acetate respiration activity in Rhodococcus JG3 across a range of temperatures.

A) 30, 20, 5, 0, -5 °C. B) -10, -15°C. Each point represents cumulative mineralization (% of $^{14}CO_2$ recovered) from triplicate assays. Open symbols denote sterilized control samples, standard error is shown here.

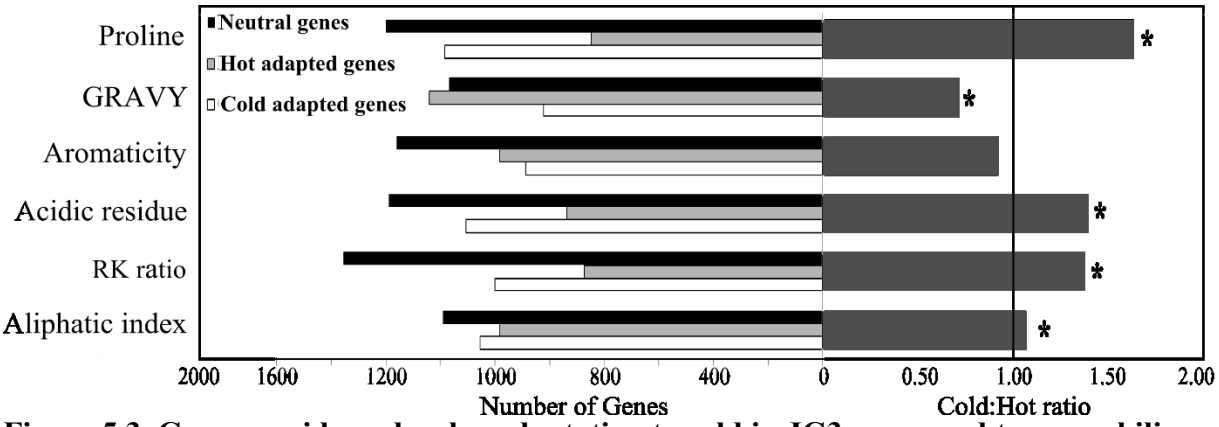

Figure 5.3. Genome wide molecular adaptation to cold in JG3 compared to mesophilic Rhodococci

Cold adaptation ratio of cold to hot genes is shown on the right. Number of hot, cold and neutral proteins is shown on the left for each index. Significant indices are indicated with * (Bonferonni corrected P< 0.00001305483).

Table 5.1. Summary of *Rhodococcus* JG3 and other mesophilic *Rhodococci* genome statistics

Genome Name (Reference)	Isolation Source	Total gene count	Total number of bases	No. of 16S rRNA	Number of genes with predicted protein product (%)	Number of genes in COG (%)	GC %	NCBI Project ID
Rhodococcus sp. JG3 (Goordial 2015b)	Antarctic dry valley permafrost	5067	5286918	2	3977 (78.49)	3296 (65.05)	65	195882
R. equi 103S (Letek et al. 2010)	Foal isolate, pathogen	4570	5043170	4	3620 (79.21)	3153 (68.99)	69	41335
R. equi ATCC 33707	Human abscess, pathogen	5116	5229298	1	3812 (74.51)	3176 (62.08)	69	31525
R. erythropolis CCM2595 (Strnad et al. 2014)	Soil	5899	6371421	4	4855 (82.3)	4021 (68.16)	62	81583
R. erythropolis PR4	Pacific Ocean seawater	6505	6895538	5	3657 (56.22)	4073 (62.61)	62	20395
R. erythropolis SK121	Wounds; skin microflora	6767	6785398	1	4820 (71.23)	4032 (59.58)	62	34067
R. jostii RHA1	hexachlorocyclohexane contaminated soil, Japan	9242	9702737	4	5673 (61.38)	5315 (57.51)	67	13693
R. opacus B4	gasoline-contaminated soil	8259	8834939	4	4861 (58.86)	5096 (61.7)	68	34839
R. opacus M213	Fuel-oil contaminated soil	8680	9194165	1	6967 (80.26)	5249 (60.47)	67	158507
R. opacus PD630	gas works plant soil sample in Germany	8721	9149864	1	6898 (79.1)	5495 (63.01)	67	30413
R. pyridinivorans AK37	Crude oil-contaminated site in Hungary	4875	5244611	1	3936 (80.74)	3045 (62.46)	68	76789
R. pyridinivorans SB3094	Oil fields	4976	5164718	1	3671 (73.77)	3028 (60.85)	68	231235
R. qingshengii BKS 20-40	Bhitarkanika National Park, Odisha, India	6216	6601618	4	5044 (81.15)	4130 (66.44)	62	185383
R. rhodochrous BKS6-46	mangrove forest soil, Bhitar Kanika National Park, Odisha, INDIA	5992	6213641	2	4694 (78.34)	3693 (61.63)	67	76791
R. sp. DK17	Crude oil-contaminated soil	8704	9107362	3	6669 (76.62)	5262 (60.45)	67	157361

