Investigating the Physiological and Metabolic Requirements of the Tramway Ridge Microbial Community, Mt Erebus, Antarctica

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Abstract

Mount Erebus is the most active volcano on the Antarctic continent, and has the most geographically and physically isolated geothermal soil on Earth. It is exposed to some of the most extreme environmental conditions on the planet. Recent preliminary genetic analysis of the microbial community present in the 65°C subsurface soil of Tramway Ridge, Mount Erebus has revealed a unique thermophilic microbial system, with the dominant members possessing little genetic similarity to any known bacteria. The aim of this project was to apply a range of techniques to investigate metabolic and physiological requirements of this poorly understood microbial system. The study included physical-chemical soil surveying, strategic cultivation, community based phenotypic arrays, nutritional enrichment experiments and pyrosequencing. This multifaceted, metabolism driven, approach incorporated both culture dependent and culture independent techniques in order to investigate the intriguing system in a way that is crucial to the understanding of this thermophilic community and the controlling environment. Overall, the results of this study have led to the hypothesis that the Tramway Ridge microbial community is driven by chemolithoautotrophic microorganisms. This hypothesis is supported by a number of observations, firstly that in the absence of carbonate in enrichments, the community shifts to being dominated by obligate heterotrophs; the microbial ecosystem exists in subsurface soils which have limited, or no, light availability; the system is nutrient poor in nature, coupled to a carbon dioxide dominated gas supply from volcanic activity; two of the keystone members identified from

pyrosequencing, phylogenetically group with bacterial phyla known for chemolithoautotrophic metabolism. Considering the detailed investigation of this apparently archaic and isolated microbial system, there is potential for this study to become an excellent model for future studies addressing the fundamental functioning and evolutionary processes associated with other thermophilic communities.

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Table of Contents

List of Figures

List of Tables

1 Literature Review

Microorganisms can be classified into three groups, categorised by their range of growth temperatures: mesophiles, psychrophiles, and thermophiles. Thermophiles, defined as organisms that can survive and reproduce in temperatures ranging from 45°C to 122°C (Rivera and Lake, 1996), possess thermostable biomolecules which allow the catalysis of metabolic processes within environments that would otherwise be detrimental to life (Madigan and Martinko, 2006). High temperature microbial ecosystems have become a focus for research, in particular for the impressive range of metabolic capabilities which expand our understanding of the upper temperature tolerances of microbial activity, and their striking parallelism to ancient microbial ecosystems (Amend and Shock, 2001; Belkin et al., 1985; Boiteau and Pascal, 2011; Bonch-Osmolovskaya, 2010; Bonch-Osmolovskaya et al., 2003; Oren, 1983; Reysenbach et al., 1999; Rozanova and Ental'tseva, 1999; Schleper et al., 1995; Slobodkin, 2005; Slobodkin et al., 1999; Wu et al., 2006; Yoshida et al., 2010; Zhang et al., 2009).

1.1 Thermophilic Metabolism

Microorganisms rely heavily on intracellular redox reactions to generate energy for essential cellular functioning. Redox reactions yield energy through the process of electron transfer from one molecule to another (Madigan and Martinko, 2006). Thermophilic prokaryotes are able to carry out redox processes

with a wider range of metabolically relevant substrates than other prokaryotes or eukaryotes, making it possible to exist in environments that restrict other life. Thermophiles can utilise hydrogen gas, Fe^{2+} and other metal ions, hydrogen sulphide, elemental sulfur, minerals, methane, ammonia, various carboxylic acids, alcohols, amino acids, and complex organic substrates as electron donors in thermophilic redox reactions, whereas nearly all eukaryotes and a large proportion of mesophilic bacteria are restricted to simple organic substrates, such as glucose (Johnson, 1998; Kozubal et al., 2008; Martens-Habbena and Stahl, 2011; Miroshnichenko and Bonch-Osmolovskaya, 2006; Orphan et al., 2003; Slobodkin et al., 1999). Thermophiles can also utilise a range of substrates, such as oxygen, Fe^{3+} and other metal ions, carbon oxides, nitrogen oxides, sulfur oxides, and elemental sulfur as electron acceptors, whereas nearly all eukaryotes and a large proportion of bacteria are largely restricted to the use of oxygen and carbon dioxide (Belkin et al., 1985; Kirk, 2011; Orphan et al., 2003; Rozanova and Ental'tseva, 1999; Slobodkin, 2005; Stepanov and Korpela, 1997).

The enormous metabolic diversity present in organisms that live in high temperature environments reflects the range of redox states, pH, mineralogy, gas composition, and solute concentrations that characterise these environments (Amend and Shock, 2001; Islas et al., 2003). Such organisms have adapted to these selective pressures, and, as a result, thermophilic microorganisms survive in the most extreme conditions of pH, salinity, desiccation, radiation, and oligotrophy described to date (Chen et al., 2004; Ferreira et al., 1997; Johnson, 1998; Mesbah and Wiegel, 2008; Mijts and Patel, 2001; Nagy et al., 2005; Priscu et al., 1999; Schleper et al., 1995; Thurmer et al., 2011).

1.2 Theory of Thermophilic Prokaryotic Origins

Thermophilic organisms form phylogenetic groups that are generally deep branching and closely related to extant ancestral organisms, suggesting thermophilic roots for most evolutionary adaptations in the prokaryotic kingdom (Amend and Shock, 2001; Boiteau and Pascal, 2011; Bonch-Osmolovskaya, 2010; Reysenbach et al., 1999). Recent studies suggest that certain high temperature, low nutrient environments, in which some of these deep branching hyperthermophiles have been found, are similar to those of early Earth at the time life first evolved (Bonch-Osmolovskaya, 2010; Cavicchioli, 2002; Miroshnichenko and Bonch-Osmolovskaya, 2006; Pikuta et al., 2007; Reysenbach et al., 1999; Russell et al., 2005; Wallis et al., 2009). If this theory is true, investigation of the relic microorganisms presently inhabiting these ancestral niche-like habitats may provide insight into how life arose on Earth. While the evolutionary origins of thermophilly remain debatable, it is clear that thermophilic microorganisms host some of the most intriguing physiological and metabolic capabilities discovered to date, and have evolved to interact with, and thrive in, incredibly harsh habitats. A deeper understanding of the capabilities of the organisms present in extreme environments will provide insight into questions central to theories of physiological adaptation and microbial evolution.

1.3 Methodologies for Studying Thermal Ecosystems

Investigating the microbiology of thermal environments can be approached using two different methodologies: culture dependent and culture independent techniques. Culture dependent techniques refer to the use of selective media to grow organisms from their natural environment (Hugenholtz et al., 1998; Janssen et al., 2002). For example, a cultivation dependent experiment by Hirayama and colleagues (2007) was conducted to investigate the microbiology of a shallow hydrothermal environment in Japan. It was initially assumed, due to the availability of sunlight, that it was driven by photosynthetic primary production. Isolates from this study however, indicated the presence of a unique microbial community that is sustained by active chemosynthetic primary production, rather than photosynthetic production. Cultivation dependent techniques are undoubtedly the most direct and information rich approach, however they come with substantial limitations and biases. Limitations include, contamination, culture resistant bacteria, expensive media and cultivation apparatus, and laborious methodology (Frohlich and Konig, 2000; Handelsman, 2004; Madigan and Martinko, 2006).

Culture independent techniques are capable of achieving a more comprehensive analysis of the composition of microbial communities (Orphan et al., 2000). They rely on molecular tools to assess community composition, usually by amplifying microbial biomarkers from the total DNA of environmental samples. For example, a culture-independent molecular phylogenetic survey by Hugenholtz et al. (1998) investigated the bacterial community in Obsidian Pool (OP), a hot spring in Yellowstone National Park. Small-subunit rRNA genes were chosen as the

molecular biomarkers and amplified directly from OP sediment DNA by PCR with universally conserved primers. Thirty percent of the gene sequences generated were unaffiliated with recognized bacterial divisions, and comprised 12 novel, division level lineages, termed candidate divisions, substantially expanding the knowledge of bacterial diversity. Cultivation independent approaches provide the most accurate information about community structure and diversity; however, these approaches are not without limitations. Most molecular based approaches, such as metagenomics and functional gene amplification, can provide information about the genetic capacity of culture resistant community members, but lack the power to determine if, or when, these capabilities are utilised in nature (Abdo et al., 2006; Aiken, 2011; Auchtung et al., 2011; Cardinale et al., 2004; Fisher and Triplett, 1999; Frostegard et al., 2011; Handelsman, 2004; Hugenholtz, 2002; Marsh, 2005; Quince et al., 2009). However, technologies such as transcriptomics can identify when particular genes or genomic regions are being actively transcribed, and hence expressed in the environment, albeit this is at the community level and lacks the phylogenetic power to decipher what organisms are expressing particular genes (Raes and Bork, 2008).

Until further advances and improvements are achieved, it is impossible to thoroughly investigate high temperature ecosystems based on either cultivation dependent or cultivation independent techniques alone. However, an integrated approach in which both techniques are simultaneously applied is becoming common in microbial ecology (Hirayama et al., 2007; Takai et al., 2004). Overall, this multifaceted strategy reduces bias, and provides a more robust analysis of complex microbial ecosystems.

1.4 Culture Dependant Techniques

The culture dependent approach is central to many biological investigations, and has been applied to a range of microbial ecosystems, as it provides direct and detailed information about the genetic and physiological capabilities of cultivable organisms. Although cultivation dependent techniques are crucial to microbial studies, they currently have several limitations. As a result, they can only be applied independently to a small proportion of the diverse range of microorganisms present on Earth. High temperature microbial ecosystems, some of the most poorly understood ecosystems on the planet, are composed of a significant percentage of members, including keystone organisms, that have proven to be incredibly resistant to cultivation efforts. However, due to advances in both culture dependent and independent techniques, some of the fundamental problems associated with cultivation based work are beginning to be resolved.

1.4.1 Advances in Culture Dependent Techniques

Information gained from studies of high temperature environments by culture dependent and independent approaches is being utilised to develop new strategies to improve cultivation techniques for these environments (Davis et al., 2005; Fujii et al., 2011; George et al., 2011; Janssen et al., 2002; Kaeberlein et al., 2002). For example, a recent cultivation study found prokaryotic organisms significantly smaller than the commonly accepted size for prokaryotes, similar to the recently detected thermophilic obligate symbiont Archaea, the Nanoarchaea. Recent studies suggest that nano-sized organisms may be cultivable using

modern media conditions, but are too small to be identified by microscopic analysis, or detected using generic molecular techniques (Brochier et al., 2005; Huber et al., 2002). There is now an array of improved techniques for successful isolation of culture resistant microorganisms. New strategies such as pre-enrichment, use of nutrient poor media, and extended incubation times, appear to be the most successful strategies for isolating culture resistant organisms from a range of environments. Other strategies such as utilizing solid media instead of liquid media, polymeric carbon sources (such as xylan) instead of easily degradable carbon sources, and Phytagel gel instead of agar, also hold promise (Dunbar et al., 1997; George et al., 2011; Janssen et al., 2002; Jensen et al., 2003; Stott et al., 2008).

When attempting to cultivate microorganisms from the environment a pre-enrichment step can be applied, where the initial community is incubated under specific laboratory conditions before media inoculation is conducted. This appears to assist microorganisms to survive in, and adapt to, an artificially simulated environment. After an enrichment period, the microorganism can be separated from the enriched community using several different approaches, including sample dilution (Janssen et al., 2002), cell encapsulation in gel micro-droplets (Kaeberlein et al., 2002), optical tweezers and micro manipulators (Frohlich and Konig, 2000), and cell sorting using flow cytometry (Hugenholtz, 2002). Using these techniques, isolation of a diverse range of thermophilic and non thermophilic prokaryotes has been accomplished (Dunbar et al., 1997; Harmsen et al., 1997; Jensen et al., 2003; Jolivet et al., 2004; Kaeberlein et al., 2002; Ward et al., 1997), revealing information about the evolution and genetic capabilities of these once poorly understood organisms.

One of the major improvements in isolating previously uncultivable prokaryotes from environmental samples is the shift from nutrient rich media to minimal media (Fujii et al., 2011; George et al., 2011; Janssen et al., 2002; Stott et al., 2008). Low nutrient media conditions may better simulate the natural conditions of microbial microhabitats. Environments, particularly hot ecosystems, are usually nutrient poor, and it has been shown that excess nutrient availability can be detrimental to the growth of fastidious microorganisms (Joseph et al., 2003; Madigan and Martinko, 2006). As a result, a diverse range of previously uncultivable microorganisms have been isolated from both well known and newly discovered microbial ecosystems (Allan et al., 2005; George et al., 2011; Lee et al., 2011; Logan et al., 2000; Oren, 1983; Stott et al., 2008; Yabe et al., 2011). For example, two novel thermoacidophiles were isolated from northern Victoria Land, Antarctica using diluted defined media (Allan et al., 2005). This study showed that all isolates recovered had better growth rates on diluted media in comparison to full strength media. Another recent study successfully isolated, on nutrient poor medium, two novel bacteria belonging to the Chloroflexi phylum, from Japanese geothermal soils (Yabe et al., 2011). These bacteria show poor phylogenetic and physiological similarities to other members of the Chloroflexi phylum, leading to the suggestion that the isolates should be placed into a new order, *Thermogemmatisporales* ord. nov., of the class *Ktedonobacteria.*

Extended incubation time has proven crucial in recovery of thermophilic culture resistant microorganisms. Extended incubation allows sufficient time for fastidious slow growing organisms that are usually found in these environments to survive transition into laboratory conditions, and build up enough biomass to

be detected by current techniques (Davis et al., 2005; Davis et al., 2011). To prevent such organisms being out competed by fast growing, opportunistic organisms, it is beneficial to use a combination of minimal media and extended incubation time (Stott et al., 2008). For example, the first isolated representative of the poorly understood OP10 phylum was cultivated from New Zealand geothermal soils, using modified cultivation techniques (Stott et al., 2008). In this case, an extended incubation period, at a minimum, two weeks, and modified minimal media with complex carbon sources, instead of simple sugars, appeared to be key elements in successfully isolating this extremely fastidious, slow growing organism.

Previously uncultivable organisms of both thermophilic and non-thermophilic nature are now being isolated, allowing new discoveries to be made regarding their physiological, metabolic, and evolutionary characteristics. The findings are continuing to broaden the current understandings of the upper tolerances of microbial life and the importance of their role in various environmental processes on Earth (Bonch-Osmolovskaya et al., 2003; Chen et al., 2004; Gocheva et al., 2009; Kimble et al., 1995; Kozubal et al., 2008; Martens-Habbena and Stahl, 2011; Rao et al., 2009; Slobodkin et al., 1999; Tarlera and Denner, 2003; Zhang et al., 2009).

1.5 Culture Independent Analysis of Microbial Communities

Advances in molecular biology have provided microbial ecologists with the necessary tools to more accurately investigate microbial ecosystems, without the need for cultivation (Johnson et al., 2009). Progress in molecular based technologies has allowed identification of numerous uncultured thermophilic and non-thermophilic organisms, which has expanded the known diversity of microorganisms (Auchtung et al., 2011; Brambilla et al., 2001; Marsh, 2005; Priscu et al., 1999; Soo et al., 2009). As a result, microbial ecology has had a boom in molecular based research, an emergent field known as molecular ecology. Molecular ecology is an exciting, rapidly evolving field defined as the application of molecular based techniques to research of ecological context (Hugenholtz, 2002; Madigan and Martinko, 2006; Marsh, 2005).