Table 5.2. Known cold and stress response proteins found in Rhodococcus sp. JG3

Category and Gene ID	Description
Cold Shock Proteins	
2529298615, 2529302660	Cold shock Protein CspA
2529299677, 2529302008	Cold shock Protein
2529300242	Cold shock Protein CspC
Osmotic Stress/ Oxidative Stress	
2529301957, 2529301959, 2529302677, 2529302678	ABC-type proline/glycine betaine transport, permease component
2529301958, 2529302676	ABC-type proline/glycine betaine transport, ATPase components
2529301960, 2529302675, 2529302674	Periplasmic glycine betaine/choline-binding (lipo)protein of an ABC-type transport system
2529299333, 2529300314	sodium/proton antiporter, CPA1 family
2529299501, 2529299598	sodium/proton antiporter, NhaA family
2529303184- 2529303189	multisubunit sodium/proton antiporter, Mrp subunits A-G
2529298910, 2529303167	choline/carnitine/betaine transporter
2529301277	choline oxidase
2529299969	trehalose 6-phosphate synthase
2529298454	trehalose 6-phosphatase
2529299958	trehalose synthase
2529302123	ectoine synthase
2529299360	Osmosensitive K+ channel histidine kinase
2529298668, 2529302479	Activator of osmoprotectant transporter ProP
2529298479	peroxiredoxin, OsmC subfamily
2529300225	peroxiredoxin, Ohr subfamily
2529299760, 2529300646, 2529301356, 2529301817, 2529302000, 2529302240	Peroxiredoxin
2529299042, 2529303192	Superoxide dismutase
2529298661, 2529299729, 2529300031	Catalase
2529300023, 2529300594	catalase/peroxidase HPI
General Stress response	
2529299492, 2529299549, 2529299876, 2529300089, 2529300868 +11 more	Universal stress protein UspA
2529301347	SOS-response transcriptional repressor, LexA
2529300811, 2529300812, 2529303143	phage shock protein C (PspC) family protein
2529301319	phage shock protein A (PspA) family protein
Membrane/ cell wall alteration	
2529299195, 2529299523, 2529300078, 2529300210, 2529300337, + 4 more	Fatty acid desaturase
2529301908	phytoene synthase
2529301906	phytoene desaturase
2529301540	3-oxoacyl-[acyl-carrier-protein] reductase
2529299166	3-oxoacyl-[acyl-carrier-protein] synthase III (KAS-III)
2529302006	3-oxoacyl-[acyl-carrier-protein] synthase II (KAS-II)
2529302533	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
Carbon Storage/ Starvation	
2529302787	Carbon starvation protein
2529301072, 2529298836	Glycogen debranching enzyme (glgX)
2529302706	Glycogen synthase (ADP-glucose)
2529300961	diacylglycerol acyltransferase
2529299485	glycerol-3-phosphate O-acyltransferase

Table 5.3. Genomic comparisons of stress response gene copy number in *R. sp. JG3* and mesophilic *Rhodoccoci*

Function	Predicted Protein	***R.* JG3**	*R. equi* 103	*R. equi* 33707	*R. erythropolis* CCM2595	*R. erythropolis* PR4	*R. erythropolis* SK121	*R. pridinivorans B3094*	*R. pridinivorans* AK376	*R. qingshengii* BKS20-40	*R. rhodochrous* BKS6-46
Osmotic stress response	Periplasmic glycine betaine/choline-binding (lipo)protein of an ABC-type transport system	**3**						2			2
	choline/carnitine/betaine transporter	**2**						2			3
	Choline dehydrogenase	**7**			1		1	8		1	8
	ABC-type proline/glycine betaine transport systems, ATPase components	**2**						1			1
	ABC-type proline/glycine betaine transport systems, permease component	**4**						2			2
	amino acid ABC transporter substrate-binding protein, PAAT family	**6**			2				1	3	
	sarcosine oxidase	**3**		1	1	1	1		1	1	
	choline oxidase	**1**									
Oxidative stress	Peroxiredoxin	**8**		2	1	1	2	5	3	1	5
Cold shock	Cold shock proteins	**5**	5	5	5	5	3	9	5	5	5
stress response	universal stress protein	**17**		8	11		8	9	2	11	14
UV stress	photolyase	**5**	1	1	2	1	1	2	1	2	2
	photorepair protein PhrA	**2**						1			1

Table 5.4. Selected cold adapted proteins in Rhodococcus sp. JG3

Role	Gene ID	Gene name	A	B	C	D	E	F	G
Fatty acid and phospholipid alteration and biosynthesis	2529302193	Short-chain dehydrogenase							5
	2529298839	Glycerol-3-phosphate dehydrogenase							3
	2529299166	3-oxoacyl-[acyl-carrier-protein] synthase III							4
	2529302006	3-oxoacyl-[acyl-carrier-protein] synthase II							3
	2529299529	1-acylglycerol-3-phosphate O-acyltransferase							3
	2529298632	acetyl-coenzyme A synthetase							4
	2529302639	Acyl-CoA dehydrogenase							4
	2529302839	Acyl-CoA dehydrogenase							4
	2529299434	Acyl-CoA synthetase (AMP-forming)/AMP-acid ligases II							4
	2529298474	Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily)							4
Cold, Osmotic and Oxidative Stress response	2529298663	Choline dehydrogenase and related flavoproteins							3
	2529298910	choline/carnitine/betaine transport							3
	2529299592	Predicted flavoprotein involved in K+ transport							4
	2529299729	Catalase							4
	2529299255	amino acid ABC transporter substrate-binding protein, PAAT family							6
	2529302676	ABC-type proline/glycine betaine transport systems, ATPase components							3
General stress response and regulation	2529299449	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain							3
	2529301347	SOS-response transcriptional repressor, LexA							4
transcription, translation and DNA repair	2529300888	ATP dependent helicase, Lhr family							4
	2529299669	Molecular chaperone							3
	2529299301	primary replicative DNA helicase							4
	2529302961	transcription antitermination protein nusG							5
	2529299297	single-strand binding protein							3
	2529303222	ATPases involved in chromosome partitioning							3
	2529299962	chaperone protein DnaJ							3
	2529301716	Formamidopyrimidine-DNA glycosylase							4
	2529301020	RNA polymerase, sigma subunit, ECF family							3
	2529301786	2'-5' RNA ligase superfamily							3
Carbon metabolism	2529301512	glucose-6-phosphate 1-dehydrogenase							5
	2529298836	glycogen debranching enzyme GlgX							4