1.5.1 Molecular Biomarkers

Culture independent approaches achieve more accurate, sensitive and reproducible representation of community structure and diversity by direct extraction of total DNA from environmental samples. Specific phylogenetic markers present in each member of the community are then simultaneously amplified by polymerase chain reaction (PCR) from the total DNA. Typically, the 16S rRNA gene is used as the phylogenetic marker in culture independent methods to study prokaryotic communities; however, other regions of the ribosomal operon, including the 5S rRNA gene, and 16S - 23S rRNA intergenic spacer (ITS) region, can be used, depending of the scope of the study (Aiken, 2011; Fisher and Triplett, 1999; Hugenholtz et al., 1998; Marsh, 1999; Olsen et al.,

1986; Ranjard et al., 2001). The 16S rRNA gene encodes the RNA portion of the small subunit of the ribosome complex, an essential cellular component necessary for biological processes of all known prokaryotes. This gene is highly conserved across all groups of prokaryotes, with universally conserved primer binding sites flanking hyper variable regions that allow closely related prokaryotes to be differentiated with sufficient sequence length to produce robust phylogenetic trees (Olsen et al., 1986; Woese, 1987). Because this is one of the most implemented culture independent biomarkers, there are large reference databases holding hundreds of thousands of 16S rRNA gene sequences, and several standardised primer sets and experimental protocols (Brambilla et al., 2001; Madigan and Martinko, 2006). Non-genetic biomarkers can also be used to determine microbial diversity, such as phospholipid fatty acids (PLFA)(Frostegard et al., 2011). PLFA profiling is a popular lipid based fingerprinting method that allows fast and sensitive analysis of microbial community structure and diversity. As with DNA based approaches, non genomic molecular biomarkers have inherent limitations. Problems include over estimation and inconsistencies of biomolecule turnover in the environment, weak phylogenetic resolution, and poor measurements of diversity (Frostegard et al., 2011; Ruess and Chamberlain, 2010; Zelles, 1999).

There is now a significant range of molecular tools that can be applied to microbial ecology studies, however each technique provides a different resolution, level of bias, and range of limitations. It is essential to utilise the approach that best suits the research objectives and questions, otherwise the data generated and downstream analysis is likely to be inaccurate and non-reproducible using other molecular approaches.

1.5.2 **DNA Fingerprinting**

A commonly used technique in molecular ecology is DNA fingerprinting, which provides a sensitive, cost effective, time efficient and reproducible picture of microbial community diversity and structure. This approach allows for the investigation of microbial communities, such as high temperature ecosystems, which are resistant to most cultivation dependent analyses. These methods generally utilise the sequence variation found within hyper-variable regions of the 16S rRNA gene, and associated ITS regions of the ribosomal operon (Madigan and Martinko, 2006). These phylogenetic markers can be simultaneously amplified by PCR from the total community DNA. Using the PCR amplicons, these methods approximate how many different community members are present in the sample and their relative abundance, giving a DNA fingerprint of the microbial community. There are several different approaches including, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (tRFLP) analysis, and automated ribosomal intergenic spacer analysis (ARISA). Both tRFLP and ARISA are now among the most commonly used high resolution fingerprinting tools within the field of molecular microbial ecology. Both use a fluorescently labelled primer to amplify variable regions within the genomes of the community members in order to generate a genetic fingerprint of the community.

tRFLP methods generally use primers for amplification of the 16S rRNA gene, which is then digested by endonuclease enzymes at specific restriction cut sites, with the labelled primer attached to the terminal digested fragment of the gene (Abdo et al., 2006; Aiken, 2011; Marsh, 1999). ARISA methods use primers that

amplify the intergenic hypervariable region located between the 16S and 23S rRNA genes of the ribosomal operon (Cardinale et al., 2004; Fisher and Triplett, 1999). In the best case, each amplified and labelled sequence varies in size, depending on the organism it originated from. This DNA is analysed using a DNA sequencer/fluorescent detector, where the sequences are differentiated based on size. From the electropherogram readout, each peak represents a community member, or group of closely related community members, and the peak intensity represents relative abundance of that organism. Both tRFLP and ARISA can be used to approximate microbial diversity and species abundance. These approaches are not without bias or limitations, which must be considered in data analysis (Blackwood et al., 2003; Danovaro et al., 2006; Kovacs et al., 2010). One major limitation of DNA fingerprinting is that, although it proves proficient at reproducibly estimating community structure and diversity, it has weak phylogenetic resolution power and should not be used for phylogenetic classifications.

1.5.3 **16S rRNA Gene Clone Libraries and Sanger Sequencing**

Construction of 16S rRNA gene clone libraries was developed for accurate and reproducible phylogenetic analysis of microbial communities without the need for cultivation. This is achieved by simultaneously amplifying the 16S rRNA gene of every member in the microbial community from total community DNA. The amplicons are then cloned into an appropriate plasmid, transformed into a bacterial cell (usually *Escherichia coli*), and grown on an agar plate. Each colony on the plate represents a unique 16S rRNA gene sequence from the original

community. DNA can then be extracted from each colony and sequenced (by Sanger sequencing) to phylogenetically identify specific members of the community (Handelsman, 2004; Pace et al., 1985; Schmidt et al., 1991).

Sanger sequencing is a chain termination sequencing method, developed by Sanger and co-workers in 1977, and requires a single stranded DNA template, DNA primer, DNA polymerase, normal deoxynucleotidetriphosphates (dNTPS) and modified fluorescently labelled dideoxynucleotidetriphosphates (ddNTPS) that terminate DNA strand elongation. Classical chain-termination sequencing relies on the incorporation of the labelled ddNTPs randomly into the newly synthesized and complementary DNA strand causing sequence termination. This process is repeated multiple times until there are newly synthesised DNA fragments of every possible length, flanked by the labelled chain terminating ddNTP. These fragments are then separated by charge using capillary electrophoresis, and detected by a fluorescent detector (Blessum et al., 1999; Martin et al., 1985; Maxam and Gilbert, 1977). This is an accurate but time consuming sequencing technology, as only one DNA template can be sequenced at a time.

1.5.4 **Pyrosequencing**

Phylogenetic analysis of diverse microbial communities can now be achieved without cloning, by high throughput pyrosequencing. This approach uses a combination of molecular techniques which allow multiple DNA templates to be sequenced simultaneously. Pyrosequencing differs from traditional Sanger sequencing, as it relies on the detection of pyrophosphate light signals generated

during the incorporation of nucleotides into a complementary DNA sequence. As DNA synthase incorporates a single nucleotide into the DNA strand complementary to the template, a pyrophosphate (PPi) is released, converted into ATP by ATP sulfurylase, then used as substrate in a reaction catalysed by firefly luciferase, which generates light as a product (Nyren, 1987). The light is detected by a luminometer in real-time. Because this method does not rely on chain termination, and hence electrophoresis, multiple DNA templates can be simultaneously sequenced (Ronaghi et al., 1996). This new technology has revolutionised molecular ecology diversity studies as it allows high throughput analysis of complex communities at a fraction of the cost and time involved with Sanger sequencing. In addition, advances in pyrosequencing platforms now allow multiple samples to be run simultaneously, and at increasingly higher read depth to provide unprecedented data output (Degnan and Ochman, 2012; Galand et al., 2009; Liggenstoffer et al., 2010; Novais and Thorstenson, 2011; Stoeck et al., 2009).

High throughput DNA sequencing has also been instrumental in the emergence of genomic research, making it feasible to sequence the genome of a single organism, or the genetic makeup of entire communities (Handelsman, 2004; Hudson, 2008). This approach allows microbial ecologists to investigate the complete genetic capacity of an organism or community, providing significantly more information than 16S rRNA based phylogenetic studies. Genomic research has evolved rapidly, revolutionising sister fields such as metagenomics, the simultaneous sequencing and analysis of all the genomes present in a given community or environmental sample (Handelsman, 2004), and meta-transcriptomics, the simultaneous sequencing and analyses of one or more

organisms' total mRNA at a given time (Raes and Bork, 2008; Wang et al., 2009). Pyrosequencing based culture independent approaches have undoubtedly transformed the way molecular ecologists are able to investigate microbial ecosystems.

1.6 Geothermal Areas

Geothermal habitats exist worldwide. However, they are highly concentrated in areas of high tectonic activity which result in major movement of the earth's crust. Such places include Japan, northern America, Iceland, Italy, Russia, New Zealand, Antarctica and its circumpolar islands (Boothroyd, 2009; Giggenbach, 1976; Kristjansson, 1992; Vila et al., 1992). At these sites, deep underground magmatic material is forced up through, or close to, the earth's surface. This uplifted volcanic material acts as the heat source for the geothermally heated habitats. The ground water in this zone can reach above boiling temperatures, but does not boil due to lithostatic pressure. When the liquid reaches high enough temperatures, the pressure forces it through pores and fissures in the earth's surface where it produces steaming geothermal soil, hot springs or geysers, depending on the water supply. If water supply is low, as generally occurs in the systems present on the volcanoes of Antarctica and its circumpolar islands, the water is vented through the earth's surface as steam. This steam results in constantly heated soil through which it is passing. As hot liquid percolates through the earth, deep surface minerals dissolve and are deposited into the subsurface and surface soil, resulting in extensive soil mineralization and alteration. To date, little is known about the diversity and ecology of the

microbial systems that exist in these geothermal habitats as few studies have investigated the microbial environment of geothermal soils.

1.6.1 **Geothermal Areas in Antarctica**

Antarctica is the southernmost continent on the planet and the 5th largest in terms of area (Stonehouse, 2002). The continent is unique because it is has some of the harshest environmental conditions that exist on the most isolated landmass on the planet. Antarctica has been isolated from other land since it separated from Gondwanaland and other sub continents over 84 million years ago (Hansom and Gordon, 1998; Veevers, 2004). Antarctica has a range of active volcanoes on the continent itself, and on its circumpolar islands, including Mount Erebus (Ross Island), Mount Rittman and Mount Melbourne (northern Victoria Land), and Deception Island volcano, one of the South Shetland Islands (Logan et al., 2000; Soo et al., 2009; Van De Vijver and Mataloni, 2008). Each of these volcanoes has geothermal soils which host extremely isolated thermophilic microbial communities. Because of the incredible isolation and unique set of evolutionary selection pressures, some researchers believe that these habitats may host some of the earliest forms of life on the planet (Soo et al., 2009). The microorganisms in these communities have been poorly characterised, or in some cases remain uncharacterised. However, every volcano investigated using culture techniques has yielded aerobic endospore forming bacterial isolates (Cameron and Benoit, 1970; Hudson et al., 1989; Logan et al., 2000; Van De Vijver and Mataloni, 2008). It is difficult to assess the significance of such bacteria; however, they are highly likely to be minor components of these

microbial ecosystems, but were the only members isolated with the techniques employed. Recently, cultivation independent studies have begun to reveal the unique and diverse microbial communities present in the geothermal soils of these volcanoes (Munoz et al., 2011; Soo et al., 2009).

1.6.2 Mount Erebus and Tramway Ridge Geothermal Subsurface Soils

Mt Erebus (3794m elevation) is the most active volcano on the Antarctic continent, hosting the only phonolitic lava lake on the planet which experiences up to ten strombolian eruptions per day (Calkins et al., 2008). Tramway Ridge is a gently sloping, extensively mineralised, ice free area of geothermally heated soil on Mt Erebus. Tramway ridge has some of the hottest soil found on Mt Erebus, at 65°C, with other soil varying in temperature from 20°C to 65°C. The soils found at Tramway Ridge are the most geographically and physically isolated geothermal soils on Earth (Figure 1) (Soo et al., 2009). It is located approximately 1.5 km northwest of the main crater (Figure 2) at an elevation of 3350 m above sea level. The area is approximately 10 000 m^2, and contained within a 40 000 m^2 Antarctic Specially Protected Area (ASPA 130). The top half of the ASPA has restricted entry, and the bottom half allows entry by permit only. Tramway ridge and the surrounding area has been designated an ASPA due to its unique temperature and ice-free ecosystem (SCAR, 2002).

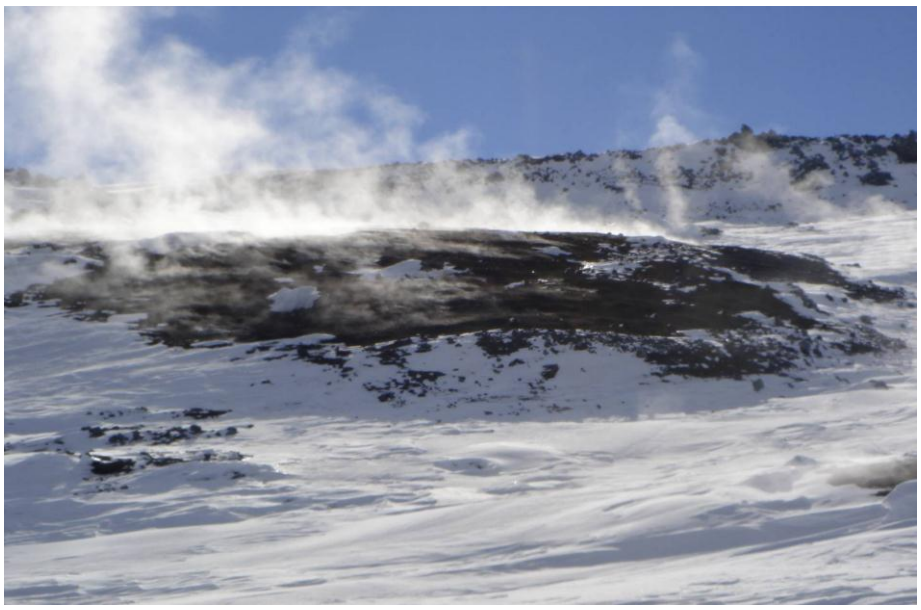

Figure 1. Steaming geothermal soil sampled from Tramway Ridge, Mount Erebus, Antarctica.

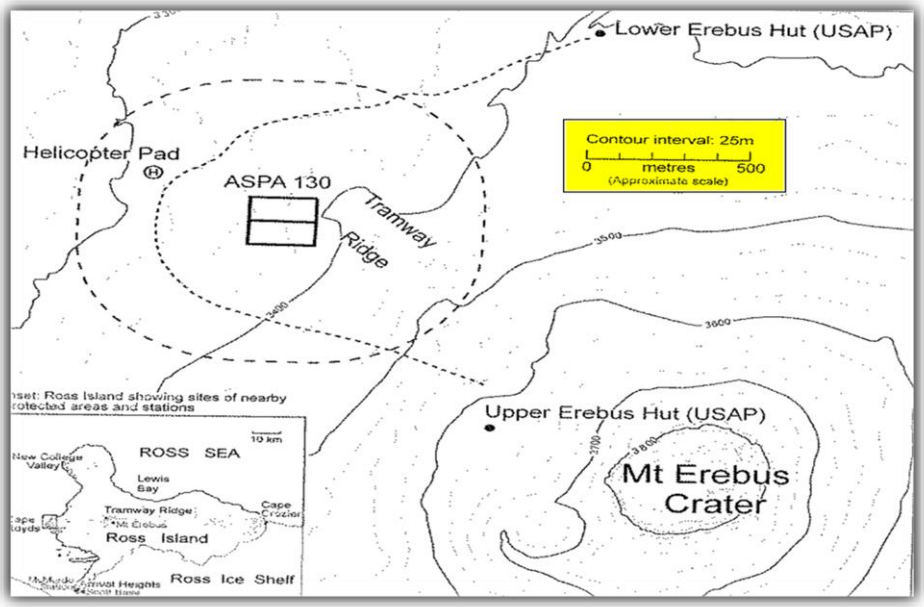

Figure 2. Location of Tramway Ridge and Upper Erebus Hut on Mt. Erebus, Antarctica.

The value of this habitat lies in the unique microbial system that was identified in early research (Broady, 1984; Lesser et al., 2002; Melick et al., 1991; Skotnicki et al., 2001). These soils have been separated from other volcanic habitats for over 30 million years, and are exposed to a rare combination of extreme

environmental conditions, including low nutrient availability, limited oxygen, prolonged periods of darkness, and extreme temperature gradients (Hansom and Gordon, 1998; Parish and Bromwich, 1991; Soo et al., 2009). The nature of this system provides a unique opportunity to study the microbial ecology of the most isolated, southern hot temperature soils on Earth.