A=aliphatic index, B= RK ratio, C=acidic residue, D=aromaticity, E=GRAVY, F=Proline %, G=Cold Adaptation score. Blue boxes indicate the protein was found to be cold adapted for that index, red is hot adapted and orange coloured boxes were not significantly different than mesophile homologs ($P<0.05$). Cold adaptation scores were given to each protein based on the 6 indices. For each index, a point of 1 was given for each index which was determined to be cold adapted, and a point of -1 for each index found to be hot adapted. No points were given for indices which were not significantly different between R.JG3 and the 14 mesophillic strains.

Table 5.5. Selected predicted multi-copy cold adapted proteins

Gene Annotation	Gene ID	Aliphatic index	RK ratio	Acidity	Aromat-icity	GRAVY	% Proline	Cold Adaptation Score
Choline dehydrogenase and related flavoproteins	2529298663							3
	2529298829							0
	2529299660							1
	2529299722							1
	2529300808							1
	2529302880							1
	2529302948							-2
choline/carnitine /betaine transporter	2529298910							3
	2529303167							1
amino acid ABC transporter substrate-binding protein, PAAT family	2529299255							6
	2529302642							1
	2529302698							0
	2529298386							-1
	2529301330							-1
	2529303211							-1
glucose-6-phosphate 1-dehydrogenase	2529301512							5
	2529301307							2
	2529303145							0
glycogen debranching enzyme	2529298836							4
	2529301072							1
alkane 1-monooxygenase	2529299852							2
	2529299421							0
	2529300951							-2
Aerobic-type carbon monoxide dehydrogenase, middle subunit	2529302354							5
	2529302645							3
	2529300899							1
	2529302509							-2

Blue boxes indicate the protein was found to be cold adapted for that index, red is hot adapted and orange coloured boxes were not significantly different than mesophile homologs ($P<0.05$). Cold adaptation scores were given to each protein based on the sum of the 6 indices. For each index, a point of 1 was given for each index which was determined to be cold adapted, and a point of -1 for each index found to be hot adapted. No points were given for indices which were not significantly different between R.JG3 and the 14 mesophillic strains.

5.8 Supplementary Information

Supplementary Figures 1-3

Supplementary Tables 1-7

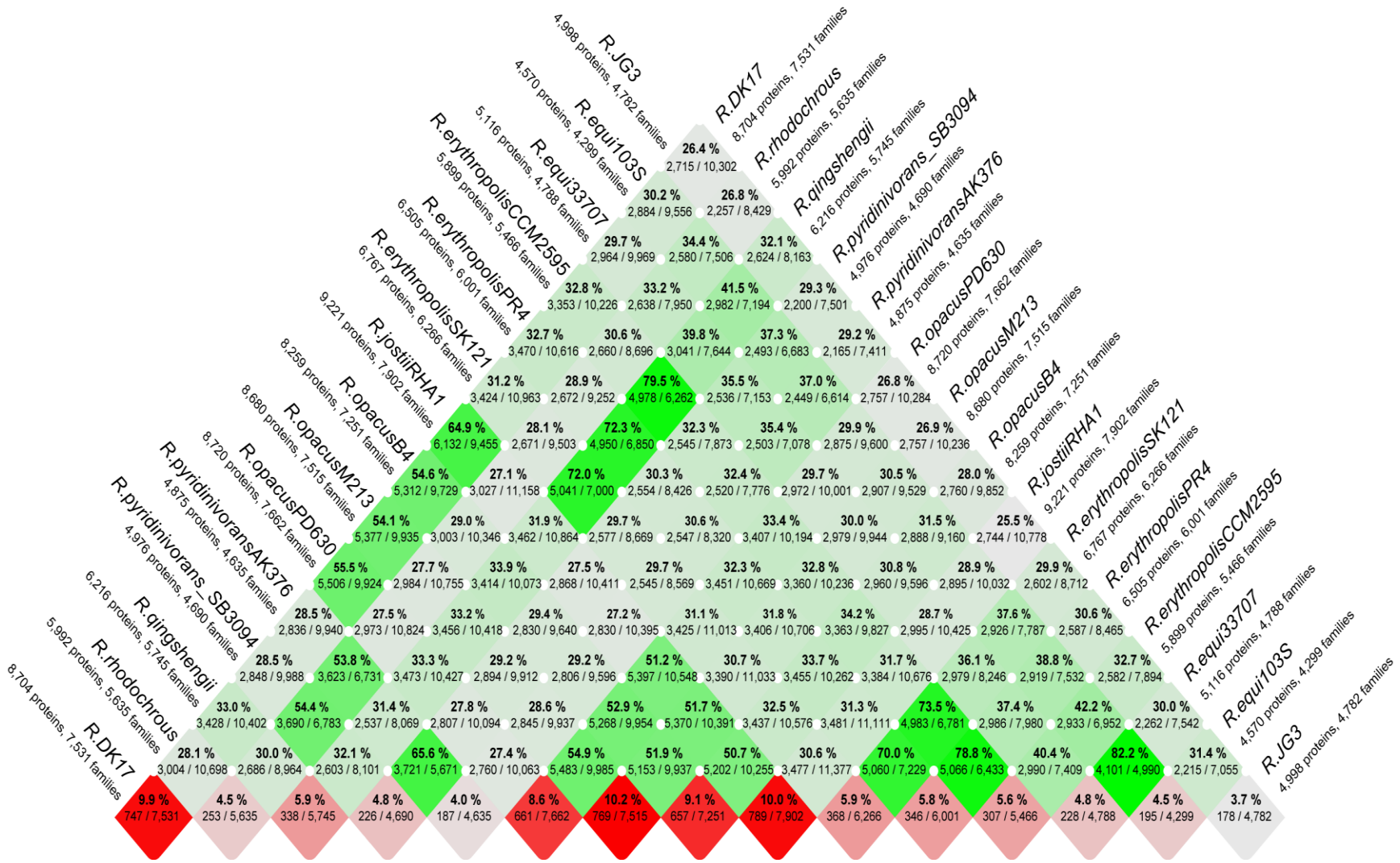

Figure S 5.1. Inter- and intra-genome similarity of all *Rhodococcus* strains

The bottom row describes within genome similarity of predicted proteins

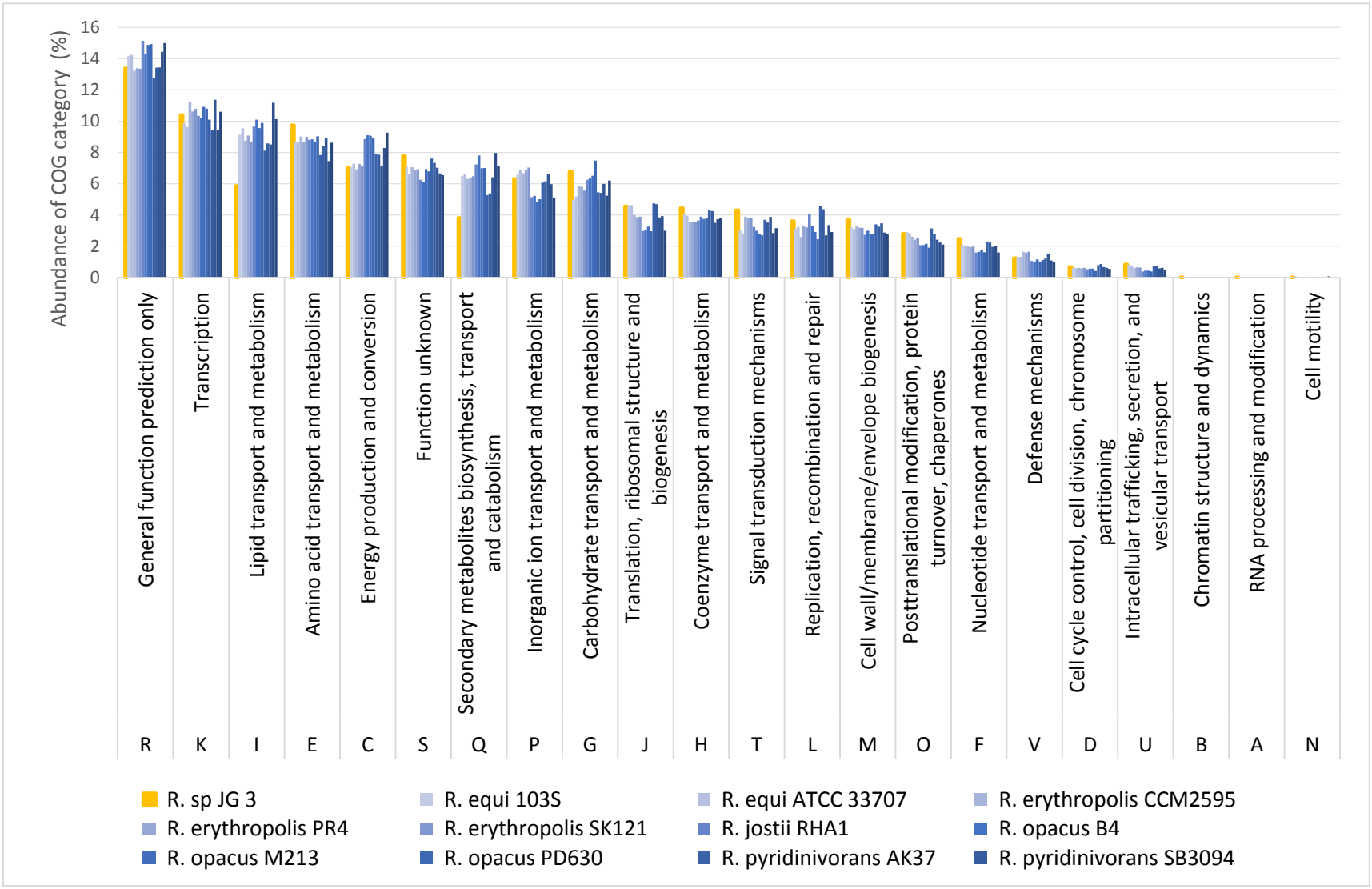

Figure S5.2. COG categories in *Rhodococcus* JG3 and mesophilic strains.