1.6.3 **Research on Tramway Ridge Geothermal Soils**

Previous 16S rRNA gene cloning efforts of geothermal soils from Tramway Ridge, by Soo and colleagues (2009), revealed a unique microbial community with all bacterial sequences showing less than 92% sequence similarity with their closest known relatives in the NCBI database. For example, the largest clade within the Bacteria of the community branched within an unknown group between candidate division OP10 and Chloroflexi phylums (Figure 3). A second highly represented clone also has poor phylogenetic resolution (less than 86%) to any sequences in the NCBI database, and is loosely grouped into the Planctomycetes phylum. Most of these loose affiliations are deep branching with organisms identified from deep-subsurface ecosystems, suggesting the Tramway Ridge community may be archaic and sub surface in origin. It has been suggested that the members of this community are relic and appear frozen in time from an evolutionary standpoint.

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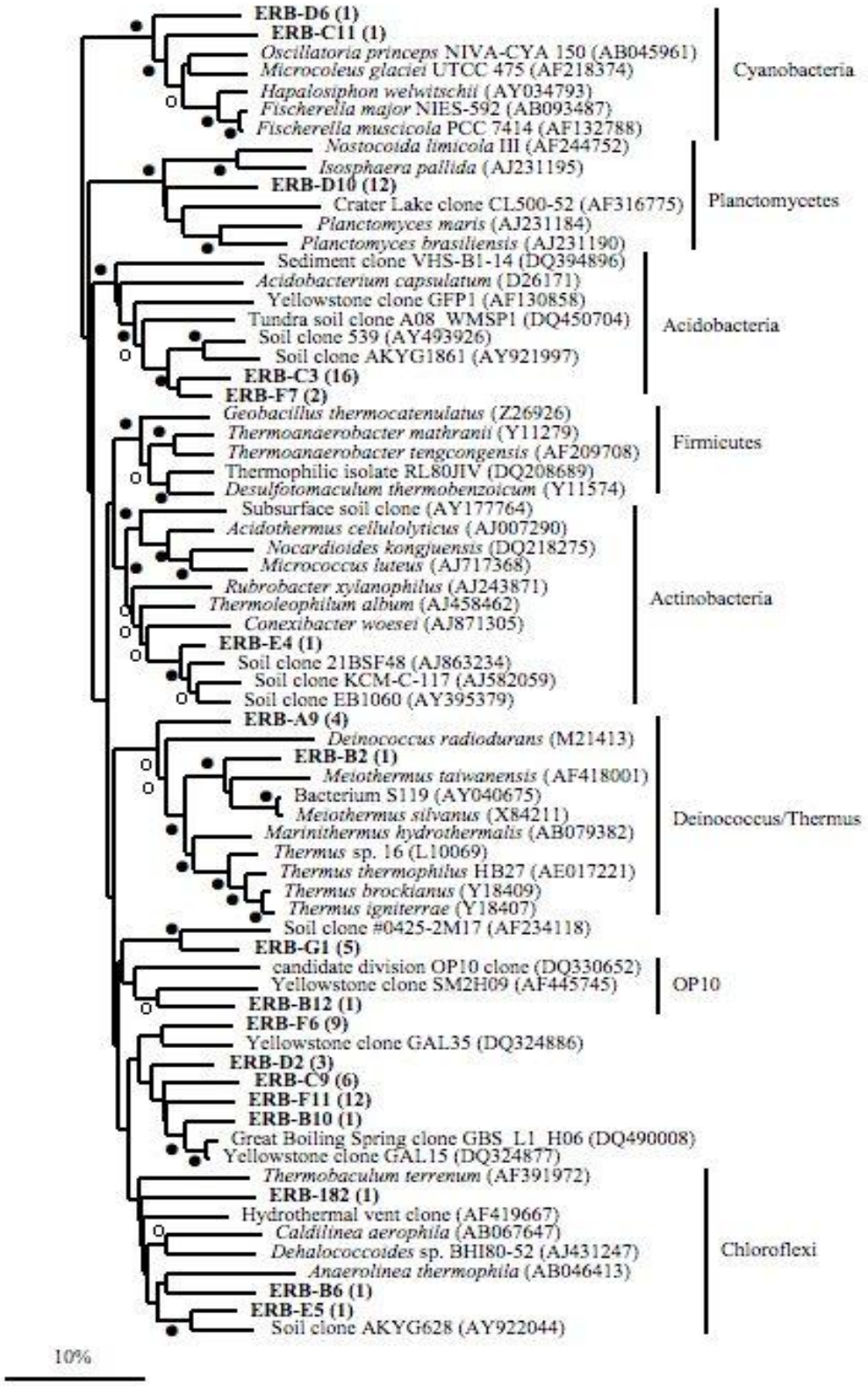

Figure 3. Phylogenetic tree showing the placement of 16S rRNA gene clones from Tramway Ridge, within the Bacteria (Soo et al., 2009). Branch points with support (bootstrap values of >90%) are indicated by filled circles; open circles show marginal support (bootstrap values of 70 to 90%). Branch points without circles are not resolved (bootstrap values <70%).

Recently, pyrosequencing has been completed on 65°C soil samples collected from Tramway Ridge (Craig Herbold per comm.). The soil was collected from the same sites sampled by Soo and colleagues (2009). Using the Roche GS Junior 454-Ti platform, community DNA was amplified and sequenced for metagenomics. Using the preliminary metagenomic data, it was possible to assess the community's putative metabolic and physiological capabilities. This has provided insight into the functioning of the community and how the microbes interact with each other, and with the environment. Mining of the metagenome is still a work in progress but has already begun to reveal interesting insight into the genetic potential of the community. Using the same sequencing platform, 16S rRNA hyper variable V5 and V6 gene regions were amplified and sequenced for in-depth phylogenetic analysis (Figure 5). Phylogenetic analysis of the 16S rRNA gene sequences identified 17 known phylogenetic lineages and two unresolved lineages using Bayesian likelihood analysis. All phylogenetic lineages were supported with >99% Bayesian posterior support.

While many of these lineages were shared between the three samples, the relative abundances of these lineages differ dramatically with depth (Figure 4). The surface sample appears to be dominated by thermophilic members of the Meiothermus Phylum, followed by high abundances of Cyanobacteria, Acidobacteria, and Bacteroidetes: organisms which are cosmopolitan in nature. The subsurface samples, however, show a community dominated by a novel archaeal sequence not detected in previous cloning efforts. Sequences have very low (<90%) sequence similarity to known archival sequences. These sequences belong to a clade that may branch from the base of the Thaumarchaeota, a

phylum that plays an important role in nitrogen cycling, and has both abundant and diverse representation in nature. Phylogenetic positioning suggests this thermophilic organism is either the deepest branching descendant of the group, or forms a new deeply branching lineage of Archaea. Other highly represented sequences from the subsurface appear significantly novel, such as unknown group 1 and 2, and cannot be phylogenetically resolved at phylum level using the classification methods employed in this study. These novel sequences appear to be deep branching and suggests endemic, and potentially ancient organisms, are dominating the subsurface soils of Tramway Ridge.

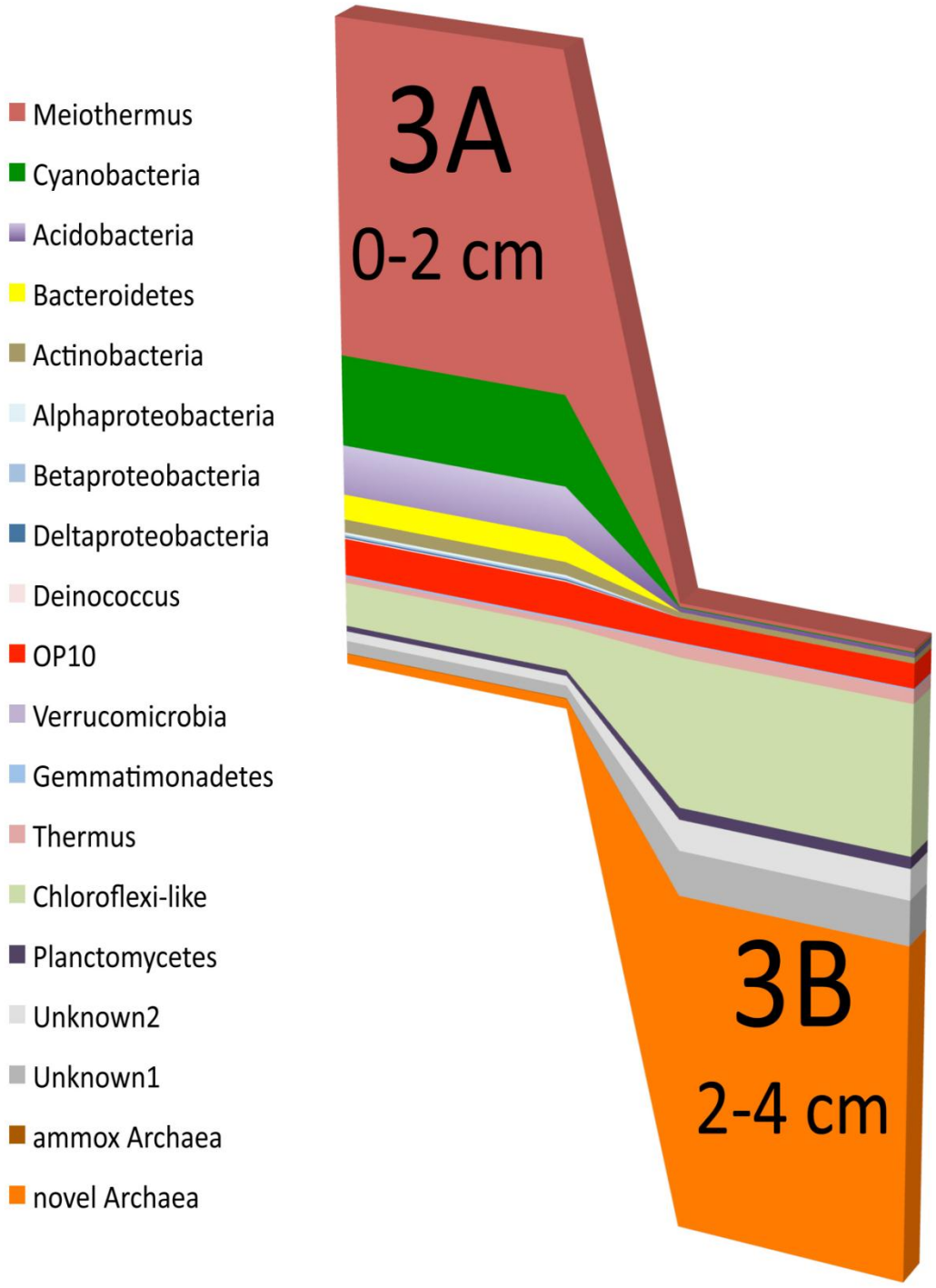

Figure 4. Representation of Tramway Ridge depth dependent microbial community structure. Data generated from 16S rRNA partial gene pyrosequencing (Roche GS Junior Ti-454 platform) from total environmental DNA. Members present at each depth are represented by a different colour, and abundance is represented by the coloured area. Surface and subsurface soil communities differ significantly, with dominant members from each depth showing poor phylogenetic grouping to any known Bacteria (Herbold, et al, per com).

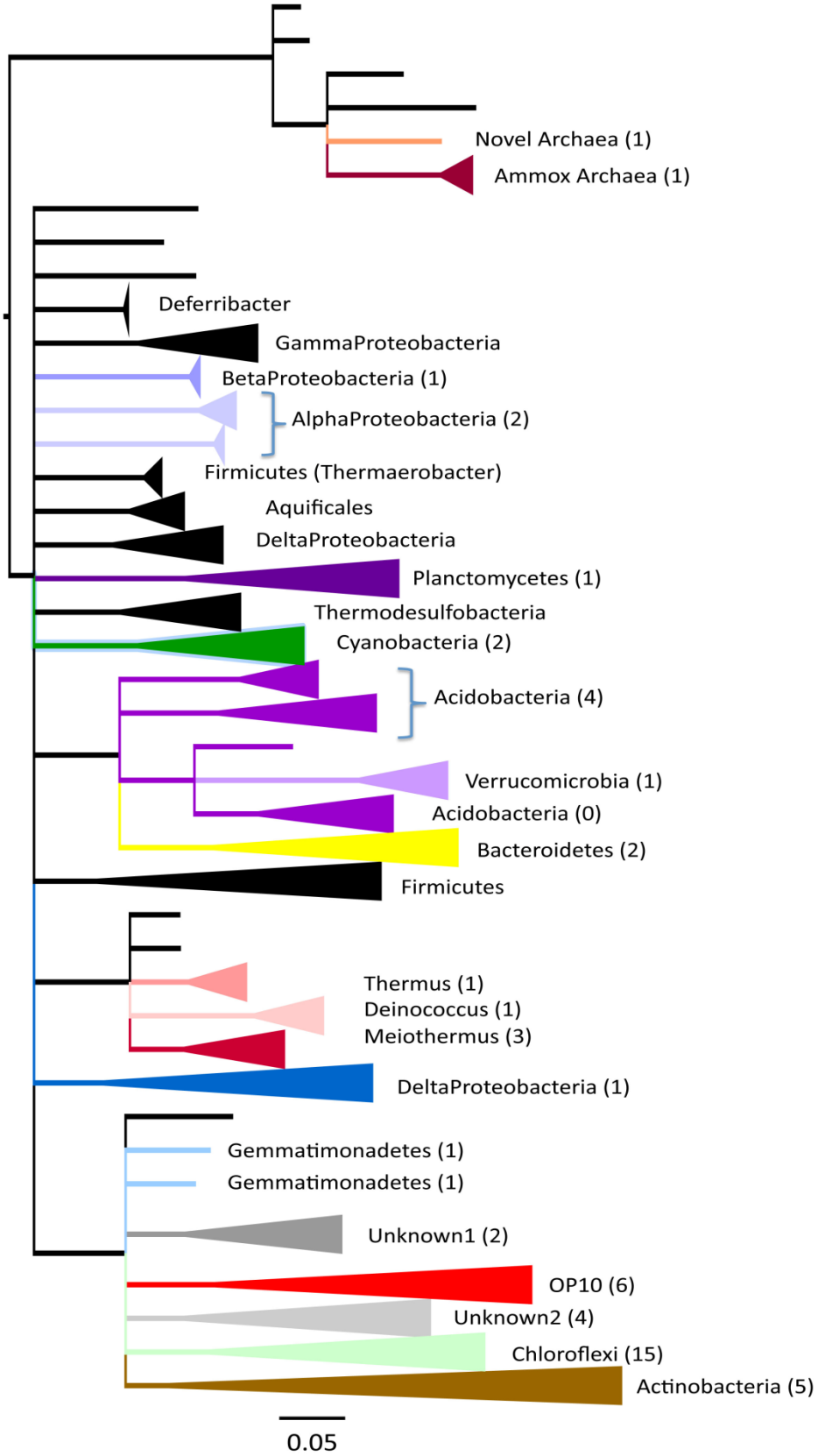

Figure 5. Phylogenetic comparison of the fifty five OTUs that exceeded 0.1% relative abundance using Mafft, to reference sequences identified using BLAST and the ribosomal database project (RDP). A Bayesian likelihood tree reveals 19 lineages with >99% Bayesian posterior support. The number of OTUs observed in each lineage is shown in parentheses.

1.7 Aims, Approaches and Outline of Thesis

This project aims to examine the metabolic and physiological requirements of the dominant Tramway Ridge thermophilic microorganisms, in order to better characterise this community, and validate assumptions generated from previous genetic analysis. Experiments include strategic cultivation, community based phenotypic arrays, and nutritional enrichment experiments. This multiplex, metabolic driven approach incorporates both culture dependent and culture independent techniques in order to investigate this intriguing system in a way that is crucial to the understanding of the thermophilic community and the environment controlling it.

The objective of this cultivation work was to investigate the organisms with novel physiological and metabolic capabilities detected by genetic analysis, and investigate their ecological significance and evolutionary relevance. As discussed previously, cultivation work has a range of limitations, and applying these techniques to such a novel microbial system is challenging. However, previous research on Tramway Ridge has yielded a substantial amount of genetic, geochemical, and physical data in order to develop a large scale strategic media design. As a supplement to the targeted cultivation experiment, a range of ecologically relevant Biolog Phenotypic Microarrays were utilised to conduct community wide metabolic screening analyses. This experiment was incorporated into the project to provide additional information about the metabolic requirements of the microbial community. For this study, the effect of pH and utilization of various Carbon, Nitrogen, Peptide, Sulphur and Phosphorus

compounds were tested on community respiration under a range of experimental incubation conditions.

The community based enrichment experiment was designed with the objective of detecting differences in community response to various nutrient treatment conditions. This experiment was designed to generate a better understanding of the nutritional requirements and metabolic functioning of the culture resistant members of the microbial community. Preliminary analysis was conducted using DNA fingerprinting (ARISA and tRFLP) followed by pyrosequencing (Roche GS Junior Ti 454 platform) to identify how individual members of the community were responding to particular treatments. Overall, an integrated approach was implemented, where both culture dependent and culture independent techmiques were used to develop a more comprehensive understanding of this intriguing, yet poorly understood microbial system.

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2 Isolation Studies and Community Based Metabolic Analysis

2.1 Introduction

Antarctica hosts a diverse range of habitats which are isolated and exposed to a combination of extreme environmental selection pressures. The continent has a number of volcanoes including Mount Erebus which is the most active volcano in Antarctica, and indeed in the entire Southern Hemisphere (Rowe et al., 2000). It has the most geographically and physically isolated geothermal soil on Earth, and is exposed to some of the most extreme environmental conditions on the planet (Wright and Pilger, 2010). These conditions provide a unique opportunity to investigate microbial ecosystems like no other on the planet. The primary objective of this project was to use both culture dependent and culture independent approaches to investigate the microbial community that exists in the geothermal subsurface soils of Tramway Ridge, Mount Erebus. The nature of this system provides a great opportunity to study novel biodiversity, unique adaptations, and infer evolutionary processes in an isolated context (Hudson et al., 1989).

2.1.1 Geothermal Soil of Tramway Ridge, Mount Erebus

The hot soils present on Mount Erebus and other volcanoes on the Antarctic continent have been separated from all other volcanic habitats, for over 30 million years (Barker and Burrell, 1977; DeConto and Pollard, 2003). These soils

are exposed to a combination of extreme environmental conditions, including low nutrient availability, limited oxygen, prolonged periods of darkness, and extreme physicochemical gradients (Hansom and Gordon, 1998; Parish and Bromwich, 1991; Soo et al., 2009). Tramway Ridge is a gently sloping, extensively mineralised, ice free area of geothermally heated soil. The ridge has some of the hottest soil found on Mount Erebus (annual average temperature of 65°C) and is located approximately 1.5 kilometres northwest of the main crater at an elevation of 3350 metres above sea level. The area is approximately 10 000 m^2 and has been designated an Antarctic Specially Protected Area (ASPA) (SCAR, 2002). The value of this habitat lies in the unique moss and cyanobacterial communities that have been identified in early research (Broady, 1984; Lesser et al., 2002; Melick et al., 1991; Skotnicki et al., 2001).

2.1.2 **Strategic Media Designs**

Previous unsuccessful cultivation attempts to isolate dominant members of the microbial community at Tramway Ridge can be attributed to the lack of fundamental knowledge of the system (Daniel and Hudson, 1988; Hudson et al., 1989; Soo et al., 2009). Extensive phylogenetic and metagenomic analyses have revealed several exciting putative capabilities and adaptations within members of the Tramway Ridge microbial community; however, without successful cultivation followed by functional studies of the members responsible for such adaptations, it is difficult to interpret these genetic findings. Although we will never be able to simulate all the exact parameters of the Tramway Ridge soil environment in the lab, we have accumulated a significant amount of data that

allows us to better understand the environment. This information has been used to modify and optimise the targeted media designs of this project.

2.1.3 **Development of Cultivation Strategies**

An abundance of information including, but not limited to, genetic, geo-chemical, and physical-chemical data has been generated from the geothermal soils of Tramway Ridge, Mt. Erebus. This information was instrumental in guiding a targeted media design with the objective of isolating the apparently endemic dominant members in the microbial community present in the 65°C subsurface soils of Tramway Ridge.

From nutrient and elemental analysis (ICP-MS), it was found that Tramway Ridge soil is significantly high in Iron and low in Sulphur. Total Carbon, Nitrogen, Sulphur and Potassium analysis revealed the soil to be very low in nutrient composition (Soo et al., 2009). This information has directed the media strategy to a variety of modified minimal media experiments. The oligotrophic nature of this habitat suggests that these organisms may be fastidious and slow growing. Previous studies that have been successful in isolating novel prokaryotes from the environment have found that both minimal media and extended incubation periods are instrumental in isolating previously uncultured organisms (Frohlich and Konig, 2000; Fujii et al., 2011; Janssen et al., 2002; Kaeberlein et al., 2002; Stott et al., 2008). For this experiment, both these approaches were incorporated.

2.1.4 **Selection of Organisms to be Targeted in Media Design**

The cultivation strategy was designed to target the dominant members of the community. The difficulty was that these targeted organisms, or even closely related organisms, have never been isolated. The result is a lack of information about the essential nutrient requirements and incubation conditions necessary to support growth of such poorly understood organisms. To overcome this lack of direct information, the dominant members of the community were thoroughly investigated using 16S rRNA gene clone and pyrosequencing amplicon data, and preliminary metagenomic information. This approach narrowed the target down to five phylogenetic groups to which the dominant members were assigned, and that appeared to be most likely amenable to isolation. Using these selection criteria, five phylum level groups were selected as targets for large scale strategic media design. Each of these phyla has high representation in the phylogenetic analyses of the Tramway Ridge microbial community, are poorly understood, and have unknown ecological roles. The objective of this cultivation work was to build on the information already known about these organisms in order to gain a better understanding of the function of this microbial system. The selected target phyla for the cultivation effort are as follows: Planctomycetes, OP10, Deinococcus-meiothermus, Chloroflexi, and a novel Archaeal lineage that at this time remains phylogenetically unresolved.

2.1.5 **Community Based Metabolic Screening**

As a supplement to our media experiment, we used Biolog Phenotypic Microarrays (PM) to provide additional information about the metabolic requirements of the microbial community of the subsurface soils at Tramway Ridge. These Biolog Microarray plates are a breakthrough technology that makes it possible to collectively assess the metabolic capabilities of microbial cells using a vast range of nutritional sources and physiological conditions (i.e. pH). Each Biolog plate consists of preconfigured well arrays in which each well tests a different metabolic phenotype. Each plate consists of 96 wells with one control well with no nutrient, and the remaining 95 wells each having a different nutrient and/or pH state to be tested on the microorganisms of interest. Each well also contains a redox dye to detect respiration, and if members of a community used to inoculate the plate are able to utilize a particular substrate to generate cellular energy, the well that contains that substrate turns from clear to purple. Redox dyes change colour when the redox potential of a cell significantly changes as a result of respiration (Berridge et al., 2005). Typically, these plates are used in a biomedical context, to test for industrial applications such as new drug candidates in cell lines (Lee et al., 2011), and optimizing cell lines and culture conditions in bio processing development (Bochner et al., 2001). It allows scientists to study the growth properties and culture condition responses of bacterial, fungal, and even human cells. As such, it is becoming a core technology for many cellular studies. For this study the effect of pH and utilization of various Carbon, Nitrogen, Peptide, Sulphur and Phosphorus compounds were tested on community respiration.

2.2 Materials and Methods

The project has a two stage approach, divided into two field seasons. The first field season (2009-2010) involved collecting soil samples from Tramway Ridge and other geothermal spots on Mt Erebus for physical-chemical and genetic analysis back in New Zealand. Samples were also collected for the first cultivation experiment and a preliminary community based metabolic profiling experiment. Both experiments were started in the field and continued back in New Zealand at the University of Waikato. The second field season (2010-2011) involved collecting more soil samples from several locations on the volcano for further genetic analysis, a larger scale cultivation effort and more directed metabolic profiling experiments.

2.2.1 Sampling

Samples were taken from both onsite (Tramway Ridge, Figure 7) and offsite (other 65°C soil) geothermal hotspots on the volcano. Soil was aseptically collected in 50 ml sterile CELLSTAR centrifuge tubes (Greiner Bio One, Germany) using sterile metal spatulas. Soil was maintained at 65°C until experiments were initiated, within an hour of collecting soils. Sites sampled in the past for preliminary cultivation and genetic analysis (Soo et al., 2009) were sampled again for this study. All initial experiments were conducted at Lower Erebus Hut (LEH) which is located 1 km north-east from Tramway Ridge (Figure 6).

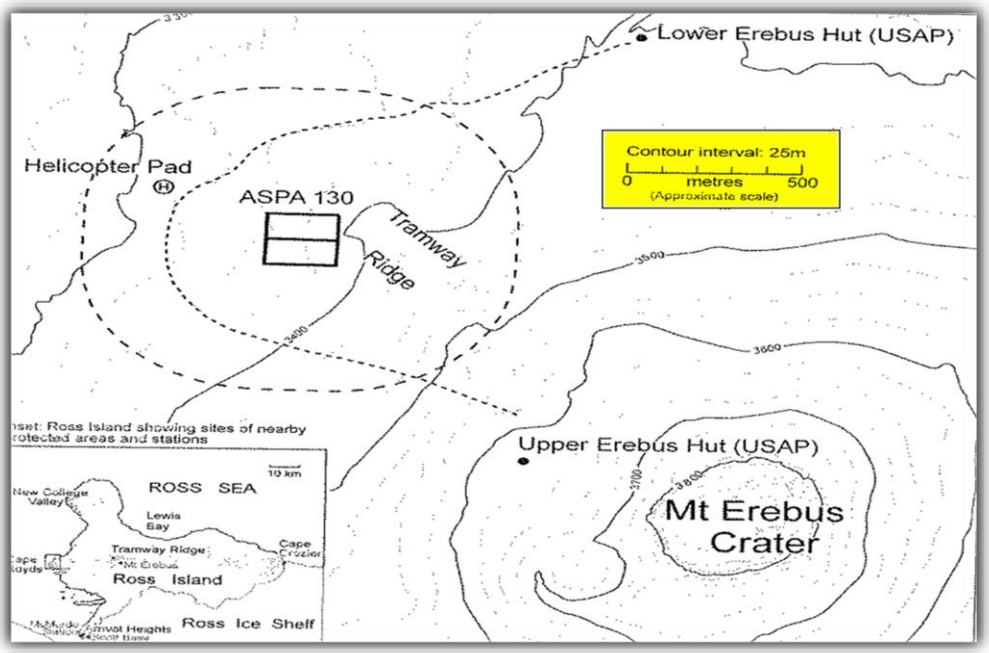

Figure 6. Location of Tramway Ridge and Upper Erebus Hut on Mt. Erebus, Antarctica.

Figure 7. Soil sampling at Tramway Ridge, Mount Erebus, Antarctica.

2.2.2 **Physical-Chemical Soil Analysis**

Soil oxygen concentration was determined on site while sampling using a Fibox 3 LCD trace minisensor oxygen meter with data-logger (PreSens Precision Sensing, Germany) calibrated for soil temperature and altitude. Subsurface gasses from the soil were drawn into a low volume chamber containing the sterilised oxygen probe which recorded the oxygen saturation in parts per million (ppm) or as a saturation percentage. Oxygen measurements were collected every 2 cm of depth. On site temperature measurements were taken using a hand-held Checktemp1C thermometer (Hanna Instruments, Rhode Island, USA), following the same profiling method outlined above for soil oxygen profiling. In field pH and conductivity measurements were conducted at LEH using the slurry technique commonly used for soil pH testing (Edmeades et al., 1985). Briefly, aliquots of 2 g from soil samples were weighed into pre-weighed vials. Mass was recorded and 5 ml of DI water was added to vials, followed by reweighing. Vials were then vortexed for one minute and left to settle for two hours. pH and conductivity measurements were taken for each vial in triplicate, with the average of the triplicate measurements used as the final value.

2.2.3 **Cultivation Experiments**

Season One

In year 1 (2009/2010 field season), a series of experiments were undertaken using five different media obtained directly from the literature (Table 1). We tested the ability of each media to grow Tramway Ridge organisms under anaerobic, aerobic, solid, and liquid conditions with fresh soil inocula at 65°C.

Table 1. Targeted media design for season one.

Medium	Description	Conditions	Target Organisms
VxylG	Minimal salt media with xylan as carbon source.	aerobic/anaerobic solid/liquid	Acidobacteria, Chloroflexi
Castenholz Medium D (CMD)	Nutrient rich Thermus media with yeast extract as carbon source.	Aerobic solid/liquid	Meiothermus Novel Archaea
AOM1	Minimal salt media. Ammonium rich. Yeast extract as carbon source.	aerobic/anaerobic solid/liquid	OP20, Chloroflexi
50% R2A	Nutrient rich. Glucose and starch as carbon source	aerobic/anaerobic solid/liquid	Actinobacteria Chloroflexi Novel Archaea Acidobacteria
Soil Media	Diluted, filter sterilised Erebus soil with no added nutrients or carbon source.	aerobic/liquid	All targeted members

All media was adjusted to pH 8.2 using 200 mM NaOH/100 mM KOH and sterilised by autoclaving at 121°C for 20 min. Media composition and preparation procedures used in the first season's cultivation experiment were as follows:

Modified VXylg liquid medium (Davis et al., 2005); to 1 L H_2O the following were added: 2.45 g TAPS buffer, 10 ml of 20 mM $MgSO_4$, 10 ml 30 mM $CaCl_2$, 10 ml of 20 mM $(NH_4)_2HPO_4$, 1 ml Se/W and SL-10 TE solutions, 0.05% (Wt/Vol) xylan (from oats spelt) as carbon source. For solid media 15 g Phytagel (with 2 g $MgCl_2$) was added. For anaerobic media 0.6 g L-Cystine and 1 ml 0.1% Resazuerin per litre was added and boiled until media colour turned from purple to light pink. Aliquots of 10 ml aliquots of media were then dispensed into Halogen screw cap test tubes and purged with nitrogen gas for 30 sec to remove remaining oxygen. Tubes were immediately sealed after purging and autoclaved. Media was allowed to cool to 60°C before adding 1.5 ml Vitamin 10 solution.

Modified AOM1 medium (Stott et al., 2008); to 1 L H_2O the following were added: 4 g $(NH_4)_2SO_4$, 0.25 g $NaHCO_3$, 0.05 g KH_2PO_4, 0.7 g $MgSO_4 \bullet 7H_2O$, 3 mg $CaCl_2 \bullet 2H_2O$. 10 mg yeast extract, 1 ml FeEDTA solution, 1 ml TE solution 2. For solid media Phytagel was added as mentioned above. After autoclaving media was allowed to cool to 60°C before adding sterilised B-vitamin mixture. Anaerobic media was made as mentioned above.

Modified 50% R2A medium (Stott et al., 2008); to 1 L H_2O the following were added: 0.25 g yeast extract, 0.25 g Difco proteose peptone, 0.25 g Casamino acids, 0.15 g K_2HPO_4, 0.25 g Glucose, 0.25 g soluble wheat

starch, 0.025 g $MgSO4•7H_20$, and 0.15 g sodium pyruvate. Solid and anaerobic media was also made as mentioned above.

Soil media; approximately 1 kg of previously collected hot Tramway Ridge soil was thoroughly mixed with 1 L H_2O. Supernatent was dispensed into 10 ml Kimax tubes and autoclaved.

Modified Castenholz Medium D (CMD) (Ramaley and Hixson, 1970); to 1 L H_2O the following were added: 0.1 g Nitrilotriacetic acid, 0.06 g $CaSO_4•2H_2O$, 0.1 g $MgSO_4•7H_2O$, 0.008 g NaCl, 0.13 g KNO_3, 0.69 g $NaNO_3$, 0.112 g Na_2HPO_4, 1 ml $FeCl_3$ (0.28 g/L) solution, 1 ml Nitsch's TE solution, 1 g trypticase peptone, and 1 g yeast extract. After autoclaving and cooling, solid media had 17.5 g agar added.