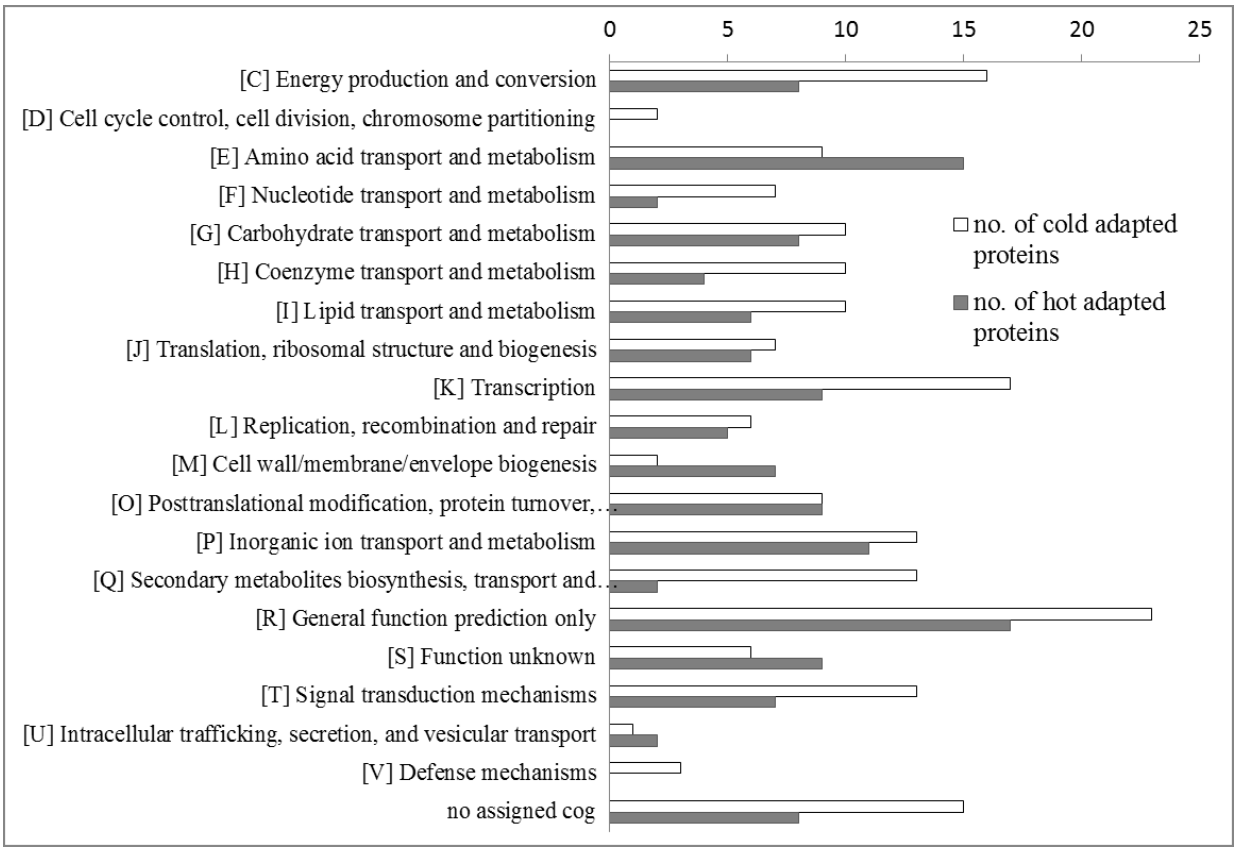

Figure S5.3. Number of Cold and Hot Adapted Proteins in COGs in *Rhodococcus* JG3

Table S 5.1 Protein percent identity of choline dehydrogenases in *Rhodococcus* JG3.

			Gene ID						
Gene Annotation	Gene ID	Cold Adaptation Score	2529300808	2529302948	2529298663	2529299722	2529302880	2529298829	2529299660
Choline dehydrogenase	2529300808	1	100						
	2529302948	-2	92.78	100					
	2529298663	3	63.06	62.52	100				
	2529299722	1	27.04	27.20	28.05	100			
	2529302880	1	26.16	26.53	26.94	34.88	100		
	2529298829	0	15.34	15.10	16.77	16.89	13.02	100	
	2529299660	1	15.05	14.43	15.46	15.79	14.85	40.04	100

Table S 5.2. Protein percent identity of choline/carnitine/betaine transporters in *Rhodococcus* JG3.

			Gene ID	
Gene Annotation	Gene ID	Cold Adaptation Score	2529298910	2529303167
choline/carnitine /betaine transporter	2529298910	3	100	
	2529303167	1	40.04	100

Table S 5.3. Protein percent identity of amino acid PAAT family transporters in *Rhodococcus* JG3.

			Gene ID					
Gene Annotation	Gene ID	Cold Adaptation Score	2529299255	2529302642	2529302698	2529298386	2529301330	2529303211
amino acid ABC transporter substrate-binding protein, PAAT family (TC 3.A.1.3.-)	2529301330	-1	100					
	2529303211	-1	32.97	100				
	2529302642	1	20.01	22.10	100			
	2529302698	0	9.197	18.75	28.42	100		
	2529299255	6	21.03	17.23	21.32	16.36	100	
	2529298386	-1	17.22	14.81	17.88	15.77	15.53	100

Table S5.4. Protein percent identity of glucose-6-phosphate 1-dehydrogenases in *Rhodococcus* JG3.

			Gene ID		
Gene Annotation	Gene ID	Cold Adaptation Score	2529301512	2529301307	2529303145
glucose-6-phosphate 1-dehydrogenase	2529301307	2	100		
	2529303145	0	41.44	100	
	2529301512	5	34.95	31.01	100

Table S5.5. Protein percent identity of glycogen debranching enzymes in *Rhodococcus* JG3.

			Gene ID	
Gene Annotation	Gene ID	Cold Adaptation Score	2529298836	2529301072
glycogen debranching enzyme	2529298836	4	100	
	2529301072	1	41.76	100

Table S5.6. Protein percent identity of alkane 1-monooxygenases in *Rhodococcus* JG3.

			Gene ID		
Gene Annotation	Gene ID	Cold Adaptation Score	2529299852	2529299421	2529300951
alkane 1-monooxygenase	2529299421	0	100		
	2529299852	2	54.64	100	
	2529300951	-2	54.26	53.45	100

Table S5.7. Protein percent identity of carbon monoxide dehydrogenases in *Rhodococcus* JG3.

Gene Annotation	Gene ID	Cold Adaptation Score	2529302354	2529302645	2529300899	2529302509
Aerobic-type carbon monoxide dehydrogenase, middle subunit	2529302354	5	100			
	2529302509	-2	35.59	100		
	2529302645	3	22.65	28.42	100	
	2529300899	1	21.97	18.08	17.56	100

Chapter 6. Discussion and Conclusions

University Valley (1700 m.a.s.l.), is one of the coldest and driest locations in Antarctica, with mean annual temperature of ~ -25ºC and no days above freezing annually. The results outlined in this thesis describe two different habitability conditions under extreme cold and dryness in University Valley: the permafrost soils that are largely inhospitable to active microbial life, and the cryptoendolithic refugia environment that hosts a simple, but functional cold adapted microbial community.

6.1 Microbial activity and diversity in University Valley permafrost and cryptoendoliths

University Valley permafrost is unique in that heterotrophic activity was undetectable *in situ*, and in laboratory microcosms at sub-zero temperatures (measured using a sensitive radiorespiration assay with ^{14}C acetate as a carbon source); this is unlike all other permafrost studied to date for heterotrophic activity at cold temperatures (Rivkina et al. 2000; Steven et al. 2008; Bakermans et al. 2014; Tuorto et al. 2014). Sulfur reduction, perchlorate reduction, and methanogenesis was also not detected using culture based methods. Though activity could not be measured at sub-zero temperatures, respiration of acetate was detected at 5°C, a temperature at which these soils have not experienced in the past ~115,000 years. In contrast to the permafrost soils, the cryptoendolithic communities' demonstrated significant heterotrophic activity down to −20°C, the lowest temperature tested, using the same radiorespiration assay. These results indicate that an active microbial ecosystem adapted to ambient *in situ* conditions resides within the

sandstone cryptoendoliths but not the University Valley permafrost. Photosystem II activity was also measured at −20°C, indicating that photosynthesis could occur at the sub-zero temperatures that the cryptoendoliths experience during the summer months in University Valley. This may be a significant advantage which contributes to the relative viability of the cryptoendoliths, with primary productivity through photoautotrophy providing the basis for an active ecosystem within the cryptoendoliths. Solute analysis of University Valley permafrost soils indicated that due to the low salt concentration, the amount of liquid water at below freezing temperatures is limited only to thin films adhering to sand grains. The activity observed only above freezing temperatures may reflect water newly available for cells, which would be otherwise dormant at *in situ* freezing temperatures.

Biomass levels in University valley permafrost was extremely low compared to other permafrost soils globally (10^3 cells/g) and culturable cells were negligible; only 6 isolates were obtained over two years of efforts using multiple medias and enrichment strategies. The use of molecular methods for assessing microbial diversity allows access to microorganisms which may otherwise be inaccessible, though these methods are not able to discern between viable, dead or dormant cells. Pyrosequencing of the 18S and 16S rRNA gene as well as metagenomic sequencing found that *Actinobacteria, Firmicutes, Proteobacteria* and *Bacteriodetes* were the most dominant phyla in University Valley permafrost, similar to other permafrost globally (Goordial et al. 2013). OTUs related to bacterial genera commonly associated with soil (*Burkholderia*, *Ralstonia*, *Sphingomonas*, *Bradyrhizobium*) and aquatic/marine environments (*Alcanivorax*, *Pelagibacter*, *Gillisia*) were found, although University Valley contains no aquatic habitats. Eukarya comprised ~12% of the soil community, mostly composed of desiccation resistant extremophillic black yeast

fungi, also found commonly in sandstone cryptoendolithic communities in the upper Dry Valleys. Overall, the bacterial diversity (H'= 1.1 - 4.3) of University Valley permafrost soils varied, but was higher than expected given the very low biomass and undetectable metabolic activity.