Season two

The results from the first season's cultivation efforts, continued metabolic screening, genetic data, geochemistry and physical-chemical analysis guided a more selective media design in season 2 (2010/2011 field season). pH ranges were also tested season one which helped us to modify the media design to better simulate the conditions of Tramway Ridge subsurface soils in season two. During field season 2, we tested the ability of each new media to grow Tramway Ridge organisms under anaerobic, micro-aerophilic, aerobic, solid, and liquid conditions with fresh soil inocula. Table 2 outlines the different media, including a description of each media, incubation conditions, and targeted organisms.

Table 2. Targeted media design for season two.

Media	Description	Conditions	Target Organisms
50% R2A	Nutrient rich. Glucose and starch as carbon source	Microaerophilic solid pH 6.0/7.5	Chloroflexi
		Aerobic liquid pH 6.0/7.5	Deinococcus-Meiothermus
		Anaerobic liquid pH 6.0/7.5	Novel Archaea
Planctomyces Media M31	Minimal media. Increased Iron and Ammonium conc.	Aerobic – solid pH 7.5	Planctomycetes
Minimal Salts Medium (MSM)	Minimal media with protein rich component. Yeast extract sole carbon source	Microaerophilic solid pH 7.2	Novel Archaea
		Anaerobic liquid pH 7.2	Novel Archaea OP10
Modified AOM1 media (OP10)	Minimal salt media. Ammonium, Iron and Potassium rich. Yeast extract and glucose as carbon source.	Anaerobic liquid pH 6.0/7.5	OP10 Chloroflexi
		Microaerophilic solid pH 7.5	
		Anaerobic liquid pH 6.0	
Castenholz Medium D (CMD)	Thermus media. Yeast extract as carbon source. Protein rich	Aerobic solid. pH 7.5	Thermus
Soil Media	Autoclaved Tramway Ridge soil. Solidified with Phytagel and supplemented with vitamins	Microaerophilic solid pH 7.5	All targeted organisms

The pH of all media was adjusted using 200 mM NaOH/100 mM KOH and 100 mM HCl, and sterilised by autoclaving at 121°C for 20 min. Media composition and preparation procedures used in the second seasons cultivation experiment were as follows:

Modified 50% R2A medium (Stott et al., 2008); prepared as described in season one with the following modifications: anaerobic media was purged with 20% CO_2/80% H_2 gas.

Modified M31 solid medium ((Kulichevskaya et al., 2009); to 1 L H_2O the following were added: 0.1 g KH_2PO_4, 1 g Chitin, 0.1 g trypticase peptone, 0.1 g yeast extract, and 15 g agar. After autoclaving, 20 ml Hutner's Basal Salts solution, and 0.5 g N-acetyl glucosamine were filter sterilised into the medium. Immediately prior to inoculation, 0.2 g/L of Ampillicin was spread on the agar plates.

Modified Mineral Salts Medium (MSM) (Dworkin, 2006); to 1 L of H_2O the following were added: 1.3 g $(NH_4)_2SO_4$, 0.074 g $CaCl_2 \bullet 2H_2O$, 0.28 g $MgSO_4 \bullet 7H_2O$, 0.28 g KH_2PO_4, 0.1 g yeast extract, 2.0 g trypticase peptone, 1 ml Nitsch's TE solution, and 1 ml $FeCl_3$ (0.28 g/L). Anaerobic media was made as described in season one, but purged with 20% CO_2/80% H_2. Solid media was made using Phytagel as described in season one.

Modified AOM1 medium (Stott et al., 2008); prepared as described in season one with the following modifications: 0.45 g KH_2PO_4, 3.4 mg $CaCl_2 \bullet 2H_2O$, 0.5 g glucose, 3 ml FeEDTA. Anaerobic media was purged with 20% CO_2/80% H_2.

CMD (Hudson et al., 1987); prepared as described in season one.

Solid Soil medium; approximately 400 g of previously collected and autoclaved 4°C Tramway Ridge soil was thoroughly mixed with 400 ml H_2O and 6 g Phytagel (15 g/l).

2.2.4 **Soil Inoculation**

All inoculation procedures were carried out aseptically and in the field. Each media was inoculated as follows:

Anaerobic liquid media: one vial of liquid anaerobic media was opened and approximately 0.5 g of soil was promptly added. The tube was closed immediately and gently mixed by swirling and left to settle for one minute. A 1 ml sterile needle and syringe was used to anaerobically transfer 0.5 ml of tube supernatant into a new anaerobic liquid vial (10^{-1} dilution). The tube was gently mixed by swirling. Both vials were then placed in a 65°C incubator.

Aerobic liquid media: one vial of liquid media was opened and approximately 0.5 g of soil was promptly added. The tube was closed immediately and gently mixed by swirling and left to settle for one minute. A 1 ml pipette was used to transfer 0.5 ml of tube supernatant into a new liquid vial (10^{-1} dilution). The tube was gently mixed by swirling. Both vials were then placed in a 65°C incubator.

Solid “dry” microaerophilic media: approximately 0.5 g soil was sprinkled into separate plates using sterile toothpicks to evenly spread soil particles on and into media. Inoculated plates were placed into an airtight box with sterile water filled vials to prevent drying out. Non catalyst, oxygen absorbing, CO_2 generating Campy Paks (Hardy Diagnostics, California, U.S.A) were placed inside box, purged with Argon and sealed. Boxes were incubated at 65°C.

Solid “dry” aerobic media: Prepared as described for solid “dry” microaerophilic inoculation, without Campy Paks and Argon purging.

2.2.5 Sub Culturing of Growth from Cultivation Experiment

Initially, all media were examined daily for growth. After one week of incubation, media was examined at four day intervals for new growth. Any growth detected was subcultured into new media on a weekly basis until it could be genetically screened back in the laboratory at the University of Waikato. For liquid media, growth was subcultured by transferring 0.5 ml of culture into a tube of fresh media. Anaerobic growth was subcultured via sterile 1 ml airtight syringes, and aerobic growth was subcultured using a 1 ml pipette and sterile barrier filtered pipette tips. Growth on solid media was transferred to new plates via sterile toothpicks using the quadrant streak method (Madigan and Martinko, 2006). Microaerophilic media was re-purged with Argon gas in the field and 20% CO_2/80% H_2 mixture back at the University of Waikato. Environmental incubation chambers sustaining microaerophilic growth had Campy Paks replaced every day for the first week, and at four day intervals thereafter.

2.2.6 DNA Extraction of Samples

DNA was extracted from media with suspected growth using a modified CTAB bead beating extraction protocol (Barrett et al., 2006) . After subculturing into fresh media, 5 ml of Liquid media was extracted when liquid became turbid and could be distinguished from the experimental control tube. Solid media was selected for extraction when visible colonies or films appeared on surface on

plate. Half of the biomass per plate was designated to subculturing, and the other half for extraction. The extracted DNA was quantified using a high sensitivity Qubit 2.0 fluorometer DNA quantification procedure (Invitrogen Ltd, New Zealand).

2.2.7 16S rRNA Gene PCR Amplification of Isolates

From each isolate the bacterial 16S rRNA gene was amplified from total DNA. Each reaction contained 0.3 µM of each 16S rRNA universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), 1X PCR buffer (Invitrogen Ltd), 0.2 mM dNTPs (Roche Diagnostics, New Zealand), 0.02 mg/ml bovine serum albumin (BSA), 1 U Platinum Taq (Invitrogen Ltd), 1.5 µM $MgCl_2$, 20 ng genomic DNA and the reaction was made up to 25 µl with Milli-Q H_2O. Thermal cycling conditions were as follows: 94°C for 3min, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 min 30 sec, and a final extension of 72°C for 5 min. All PCR reactions were run on a Bio-Rad DNA Engine (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA). Once amplified all PCR reactions were run on a 1.5% agarose gel at 70 V to ensure amplification success and then purified using a 5M Quickclean PCR Purification Kit (Genscript Corporation, New Jersey, USA).

2.2.8 RFLP Screening, Sequencing and Phylogenetic Analysis of Isolates

All isolate amplicons were first screened using the restriction fragment length polymorphism (RFLP) analysis to reduce redundancy and identify unique isolates. The PCR amplified 16S rRNA genes were digested using 2 different four base pair

cutting restriction endonuclease enzymes MspI and Sau96I (Roche Diagnostics GmbH, Mannheim, Germany). Each sample was digested using 1x NE buffer 4 (Roche, Germany), 2.5U Sau 96, 2.5 U MspI, 20 ng DNA and Milli-Q H_2O to make each reaction up to 10 µl. All PCR digests were run on a Bio-Rad DNA Engine (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA). Thermocycling conditions were 37°C for 3 hours, and 95°C for 20 minutes. Digested products were run on a 2.0% agarose gel at 40 V to identify banding patterns. Each different banding pattern identified was used to define an operational taxonomic unit (OTU). A representative PCR product from each OTU was sent to the University of Waikato DNA Sequencing Facility for bidirectional, pair end 16S rRNA Sanger sequencing. This gave 600 bp long pairwise sequences which were edited using Genesis (Drummond et al., 2009) and genetically identified by conducting a sequence alignment using the nucleotide (nt) BLAST search within the NCBI database.

2.2.9 **Biolog Community Based Phenotypic Microarray Experiment**

For the preliminary metabolic screening experiment, we used the following five different phenotypic microarray (PM) plates (Biolog Inc., Hayward CA, USA): two different Carbon plates (PM 1 and 2), one Potassium and Sulfur plate (PM 3), one Nitrogen plate (PM4), and one pH plate (PM10). The PM plates were inoculated in triplicate to ensure reproducibility. Plates were incubated at 65°C under both oxic and sub-oxic conditions and examined each day for evidence of respiration. Sub-oxic incubation was achieved by placing the plates in an airtight box, purging the box space with Argon gas and placing non catalyst, oxygen-consuming, CO_2

generating Campy Packs (Hardy Diagnostics, California, U.S.A) in the box before it was sealed.

Table 3. Biolog PM preparation for inoculum fluid. All solutions were filter sterilised.

Biolog Solution	**Ingredients**	**Concentration mg/ml**
Salts solution	$MgCl_2 \bullet 6H_20$	49.00
	$CaCl_2 \bullet 2H_2O$	17.60
N-standard solution	L-arginine	0.05
	L-Glutamic acid	0.10
Sulphur solution	L-cysteine	0.12
	5'AMP Na salt	0.37
	Sodium thiosulphate	0.24
	Adjusted to pH 8.5 with 200mM NaOH/100mM KOH	
Yeast extract/Tween Solution	Yeast extract	6.00
	Tween 80	3.00
Carbon Solution	Sodium pyruvate	13.20
	D(+)Galactose	7.20
	D(+)Cellobiose, Xylan (from oats spelt)	3.60

The following are the preparations of the microarray plate additive solutions which were filter sterilised and pippetted equally into each well of the appropriate microarray plates prior to inoculation:

PM additive solution 3; 5 ml of Biolog Salts, Carbon, yeast extract/Tween, and N-standard solutions were added to 30 ml sterile H_2O.

PM additive solution 1,2 and 10; 5 ml of Biolog Salts, Yeast extract/Tween, N-standard, and salts solutions were added to 30 ml sterile H_2O.

The soil inoculum was prepared by adding 15 g of 65°C Tramway Ridge soil to a 50 ml sterile CELLSTAR centrifuge tube (Greiner Bio One, Germany). Soil was thoroughly homogenised by adding 40 ml of sterile H_2O and vortexing for 30 min. The inoculum was left to settle for 5 minutes before transferring 30 ml of the supernatant to a new centrifuge tube. To all remaining PM inoculum fluids, 20 ml IF-Oa, 0.24 ml dye mix, 2 ml cellular inoculums, and 2 ml of relevant PM additive solution were added. To each corresponding PM plate, 100 µl of each inoculum fluid was dispensed into individual wells. Each plate type was placed under both aerobic and microaerophilic conditions for incubation. Preparation of microaerophilic conditions were as described in section 2.2.4 of this thesis. To all PM inoculum fluids, 20 ml IF-Oa, 0.24 ml dye mix, 2 ml cellular inoculums, and 2 ml of relevant PM additive solution were added. To each corresponding PM plate, 100 µl of each inoculum fluid was dispensed into individual wells. Each plate type was placed under both aerobic and microaerophilic conditions for incubation. Preparation of microaerophilic conditions were as described in chapter 2.2.4 of this thesis.

2.2.10 **Biolog Plate Collection of Positive Wells**

Biolog plates were visually checked each day for positive respiration (colour development in wells from clear to purple). Plates were removed individually and placed on white background to allow identification of positive wells, and any colour changes were documented daily. This process was carried out over one week, or until positive results ceased to continue. Plates were then transported back to New Zealand to be disposed of under the guidelines of the ERMA approval for this project.

2.3 **Results**

2.3.1 **Soil Oxygen Measurements**

Measurements of the degree of soil oxygen saturation at four distinct sites at Tramway ridge revealed, in all cases, that oxygen availability is low in the subsurface soils after correction for altitude and temperature (Table 4). The measurements show that the steam vents subsurface soils are significantly oxygen depleted but not strictly anaerobic i.e. <1 ppm oxygen within soil, compared to >9.5 ppm on average oxygen in atmosphere outside of soil site.

Table 4. Soil oxygen results of Tramway Ridge subsurface soils.

	Old site 1	Old site 2	Old site 3	New Site 4
Outside temperature (°C)	1	-11	-16	-1
Outside oxygen concentration (ppb)	9.7	11.1	13.4	9.7
Temperature (1-5cm)(°C)	65.5	63.5	62.7	63.7
Inside (soil) oxygen concentration (ppb)	1.03	1.2	1.2	1.2
Temperature (5-10cm)(°C)	64	62.4	NA	62.8
Inside (soil) oxygen concentration (ppb)	1.12	1.2	NA	1.2

2.3.2 **pH and Conductivity Measurements**

Preseason pH readings of the nine 65°C soil samples ranged from pH 7.5 to 8.1 (Table 5). These results determined the pH for the media used in season one. ERB old site 2 sample site consistently gave the highest pH readings. ERB1 sample site showed the lowest pH readings, with ERB old site 1A averaging pH 7.54, and ERB old site 1C averaging pH 7.87. The average for Tramway ridge 65°C soils collectively was pH 7.95 (Table 5).

Table 5. Preseason pH measurements of Mt Erebus soil samples used for cultivation and enrichment experiments. Sample ERB old site 3 adjacent refers to soil collected from a lower temperature site beside the old 65°C Tramway Ridge site 3. ERB old site 3 adjacent and ERB 10 cool were both depth profiled.

Sample	pH				
	1	2	3	4	Average
ERB old site 1A	7.05	7.93	7.64	7.55	7.54
ERB old site 1B	8.09	8.13	7.7	7.79	7.93
ERB old site 1C	8.03	8.12	7.67	7.67	7.87
ERB old site 2A	8.33	8.42	7.94	7.84	8.13
ERB old site 2B	8.38	8.27	7.83	7.85	8.08
ERB old site 2C	8.34	8.45	7.91	7.88	8.15
ERB old site 3A	8.29	8.25	7.75	7.79	8.02
ERB old site 3B	8.22	8.15	7.78	7.66	7.95
ERB old site 3C	8.16	8.03	7.78	7.67	7.91
Total average					7.95

Table 6 shows the results of infield pH and conductivity testing on both Tramway Ridge (onsite) and offsite soil samples. Infield conductivity measurements showed generally low values ranging from 44.2 µS to 382.3 µS. Depth profiling of soil sites showed that conductivity decreased with depth: the surface soil (0-5cm) for every site sampled, yielded the highest conductivity value, which consistently decreased with the depth of the sample. All off site samples showed dramatically lower conductivity values compared to Tramway ridge samples. Comparison of pH values between preseason and in field soil samples showed a drop in pH values for in field soil samples with all ERB old site in field samples averaging pH 7.83. On average, soil samples collected from low temperature sites showed relatively high pH, and non Tramway Ridge soil samples showed the lowest pH readings (i.e. all offsite samples).

Table 6. In field pH and conductivity testing Mt Erebus soil samples.

Sample	pH			Conductivity (μS)		
	1	2	Average	1	2	Average
ERB old site 1	7.76	7.71	7.74	183.2	168.0	175.6
ERB old site 2 (0-5cm)	7.86	7.81	7.84	230.6	183.1	206.9
ERB old site 3 adj (0-1cm)	7.78	7.83	7.81	243.9	206.0	225.0
ERB old site 3 adj (1-4cm)	7.93	7.9	7.92	197.2	182.0	189.6
ERB old site 3 adj (4-8cm)	7.87	7.83	7.85	172.3	189.2	180.8
ERB new site 4	7.69	7.78	7.74	211.1	184.7	197.9
ERB new site 5 (0-5cm)	7.52	7.65	7.59	549.0	215.5	382.3
ERB 10 cool (0-4cm)	7.92	7.95	7.94	160.1	160.6	160.4
ERB 10 cool (1-4cm)	7.81	7.79	7.80	154.3	150.9	152.6
ERB 10 cool (4-8cm)	8.05	8.02	8.04	115.7	129.1	122.4
ERB 10 (0-5cm)	8.01	8.08	8.05	164.5	136.2	150.4
ERB 11 (0-1cm)	8.07	8.11	8.09	125.5	132.0	128.8
ERB 11 (1-4cm)	7.93	7.98	7.96	120.5	97.5	109.0
Offsite 1	7.2	7.19	7.20	43.4	45.0	44.2
Offsite 2 western crater	7.02	7.35	7.19	85.8	89.4	87.6
Offsite 3 western crater	6.61	6.48	6.55	47.2	43.7	45.5
Offsite 4 western crater	7.15	7.09	7.12	54.0	54.9	54.5

2.3.3 **Cultivation Experiments**

During field season one, each experimental medium was tested under anaerobic, aerobic, solid, and liquid conditions with fresh soil inocula. All media showed growth, with aerobic solid conditions showing the most growth, in terms of both number of colonies and optical density (OD). In particular, growth was observed on 50% R2A solid agar plates under all conditions (i.e. solid, liquid, anaerobic, and aerobic). Forty pure-culture isolates selected from each media were macroscopically characterised and prepared for sequencing at the University of Waikato. The 16S rRNA phylogenetic analyses of the isolates showed that all organisms grouped within the Firmicutes phylum. Aerobic Isolates were assigned to the subgroup Bacilli and anaerobic isolates were assigned to the subgroup Clostridia. One isolate in particular was found on all solid and most liquid media and was phylogenetically assigned to the Bacillus subgroup, with 93% similarity to the previously cultivated organism *Geobacillus* sp., (Wu et al., 2006). This organism and a large proportion of the isolates grown under these experimental conditions have not been previously isolated from Tramway Ridge, or any Mt Erebus hot soils.

During field season two, we tested a new set of modified media to grow under anaerobic, microaerophilic, aerobic, solid, and liquid conditions with fresh soil inocula. All media supported growth, with anaerobic liquid conditions showing the best response for novel growth, as it was the only media conditions that were successful in growing a non Firmicute related organism. Overall, 25 isolates were visually characterised and prepared for RFLP screening. Using reference digest patterns from season one and 16S rRNA sequencing, RFLP revealed the

majority of isolates belonged within the Firmicutes phylum, with *Geobacillus* again being identified in most media conditions. Any new Firmicute isolates that were not detected in season one had a frozen stock prepared. RFLP screening revealed an anaerobic isolate with a different banding pattern to all other isolates from both season one and two. The isolate grew on 50% modified R2A media under anaerobic (20% carbon dioxide and 80% hydrogen gas mixture) conditions. After 16S rRNA sequencing, the isolate was phylogenetically assigned to the Beta-Proteobacteria, with the highest alignment score of 96% (with Query coverage of 82%) to all sequences within the NCBI database. All recorded closely related organisms show mostly aerobic growth with no apparent strictly anaerobic members. Both field seasons on Mt Erebus have proven successful in using a strategic media design to isolate organisms previously not grown from Mt Erebus, Antarctica. The result of this experiment is a culture library of previously uncultivable organisms from Tramway Ridge subsurface soils.

2.3.4 **Biolog Community Based Phenotypic Array Experiment**

Our duplicated PM microarray plates showed that most respiration of the Tramway Ridge microbial community occurred under strongly alkaline conditions (Figure 8) in both sub-oxic environments (Table 7 and Table 8). The highest growth pH examined was pH 10.

In this experiment anaerobic growth supported a broader range of metabolic capability compared to aerobic conditions. These results have identified high rates of microbial activity on previously unexamined nutritional compounds such as inulin, 2,3-Butanone, urea and several amino acids including tryptophan,

histidine, tyrosine, serine, alanine, arginine, leucine, lysine, methionine, phenylalanine, proline, Threonine, Valine, and Ornithine. This experiment also found the highest metabolic activity of the community to be within pH range 9-10 for both aerobic and microaerophilic conditions.

Figure 8. Positive results in wells on a Biolog PM pH plate. Each well in row A has a different pH value with A1 at pH 3.5 and each well to the right having a pH value incremented by pH 0.5. Well A12 has pH 10. Each well in rows B through D contain a variety of nutritional sources at pH 4.5. Rows E through G contain the same well arrangements of nutritional sources at pH 9.5. Row H 1-10 contains wells of different carbon sources with no predefined pH state. H11 contains phosphate, and H12 contains sulphate and also have no predefined pH state.

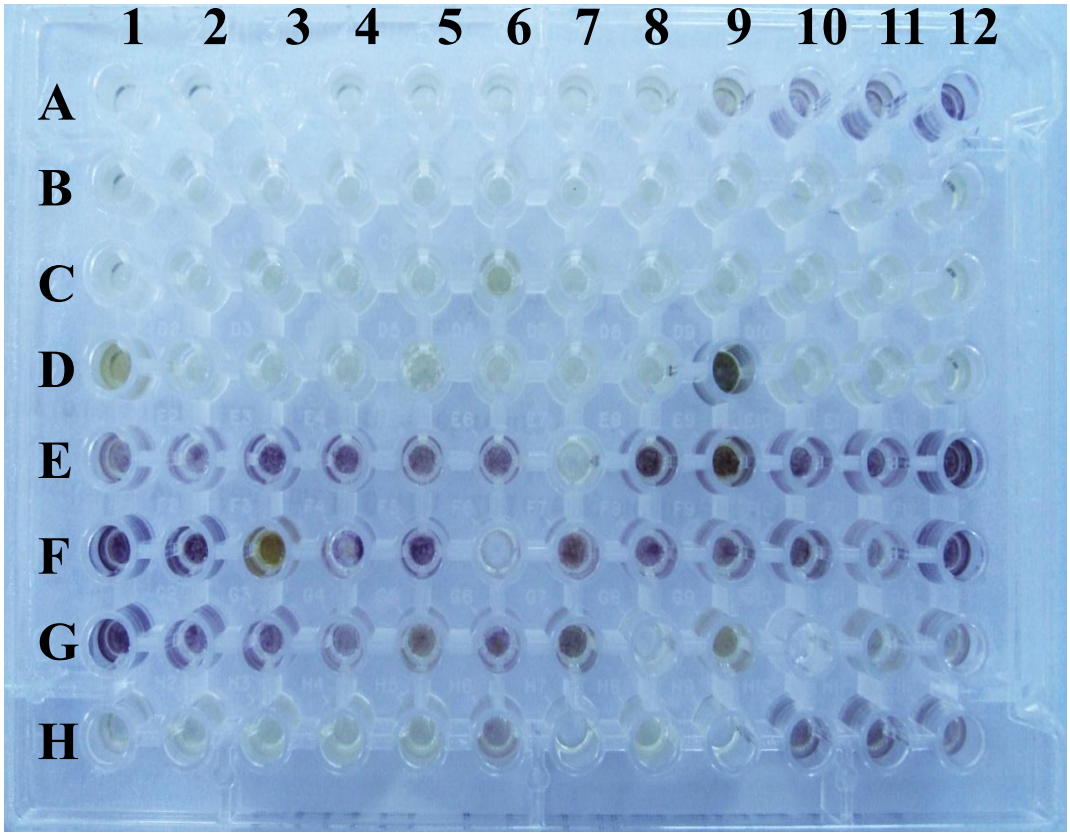

Table 7. Biolog PM duplicated positive respiration response to substrates under aerobic conditions.

Aerobic responses		
PM #	**Time (hrs)**	**Substrate**
10	26	pH 4.5 + 5-hydroxy tryptophan
		pH 9.5 + L-Histidine, L-Tryptophan, L-Tyrosine, phenylethylamine
2	26	2-Deoxy D-Ribose, D-Glucosamine, Di-hydroxyAcetone

Table 8. Biolog PM duplicated positive respiration response to substrates under microaerophilic conditions.

Microaerophilic respiration		
PM#	**Time (hrs)**	**Substrate**
10	26	pH 4.5 + 5-hydroxy tryptophan
		pH 9.5 + L-Serine, L-Tyrosine, L-Tryptophan
	50	pH 9, 9.5, 10
		pH 9.5 + L-Alanine, L-Arginine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, L-Glycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Threonine, L-Valine, Hydroxy-L-Proline, L-Ornithine, L-Homoarginine, L-Homoserine, Anthranalic acid, L-Norleucine, L-Norvaline, Agmatine, Cadaverine, Putrescine, Histamine, Trimethyl amine-N-oxide, Urea, X-alpha-D-glucuronide, X-alpha-D-mannoside
		X-PO4,X-SO4
	120	X-alpha-D-glucoside, X-beta-D-glucoside, X-beta-D-Galactoside
2	26	Inulin, D-Glucosamine, Di-hydroxyAcetone, 2,3-Butanone
	50	5-Keyo-D-Gluconic Acid
	120	D-Tagatose, Oxalic acid
1	26	D-Melezitose
	120	Tyramine

2.4 Discussion

2.4.1 Cultivation Experiments

The 16S rRNA gene phylogenetic analyses of all successfully stored isolates have shown a laboratory enrichment for Tramway Ridge members that are poorly represented in the natural environment. Although the organisms isolated in this study have not been isolated from Mount Erebus before, they are very well represented in literature and show no endemic properties. The Firmicute phylum is highly established and has numerous isolated representatives, as the organisms are easily cultivated and found ubiquitously in soils and other environments (Aislabie et al., 2008; Blanc et al., 1999; Hansel et al., 2008; Maron et al., 2005). Although Firmicutes can be identified in nearly all soil samples, they tend to represent only a very small proportion of the total microbial community (Floyd et al., 2005; Janssen, 2006), and as shown by Soo and colleagues (2009) in Tramway Ridge soils.

A distinguishing features of all Firmicutes is the ability to sporulate (Vos et al., 2009). This condition is induced under extreme environmental stress, such as nutrient deprivation, and allows the bacteria to exist in a dormant metabolic state until conditions improve (Nicholson, 2002; Nicholson et al., 2000). Bacteria that exist in spore form are substantially smaller and lighter than metabolically active cells (Madigan and Martinko, 2006). These properties allow greater dispersal ability, and result in spore forming bacterial groups, such as the Firmicutes, to be the most naturally observed cosmopolitan groups within the Bacterial kingdom (Maron et al., 2005; Wilkinson et al., 2012). Dramatic changes in environmental factors can be detrimental to the survival of most soil

microorganisms that do not have the ability to sporulate. Sporulation provides an explanation as to why Firmicutes have the ability to better survive the transition from environmental habitats to laboratory based cultivation conditions compared to other microorganisms, even when they are observed naturally at low abundance.

From the second season's strategic media design, we did manage to isolate one anaerobic organism which did not phylogenetically group within the Firmicute phylum. The closest relative in the NCBI database is an aerobic isolate that phylogenetically grouped within the Beta-Proteobacteria phylum, in subgroup Aquabacterium (Kalmbach et al., 1999). It appears that the organism isolated in this study is one of only a few isolated Beta-Proteobacteria sub phylum that are strictly anaerobic (Weelink et al., 2009). The sequence was also represented in the Tramway Ridge genetic database generated from several 454 genetic analyses on the same sample (Craig Herbold, et al per com). The DNA used to generate the Tramway Ridge dataset was extracted from the same soil used for inoculation in cultivation experiments. This information suggested that this isolate is a member of the Tramway Ridge community, and worthy of further genetic and physiological characterisation; however, the isolate could not be maintained under laboratory conditions. As a result, we could not fully characterise or successfully store the isolate. Further studies, guided by the information gained in this project look promising in isolating more representative organisms from Tramway Ridge soils.

2.4.2 **Biolog Community Based Phenotypic Array Experiments**

Several insights were gained from our metabolic profiling using Biolog PM plates. The most exciting results involve pH and oxygen effects. Our pH plates showed the most respiration occurred at a pH between 9 and 10 under sub-oxic conditions. The highest pH examined was pH 10. This is interesting because the pH of Tramway Ridge has previously been measured as slightly acidic (Hudson et al., 1989). Our work has validated previous work that identified a neutral to alkaline pH for the 65°C sites (Soo et al., 2009), with the highest pH measured thus far having been a pH of 8.2. Why the community responds positively to higher pH is intriguing and will require future work. The observation that this effect is most profound under suboxic conditions suggests a synergistic effect of suboxic conditions and high pH. This finding certainly corroborates our subsurface oxygen work and suggests a combined role of oxygen and pH in maintaining the community structure at Tramway Ridge. Future work will focus on elucidating the finer points of this control. The results of this experiment providing unprecedented nutritional and physiological information about the microbial system and can be used in the future to guide more extensive cultivation efforts.

The results from both season one and two metabolic experiments validated the putative metabolic potential discovered from the preliminary metagenomic data of the Tramway Ridge microbial community: data that was used to optimise cultivation experiments. We found high rates of microbial activity in the presence of compounds such as inulin, 2,3-Butanone, urea and several amino acids which differ substantially from the carbon and nitrogen sources commonly

used for enrichment of geothermal soil isolates. These findings were unexpected and emphasised how unique and poorly understood the metabolic requirements of this microbial system actually are: for example, the extensive utilisation of amino acids, unusual peptide molecules, and complex polysaccharides for microaerophilic respiration. The results of the first seasons experiment allowed us to modify our media strategy by altering the pH and incorporating the nutrients identified in the phenotypic Biolog array that appeared to enrich for growth. These findings were very helpful in deciphering the actual pH of the Tramway soil. Because pH is a strict factor in determining growth in bacteria (Fierer and Jackson, 2006), we found it fundamental to validate our earlier preseason pH readings. We found a strong association between soil temperature and soil pH. Geothermal soil collected from 65°C hot spots had readings between pH 7.5 – 9.0. Soil collected from cooler temperatures had lower pH readings. This temperature:pH relation was also found by Soo and colleagues (2009).

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3 Targeted Enrichment of Tramway Ridge Microbial Community Members: a Novel Approach to Deciphering Physiological and Metabolic Drivers of Cultivation Resistant Microbial Systems

3.1 Introduction

Enrichment of microorganisms from the natural environment is instrumental to a wide range of microbial applications, including agriculture, industry, medicine, and environmental studies (Feria-Gervasio et al., 2011; Montealegre et al., 2002; Santegoeds et al., 1996; Taskila et al., 2009; Wawrik et al., 2005). Enrichment experiments provide an opportunity to examine how cultivation resistant organisms in a community respond to different treatment conditions (Santegoeds et al., 1996; Wawrik et al., 2005), including nutritional and physico-chemical conditions (e.g. pH, salinity, conductivity, temperature, and humidity) (Avrahami et al., 2011; Frey et al., 2004). Cultivable bacteria are said to account for only 1% of microorganisms present in environmental samples (Pace et al., 1986). Culture dependent techniques refer to the use of selective media to grow organisms from their natural environment (Hugenholtz et al., 1998a; Janssen et al., 2002). Culture independent techniques, however, are capable of achieving comprehensive analysis of microbial communities without the need for cultivation, through the application of molecular techniques (Blackwood et al., 2003; Handelsman, 2004; Hugenholtz et al., 1998; Orphan et al., 2000). As a

result, studies investigating microbial diversity generally find discrepancies between culture dependent and culture independent based approaches (Amann et al., 1990; Barns et al., 1994; Hugenholtz et al., 1998; Hugenholtz and Pace, 1996).