The cryptoendolithic community was simple in trophic structure, and was comprised of photoautotrophic algae, and heterotrophic fungi and bacteria. The community was dominated by Eukarya (54% of community), composed of fungi and algal phyla reflective of a lichen dominated community. In the cryptoendolithic community, the black yeast fungi can serve an important role in UV protection by providing an opaque barrier above the community. Algal diversity was higher than expected based on a previous molecular survey of Dry Valley sandstone cryptoendoliths (de la Torre et al. 2003); we observed over 300 OTUs of the algal genus *Trebouxia* in two cryptoendolith samples. Negligible Archaea were identified in both niches (0.4% of the cryptoendolith and 0.01% of permafrost soil community) consistent with previous reports that Archaea are absent, or difficult to detect in Dry Valley soils and lithobiontic communities (Pointing et al. 2009; Lee et al. 2012). Viruses were relatively more abundant in the permafrost soils (2%) than in the cryptoendolithic community (0.5%), in contrast to previous comparisons of lithic niches and open soils in the Dry Valleys (Zablocki et al. 2014). Little is currently known about viral roles in community ecology in the Antarctic Dry Valleys, though viruses exerted top down control on soil communities in Arctic soils, decreasing both microbial biomass and activity (Allen et al. 2010).

Since the valley floor of University Valley is derived from erosion of the valley walls, we hypothesized that the source of the high diversity seen in University Valley surface soils would

be the cryptoendoliths inhabiting the valley walls. However, pyrosequencing of two cryptoendolith samples showed they shared few bacterial, archaeal or fungal OTUs in common with University Valley permafrost soils, and the most abundant taxonomic groups in the sandstone were poorly represented (<1%) or completely absent. Thus, the microorganisms found in the soils in University Valley are likely derived from a mixture of cryptoendolithic sources, as well as from aeolian deposition via strong katabatic winds which occur in the Dry Valleys.

6.2 Functional Diversity in University Valley permafrost and cryptoendoliths

In the highly oligotrophic soils found in University Valley, carbon and nutrient sequestration is important. Overall we found little evidence that the permafrost community is capable of supporting itself without exogenous carbon supplied since no full carbon fixation pathways were found in the soils using metagenomic analysis. The cryptoendolithic communities are capable of photoautotrophy through the Calvin-Benson cycle. It is possible that carbon fixed by the cryptoendolithics could percolate into nearby soils (Friedmann et al. 1993b) or be deposited onto soils as the valley walls weather and erode. Genes required for heterotrophy were abundant in both metagenomes including genes required for acetate metabolism, the substrate used in the respiration activity assays used in this study. Additionally, the genes responsible for the degradation of cellular material were more abundant in the permafrost soil and included several involved with murein recycling, and N-acetylglucosamine and chitin utilization. These genes could be advantageous for using cellular material as a nutrient source, including potential biomass from eroding cryptoendolithic communities.

A number of stress response genes for adaptation to cold, osmotic, and oxidative stress were found in metagenomes from both niches, though the diversity of stress response genes was higher in the cryptoendolith metagenome than in the permafrost metagenome (with 87 and 34 different stress response related genes respectively). The two niches shared 25 of 96 stress response genes in common; the shared genes were primarily involved in cryoprotectant production and compatible solute transport across membranes - general strategies used by cryophilic microorganisms to adapt to subzero environments (Bakermans et al. 2009; Bakermans et al. 2012)

Though the permafrost soils in University Valley have a lower diversity of stress response genes, the permafrost is enriched with genes involved with dormancy and sporulation when compared with other publically available Arctic permafrost metagenomes. Thus, the permafrost soils select for traits which emphasize survival and dormancy, rather than growth and activity, while the cryptoendolithic environment selects for organisms capable of growth under extremely oligotrophic, arid, and cold conditions.

6.3 Characterization and cold adaptation of a University Valley permafrost isolate

Only one bacterium from University Valley permafrost was found to grow at the subzero temperatures relevant to those *in situ* (out of 6 isolates total, including a subzero growing yeast). By sequencing the genome of *Rhodococcus* sp. JG3 and using comparative genomics with mesophilic *Rhodococci* we gained insight into the molecular traits that may allow for survival in the cold, arid and oligotrophic University Valley soils on long timescales. *Rhodococcus* sp. JG3

employed several complementary strategies for adaptation to the cold and oligotrophic conditions in University Valley. Similar to other psychrophiles (Breezee et al. 2004; D'Amico et al. 2006; Ayala-del-Río et al. 2010b; Mykytczuk et al. 2011a), R. sp. JG3 carries the necessary genes for cell wall and membrane alteration, cold shock proteins, and osmotic and oxidative stress response including increased copy number of genes involved in compatible solute synthesis and uptake. We found that many proteins that are functionally associated with cold adaptation also had amino acid substitutions which could allow for better functionality at low temperatures. Crucially, in a highly oligotrophic environment such as University Valley permafrost, R. sp. JG3 is equipped with genes for the synthesis and accumulation of carbon and energy storage molecules such as triacylglycerols (TAGS) and glycogen. These molecules are known to serve as carbon and energy reserves in *Rhodococcus* species under starvation conditions (Alvarez et al. 2000; Alvarez et al. 2013), (Hernández et al. 2008). Intriguingly, R. JG3 also possessed carbon monoxide dehydrogenases, indicating the possibility of carbon fixation, an adaptation that would be advantageous in Dry Valley permafrost soils. Future transcriptomic work with this strain is the next step to understanding whether the cold-adapted genes identified in this work are actually transcribed at cold and subzero temperatures.