Antarctica has a number of volcanoes including Mount Erebus. The hot soils present on the Antarctic continent have been separated from all other volcanic habitats for over 30 million years (Barker and Burrell, 1977; DeConto and Pollard, 2003) and are exposed to a rare combination of extreme environmental conditions, including low nutrient availability, limited oxygen, prolonged periods of darkness, and extreme temperature gradients (Hansom and Gordon, 1998; Parish and Bromwich, 1991; Soo et al., 2009). Mount Erebus hosts the only phonolitic lava lake on the planet and experiences up to ten strombolian eruptions per day (Calkins et al., 2008). Tramway Ridge is a gently sloping, extensively mineralised, ice-free area of geothermally heated soil on Mount Erebus. The ridge has some of the hottest soil found on Mount Erebus (annual average temperature of 65°C) and is located approximately 1.5 kilometres northwest of the main crater at an elevation of 3350 metres above sea level (Soo et al., 2009). The area is approximately 10 000 m^2 and has been designated an Antarctic Specially Protected Area (ASPA) (SCAR, 2002). The value of this habitat lies in the unique biological systems identified in early research (Broady, 1984; Lesser et al., 2002; Melick et al., 1991; Skotnicki et al., 2001). The nature of this system provides a great opportunity to study novel biodiversity, unique adaptations, and evolutionary processes in an isolated context (Hudson et al., 1989).

Previous 16S rRNA gene cloning efforts by Soo and colleagues (2009), of geothermal soils from Tramway Ridge revealed a unique microbial community, with all bacterial sequences showing less than 92% sequence similarity with their closest known relatives in the NCBI database. The largest bacterial clade within the community branched within an unknown group between candidate division OP10 and Chloroflexi phyla. A second highly represented clone also has poor phylogenetic resolution and low identity (less than 86% identity) to any sequences in the NCBI database, and is loosely grouped with the Planctomycetes phylum. Most of these loose affiliations are deep branching with organisms identified from deep-subsurface ecosystems, suggesting the Tramway Ridge community may be archaic and of sub surface origin. It has been suggested that the members of this community are relic and appear frozen in time from an evolutionary standpoint. Recently, metagenomic and 16S rRNA pyrosequencing has been completed on 65°C soil samples collected from Tramway Ridge (Craig Herbold per comm.). A number of 16S rRNA gene sequences from subsurface soils have very low (<90%) sequence similarity to any known sequences. These novel sequences appear to be deep branching and suggest endemic, and potentially ancient, organisms are dominating the subsurface soils of Tramway Ridge. Previous cultivation dependent approaches applied to Tramway Ridge have been unsuccessful in isolating the novel microbes that dominate this ecosystem (Daniel and Hudson, 1988; Hudson et al., 1989).

In this study, a community based enrichment experiment was conducted with the objective of identifying the response of the Tramway Ridge microbial community to different nutrient treatments. Preliminary analysis was conducted using DNA fingerprinting (ARISA and tRFLP) to detect basic variation in

community responses between treatments. Clustering of ARISA data provided preliminary information on the community response and was used to select treatments for further genetic analysis using tRFLP. A subset of samples were then chosen for pyrosequencing (Roche GS Junior Ti 454 platform) to determine which members of the community were responding to particular treatments. This study has provided novel insight into this unique microbial system, while creating a hypothesis regarding the keystone members, their metabolic requirements, and how they drive the community.

3.2 Materials and methods

3.2.1 Sample Collection

Samples were taken from geothermal hotspots (65°C) on Tramway Ridge (65°C). Soil was aseptically collected in 50 ml sterile CELLSTAR centrifuge tubes (Greiner Bio One, Germany) using sterile metal spatulas. Soil was maintained at 65°C by a thermally regulated transport container until experiments were initiated, within an hour of collecting soils. Sites sampled in the past for preliminary cultivation and genetic analysis (Soo et al., 2009) were sampled again for this study. Soil samples were then homogenised by initially mixing with a sterile spatula, followed by vortexing for 10 seconds. From the homogenized samples, 5 g aliquots were weighed and dispensed into thirty 15 ml sterile CELLSTAR centrifuge tubes (Greiner Bio One) for nutrient treatment and incubation. All initial experiments were conducted at Lower Erebus Hut (LEH), located 1 km north-east from Tramway Ridge in the 2010/2011 field season.

3.2.2 Community Enrichment

A range of 14 different nutrient treatments were selected for community enrichment studies (Table 9). Nutrients were selected for their potential to enrich for the dominant members of the community as identified by previous cultivation efforts, metagenomic analysis, 16S rRNA gene phylogenetic analysis and community based metabolic profiling experiments. All nutrient solutions were prepared in sterile Milli-Q water, with each treatment being tested in duplicate.

Table 9. Nutrient solutions prepared for enrichment experiments.

Nutrients	Abbreviation	Description
Milli-Q (control)	MQ	Sterile Milli-Q water. Experimental control
Nitrite/Nitrate	NO	27 mM sodium nitrite, 45 mM calcium dinitrate
N-acetyl Glucosamine	Glu	5 mM D-Glucosamine hydrochloride
Carbonate	Carb	30 mM $HKCO_3$
Ammonium	NH_4	36 mM ammonium chloride
Chitin	Chitin	Saturated Chitin (saturated by autoclave)
12.9 salt mS/cm	$NaSO_4$	5 mM Sodium sulphate. Conductivity
Potassium sulphate	KSO_4	5 mM potassium sulfate
Inositol	Inos	5 mM inositol
Inulin	Inulin	0.1% inulin
Trypticane peptone	Peptone	0.1% tryptone
Casamino acids	Cas	0.1% casamino acids
Sodium sulfide	NaS	5 mM sodium sulfate
Ammonium oxalate	NH_4Ox	5 mM ammonium oxalate

The two time zero (T0) tubes were immediately frozen at -20°C. The remaining duplicated tubes were then supplemented with 1 ml of the corresponding nutrient solution. Tubes were then sealed and incubated at 65°C for nine days. After the ninth day, 1 g of soil was removed from each tube using a sterile spatula, transferred to a sterile 2 ml screw cap tube, and frozen at -20°C. Each treatment tube was then supplemented with another 1 ml of nutrient solution, and incubated again for a further 13 days, until day 22. After incubation, another 1 g of soil was removed from each tube and frozen, as described above. Samples were transported back to New Zealand for DNA extraction and molecular analysis. DNA extraction was conducted as described by Barrett and colleages (2006). The extracted DNA was quantified using a high sensitivity Qubit 2.0 fluorometer DNA quantification procedure (Invitrogen Ltd, New Zealand).

3.2.3 **Automated Ribosomal Intergenic Spacer Analysis (ARISA)**

ARISA was used as a preliminary fingerprinting tool to identify the differences in compositions of bacterial communities after the different enrichments. The internal transcribed spacer (ITS) region in the bacterial rRNA operon was amplified using PCR from total community DNA of each sample. Each reaction contained 0.3 μM of each primer (hexachlorofluorescein (HEX) fluorescent dye labelled ITSReub-Hex (5`-GCCAAGGCATCCACC-3`) and ITSF (5`-GTCGTAACAAGGTAGCCGTA-3`) (Cardinale et al., 2004)), 1x PCR buffer (Invitrogen Ltd, New Zealand), 0.2 mM dNTPs (Roche Diagnostics, New Zealand), 0.02 mg/ml bovine serum albumin (BSA), 1 U Platinum Taq (Invitrogen Ltd), 0.5 μM $MgCl_2$, 20 ng genomic DNA, and the reaction was made up to 25 μL with

Milli-Q H_2O. Thermal cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 2 min, followed by a final extension of 72°C for 7 min. All PCR was run on a Bio-Rad DNA Engine (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA). Once amplified, all PCR products were run on a diagnostic 1.5% agarose gel to ensure amplification success. The PCR products were diluted 1:20 in Milli-Q H_2O and analysed at the University of Waikato DNA Sequencing Facility.

3.2.4 **Terminal Restriction Fragment Length Polymorphism (tRFLP) Analysis**

tRFLP was the second DNA fingerprinting tool used to validate the sample clustering found using ARISA. Amplification of 16S rRNA genes from total community DNA of each sample was completed in triplicate. Each reaction contained 0.4 μM of fluorescently labelled universal primer Tx9F (5'-GGATTAGAWACCCBGGTAGTC-3') and 1391R (5'-GACGGGCRGTGWGTRCA-3') (Ashby et al., 2007), 1x PCR buffer (Invitrogen Ltd), 0.2 mM dNTPs (Roche Diagnostics), 1 U Platinum Taq (Invitrogen Ltd), 2.5 μM $MgCl_2$, 20 ng genomic DNA, and the reaction was made up to 30 μL with Milli-Q H_2O. Thermal cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 20 sec, 53°C for 20 sec, 72°C for 45 sec, followed by a final extension of 72°C for 3 min. All reactions were run on a Bio-Rad DNA Engine (PTC-200) Peltier Thermal Cycler. Once amplified, triplicate PCR reactions were combined, run on a 2% TAE agarose gel stained with "SYBR Safe" (Invitrogen Ltd). Bands containing the PCR products were excised, and DNA retrieved using the MO BIO UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA,

USA) as per manufacturer's instructions, modified to run the final spin step twice. DNA concentrations of the PCR products were determined using a Qubit 2.0 fluorometer. Each sample was then digested using 1x NE buffer 4 (Roche, Germany), 8 U MspI (Roche), 78 ng DNA, and Milli-Q H_2O to make each reaction up to 20 µL. DNA was digested at 37°C for 3 hours, and terminated by incubating at 65°C for 20 minutes. Digested PCR products were diluted 1:20 in Milli-Q H_2O and analysed at the University of Waikato DNA Sequencing Facility.

3.2.5 **DNA Fingerprinting Data Processing and Analysis**

To compare and interpret both ARISA and tRFLP fingerprints, the sequencing output was run through an informatics pipeline (Abdo et al., 2006), and analysed using Primer 6 software (Clarke and Gorley, 2006). For analysis of ARISA profiles, all peaks present between 100 and 1200 bp that were larger than 50 relative fluorescent units (RFU) were accepted as peaks, and peaks that were called within one base pair in length of one another were considered the same peak and "binned" together. For analysis of tRFLP profiles, all peaks present between 20 and 600 bp that were larger than 10 relative fluorescent units (RFU) were accepted as peaks, and peaks that were called within one base pair in length of one another were "binned" together. In each community profile, the abundance of each accepted peak was expressed as a proportion of the total of all peak heights accepted in the profile. The processed text files were then imported into PRIMER 6 where a pairwise distance matrix was constructed based on Bray-Curtis distance between each sample. The total number of peaks from each

sample was used as a proxy for biodiversity, and peak distribution and intensity was used as an indicator of community structure.

3.2.6 **454 Pyrosequencing**

Six samples that best represented the trends apparent in the fingerprint data were selected for sequencing using the Roche GS Junior Ti-454 pyrosequencing platform. The 16S rRNA gene was amplified using 0.4 μM of each unadapted primer Tx9F (5'-GGATTAGAWACCCBGGTAGTC-3') and 1391R (5'-GACGGGCRGTGWGTRCA-3') (Ashby et al., 2007). Each sample was amplified in triplicate. Each reaction contained 1x PCR buffer, 0.2 mM dNTPs, 0.02 mg/mL BSA, 0.02 U Prime star Taq (Takara Holdings Inc, Japan), 2 mM $MgCl_2$, 20 ng genomic DNA and the reaction was made up to 30 μL with Milli-Q H_2O. Thermal cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 20 sec, 55°C for 10 sec touching down 0.2°C per cycle, 72°C for 20 sec, followed by a final extension of 72°C for 3 min. All reactions were run on a Bio-Rad DNA Engine (PTC-200) Peltier Thermal Cycler. Triplicate reactions for each sample were combined and cleaned using the MO BIO Ultraclean purification kit. Samples then went through a second cleanup step using the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA, USA) as per the manufacturer's instructions. Sample DNA content was quantified using a Qubit Fluorometer (Invitrogen Ltd, New Zealand).

A second round of triplicate PCR was run as above but with only 10 cycles and using 25 ng of the purified DNA from the previous step per reaction (Milli-Q H_2O volume adjusted accordingly). The primers used were adapted for one-way reads

according to the Roche GS Junior System Guidelines for Amplicon Experimental Design Manual (August 2010), including unique MID identifiers for each sample (BacX-Tx9F (5`-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-GGATTAGAWACCCBGGTAGTC-3`) and BacB-1391R (5`-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-GACGGGCRGTGWGTRCA-3`)). A second gel extraction and AMpure clean up was performed as above. Sample DNA content was quantified using a Qubit Fluorometer, and then diluted to $1x10^9$ molecules/μL as per the Roche Amplicon Library Preparation Method Manual (GS Junior Titanium Series, May 2010 (Rev. June 2010)). QPCR using a KAPA Library Quantification Kit for Roche 454 Titanium/Universal (Kapa Biosystems, Woburn, MA, USA) was used to check the $1x10^9$ dilution, and was adjusted accordingly for making the amplicon library. The diluted amplicons were mixed together in the desired proportions to create the $1x10^9$ amplicon pool. Sequencing was performed using the GS Junior Titanium emPCR Kit (Lib-L), the GS Junior Titanium Sequencing Kit, PicoTiterPlate Kit and GS Junior System (Roche 454 Life Sciences, Branford, CT, USA) at The University of Waikato DNA Sequencing facility.

3.2.7 **454 Pyrosequencing Data Processing**

454 PCR amplicon pyrosequencing data was analyzed using AmpliconNoise v1.0 (Quince et al., 2011). Briefly, raw flowgrams (sff files) with perfectly matching primer and barcode sequences were filtered for a minimum flowgram length of 360 cycles (including primer and barcode sequences) before the first noisy signal (i.e., 0.5-0.7 or no signal in all four nucleotides). All flowgrams were then

truncated at 360 bases and clustered to remove sequencing noise using PyroNoise (Quince et al., 2009; Quince et al., 2011). PCR noise was removed using SeqNoise (Quince et al., 2011), and PCR chimeras were removed using Perseus (Quince et al., 2011). The resulting sequences were processed using Mothur 1.17.0 (Schloss et al., 2009) to create a unique sequence and names file. Samples were sequenced at variable depths, a common artefact of 454 sequencing platforms, resulting in different sequence read counts per sample. Distances between unique reads were calculated using pairwise Needleman alignments in Espirit (Sun et al., 2009). Mothur was again used to cluster reads into operational taxonomic units (OTUs) defined at a distance of 0.03 using furthest neighbor clustering ($OTU_{0.03}$). To prevent the inconsistency in sample read depths affecting data analysis, a Monte Carlo simulation was used to resample the data set. The dataset was re-sampled (bootstrapped) 10,000 times with a sample size of 1000 from each sample. For phylogenetic assignments, representative sequences of all identified $OTU_{0.03}$ were analyzed using both the Classifier function provided by the Ribosomal Database Project (RDP) Release 10, Update 15 with taxonomic assignment threshold set at 80% (Wang et al., 2007), and ntBLAST algorithm for sequence alignment in the NCBI database.

3.3 **Results**

3.3.1 **Community diversity measurements**

Using total observed peaks in both ARISA and tRFLP, in combination with total observed unique OTUs in the sequencing dataset, it was observed that all three techniques were in agreement in ranking the diversity of each sample (Table 10). Each technique showed that microbial diversity decreased in all treatment communities when compared to the T0 community. Each technique also shows that Carb treatment samples have the highest observed diversity of all treatment communities for this study. All treatments other than Carb samples showed a greater than two-fold reduction in diversity compared to the T0 community, as estimated by all three techniques (Table 10).

Table 10. Diversity measurements of treatment samples. For each sample, diversity was measured using both ARISA, tRFLP, and sequencing analysis. In fingerprinting analyses, community diversity was measured by total number of peaks observed per sample. In sequencing analysis, community diversity was measured by total number of unique OTUs observed per sample.