6.4 Conclusions

Microorganisms are found virtually everywhere on the Earth's surface, with perhaps the exception of surfaces which have purposefully sterilized by humans such as medical equipment. However, in spite of the ubiquitous distribution of microorganisms, the environmental conditions must exist for their viability and activity. The environmental constraints on microbial life are a

fundamental question in microbiology as well as in the field of astrobiology, where the limits of life on Earth may be used to inform hypotheses about the potential existence of life on other planetary bodies within our solar system, especially significant astrobiology targets such as Mars, Enceladus, and Europa, all of which are also essentially surface cryoenvironments. This thesis outlines a natural setting in the high elevations of the Antarctic Dry Valleys, University Valley, which is pushing the boundaries of terrestrial life on Earth. The permafrost soils of University Valley are not completely sterile, but they do not appear to support an active microbial ecosystem and are therefore uninhabitable; traits which favour dormancy and sporulation are enriched in these soils rather than those for activity and growth. In contrast, the more clement conditions provided by the porous sandstone rock structure has fostered a thriving cryptoendolithic microbial community living within the same valley. These cryptoendolithic communities are living on the edge of the cold-arid limits of life; photoautotrophic algae provide crucial carbon and forms the basis of the cryptoendolithic ecosystem which also includes heterotrophic fungi and bacteria, and the community as a whole is adapted to the fluctuating harsh conditions of the Antarctic Dry valleys. In cold and hot deserts globally, microbial life can be observed limited to lithic substrates, pointing to both the tenacity of microbial life, and the ecological importance of even the smallest differences in environmental conditions. The narrow stratified band of biomass beneath the cryptoendolithic rock surface is a visual example of the significance of microscale differences in temperature, humidity, and light. The sandstone environment is seemingly the difference between life and death in University Valley; cold adapted microorganisms which are able to grow in the cryptoendolithic habitat are not able to maintain activity when the sandstone rocks erode onto the valley floor and form the surface permafrost of University Valley.

The absence of detectable heterotrophic activity in University Valley permafrost soil may potentially be due to the very low biomass found in the soils, where any metabolic activity could be below the detection limits of current methodology. The isolation of a sub-zero growing *Rhodococcus* isolate which does not form spores, hints at the possibility of active microbial life *in situ*. Extremely cold, desiccating, oligotrophic, and relatively old environments such as University Valley permafrost are ideal testing beds for life detection methodology for other planetary bodies such as Mars which contains large areas of surface permafrost and which is considered a primary astrobiology target on Mars (Tamppari et al. 2012). If it is difficult to unambiguously detect viable microbial life in these environments on Earth, it will be challenging to accomplish in future robotic missions with life detection instrumentations. Indeed, the lack of a detectable active microbial ecosystem in University Valley permafrost brings into question whether or not Martian surface permafrost could support microbial life as we know it, given that Martian surface permafrost environments are colder, drier, older, appear to have destructive soil chemistry (highly reactive oxidative agents), receive much more radiation, while also receiving much less input of organic C, N, P or seeded with allochthonous organisms through aeolian deposition. Ultimately, understanding the abiotic factors which contribute to habitability, even on microscales, is crucial to informing the search for life on other planetary bodies as well as identifying the limits of life on Earth.

6.5 Future Directions

The results of thesis point to a potential place on Earth, where active microbial life is either non-existent, or below current detection limits. This is a surprising result, and indicates that

University Valley is an ideal testing bed for new life detection methodologies, as well as the search for novel microbial metabolisms and adaptations to the cold, arid and oligotrophic conditions. The presence of carbon fixing genes in a University Valley permafrost bacterial isolate point to this possibility, and would not have been detected using the activity assays employed in this study. Alternative activity assays should be employed to detect whether such metabolisms could be utilized *in situ*. The assays used in this study were carried out with the hypothesis that permafrost soils in University Valley would be similar to those encountered elsewhere, with possibly even more cold adapted organisms. Instead, this site is more similar to the deep subsurface environment, and future activity assays should borrow from methodology being employed and innovated for this environment. Sub-seafloor environments have similar low cell biomass (10^3cells/ cm^3), are energy starved, and turnover time for cells is modeled to be between 100-10,000 years (Jørgensen 2011). Nanoscale secondary ion mass spectrometry (nanoSIMS) has been used to detect activity in such deep sub-seafloor environments, and could even identify the amounts of ^{13}C and ^{15}N substrates that were taken up and incorporated into biomass (Morono et al. 2011). NanoSIMS methodology in combination with stable isotope probing (SIP) and fluorescence in situ hybridization (FISH) has been used to detect specific metabolic activity in individual microorganisms, and can identify interspecies interactions and trophic relationships (Lever et al. 2013; Dekas et al. 2014). Utilizing such methodologies may add to the our understanding of the microbial ecology of high elevation Antarctic Dry Valley permafrost, including whether the microorganisms present in soils are dead, dormant, or active with extremely low metabolic rates.

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