Sample	ARISA (No. of peaks)	tRFLP (No. of peaks)	Pyrosequencing (No. of observed unique OTUs)
T(0)	67	18	420
Carb_2 day22	28	14	359
Carb_1 day9	29	13	311
NH4_2 day9	27	9	N/A
MQ_2 day9	20	5	131
Glu_1 day9	16	8	122
NO_2 day9	22	4	62

3.3.2 **ARISA**

ARISA analysis of samples showed that the communities from each treatment clustered into three groups, with the exception of the NO day22 sample (Figure 9). The T0 community clusters closely with three of the four Carb treatments and also with one NH_4 day9 treatment samples. The second group is composed of 25 samples, 22 of which represent day22 enrichment communities. This group contains two of the NO samples used in data analysis, with the third, the duplicate day22 sample, appearing as an outlier in this analysis. The third group, which clusters furthest from the T0 group, contains 22 samples, 21 of which

represent day9 enrichment communities, with the exception being the only Carb sample not in the T0 group.

Results of this analysis show that the Carb treatment, in general, appeared to maintain a community similar to the T0 community. Groups two and three, however, show that multiple enrichment conditions induce a similar community response, resulting in communities clustering together based on enrichment period (9 or 22 days) rather than nutrient treatment. This is illustrated by the fact that 8 of the 14 duplicated treatments have day 9 samples in group two, and day22 samples in group three. The exception (not including the outlier) is the NO treatment which clusters only within group two. Seven samples representing the different clusters identified by ARISA were selected for further analysis by tRFLP. The following samples could not be successfully analysed using ARISA and were excluded from data analysis: NO day9, Salt day22, and KSO_4 day22.

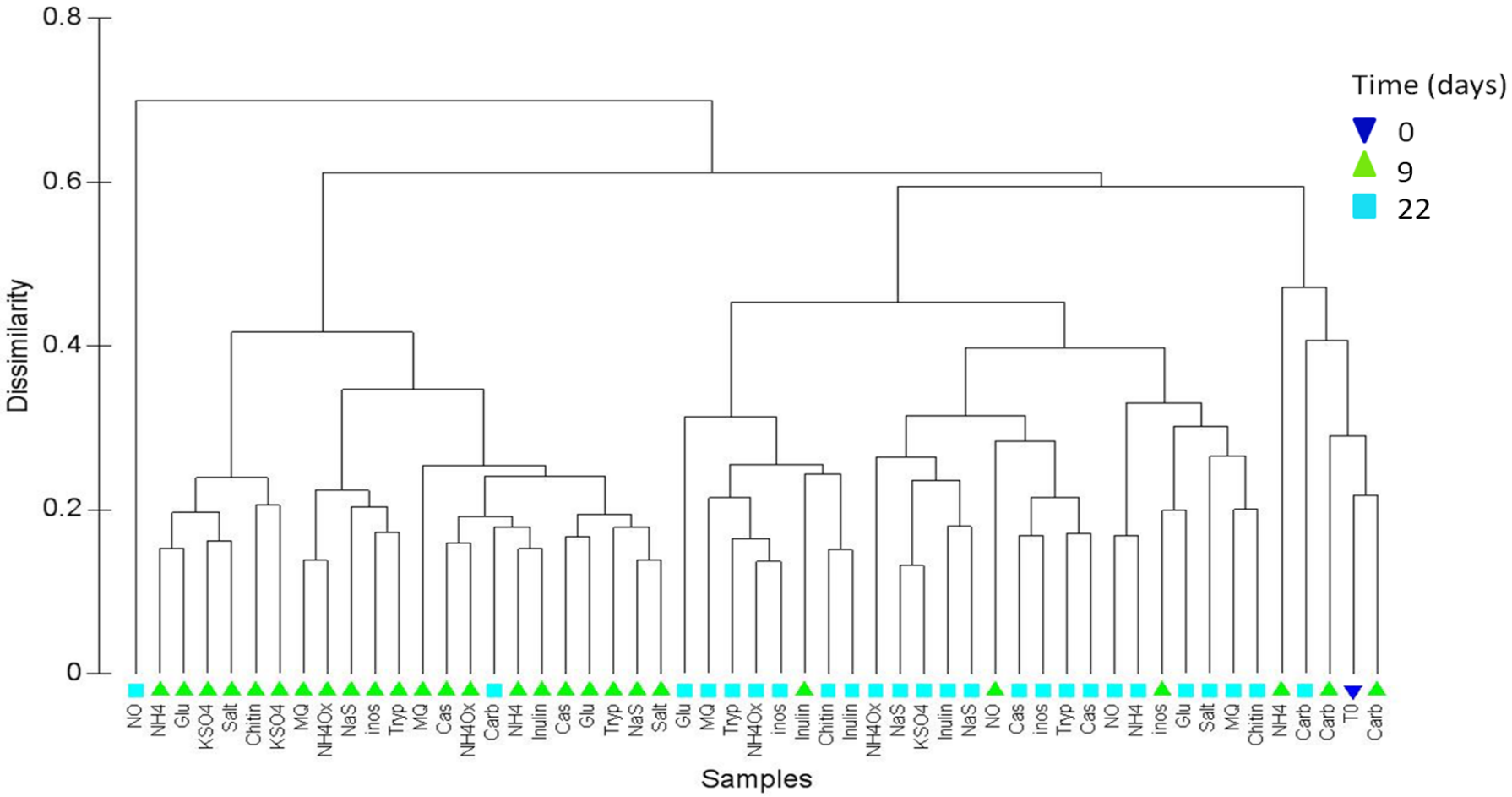

Figure 9. Clustering of ARISA peak distributions for each sample.

3.3.3 **tRFLP**

tRFLP was applied to the seven samples (including T0) selected from the ARISA analysis. These samples were selected based on their clustering in relation to the T0 community. The clustering relationships were determined by comparing similarities and differences of peak profiles between samples. Three samples (NO2-day9, MQ2-day9, and Glu1-day9,) were selected because they showed obvious divergence from the T0 community, representing the two divergent groups identified from ARISA analysis. The remaining three samples were chosen as they showed close clustering to the T0 community (Carb1-day9, Carb2-day22, and $NH_4$2-day9). tRFLP analysis was consistent with both the diversity rankings of samples (Table 10) and clustering relationships revealed from the initial ARISA analysis (Figure 10). Supporting the ARISA analysis, all Carb and the NH_4 enriched community samples show the highest treatment community diversity, and cluster with the T0 community. The remaining 3 samples, which in the ARISA analysis separated into two groups, form only one cluster. The NO sample, which initially clustered in group 2 by ARISA analysis, forms a cluster with MQ and Glu. Using tRFLP analysis NO and MQ appear to cluster together more closely than either does with Glu. This analysis supports the findings of the ARISA analysis, where half the treatments analysed enriched for an emergent community, while the other half supported a community similar to that of T0.

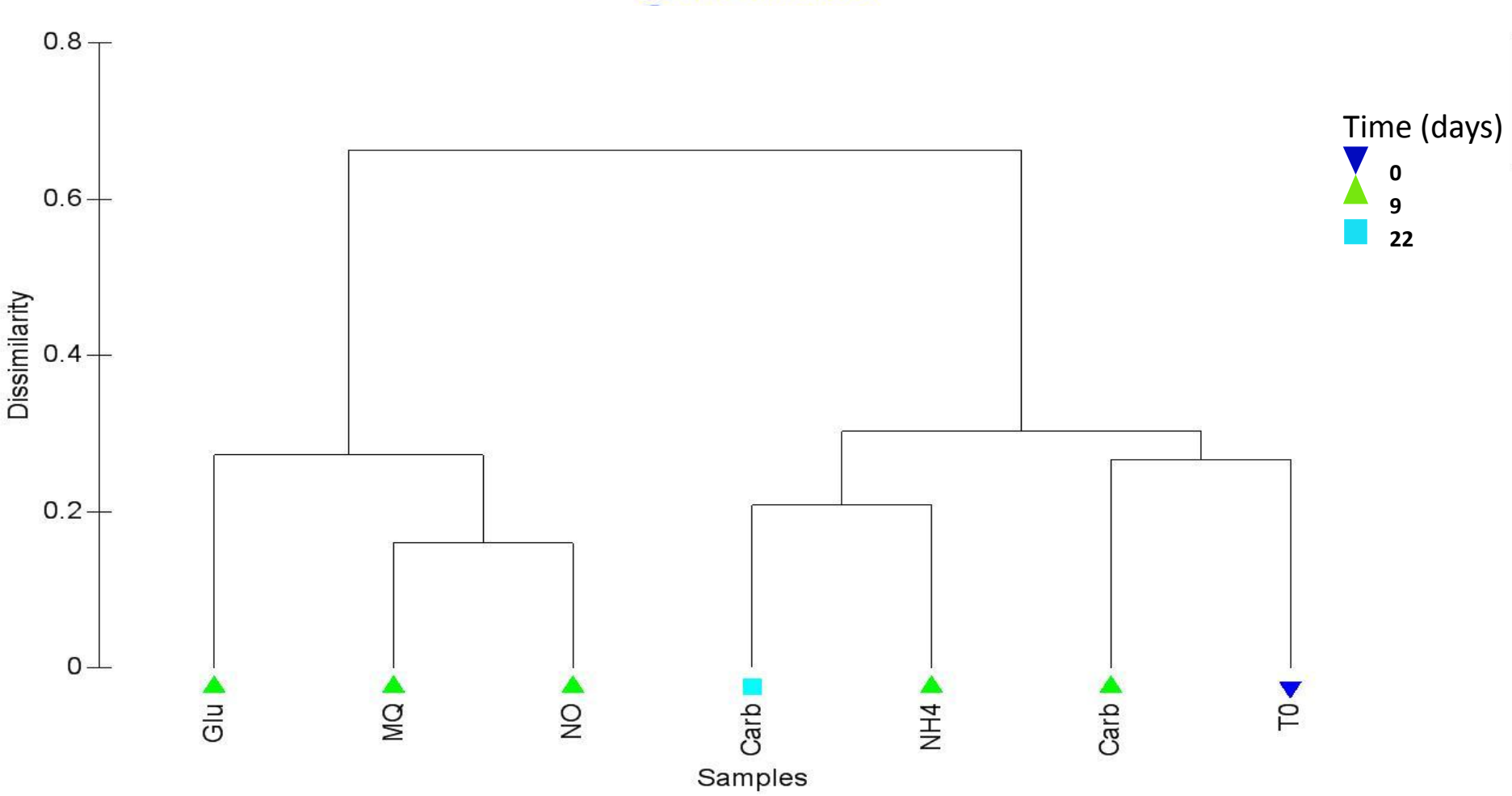

Figure 10. Clustering of tRFLPtRFLP peak distributions

3.3.4 **454 Analysis**

Six samples were selected from the fingerprinting analysis for sequencing using the Roche GS Junior Ti-454 pyrosequencing platform (T0, Carb day9, Carb day22, Milli-Q day9, NO day9, and Glu day9). The samples cluster into the same two groups identified with tRFLP (Figure 3), after re-sampling of the data using R (R development Core Team, 2011). Applying the Monte Carlo statistical test with Bonferroni correction, reveals that community diversity (number of observed OTUs) in T0 and both Carb samples is significantly higher than all non Carb treatment samples, with all P values <0.001, as shown in Figure 11 and Table 11.

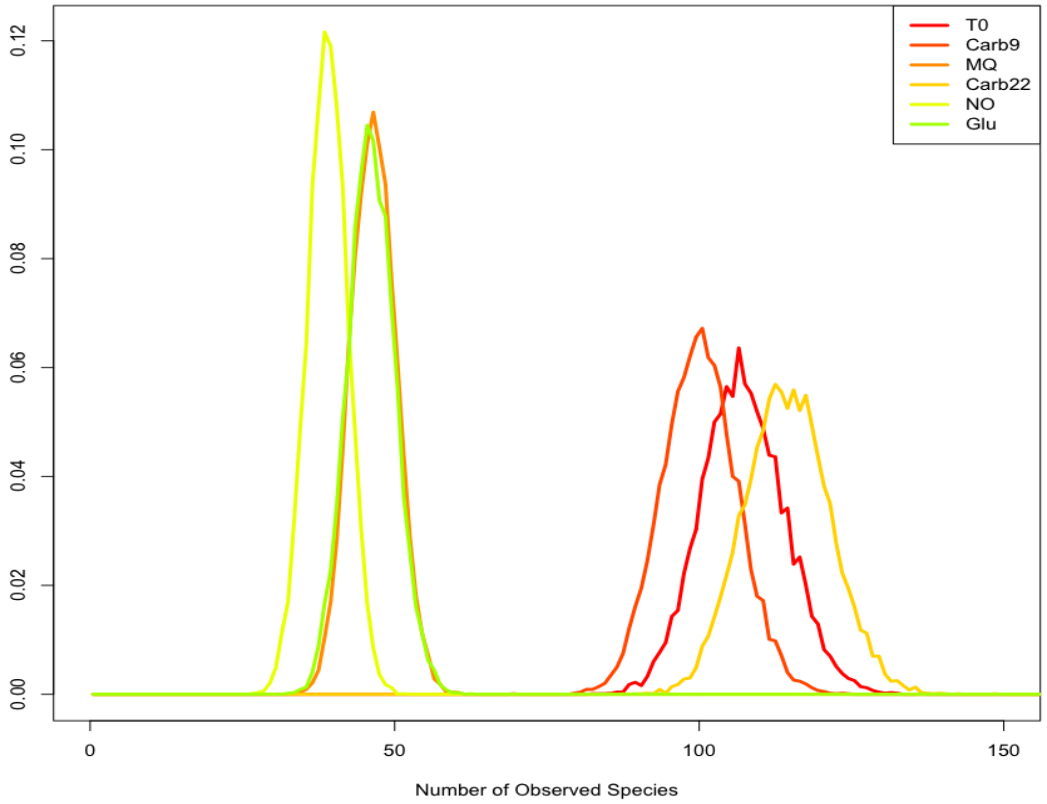

Figure 11. Diversity index of each sample based on 454 16S rRNA amplicon sequencing and Monte Carlo simulation. The Y axis represents the probability density with the area under the curve equal to one.

Table 11. P values for diversity comparisons after Monte Carlo simulation and Bonferroni correction.

Probability that:	**Glu day9**	**NO day9**	**MQ day9**	**Carb day22**	**Carb day9**	**T0**
Glu diversity is greater than	1	0.094	-	-	-	-
NO diversity is greater than	-	1	-	-	-	-
MQ diversity is greater than	0.506	0.075	1	-	-	-
Carb day 14 diversity is greater than	<0.001	<0.001	<0.001	1	0.07	0.251
Carb day 7 diversity is greater than	<0.001	<0.001	<0.001	-	1	-
T0 diversity is greater than	<0.001	<0.001	<0.001	-	0.242	1

At the phylum level, the T0 community is dominated by a group of novel organisms, each showing generally poor 16S rRNA gene sequence alignment with other known organisms, using both the RDP classifier, and the BLAST search algorithm within the NCBI database. The most represented OTU in the T0 community, named TW1 for the purpose of this study, cannot be phylogenetically resolved using the classification methods adopted in this study.

Phylogenetic grouping of TW1, using a representative sequence, is made with poor confidence (24%) at the phylum level using the RDP classifier to the phylum Aquificae, and has poor affiliation (13%) to all sequences in the NCBI database. The closest sequence similarity is to a poorly resolved Obsidian Pool 11 (OP11) bacterial 16S rRNA environmental clone sequence. The second most abundant OTU, constituting 9.7% of the total community, was assigned with 52% confidence using RDP classifier to the phylum Chloroflexi, and had <93% sequence identity to environmental clones in the NCBI database. Because this OTU cannot be resolved, for the purpose of this study it has been named TW2. Another highly represented OTU (composing 4.4% of the total T0 community) has high (>98%) sequence identity to OP10 environmental clones in the NCBI database, and was classified with 99% confidence to the same phylum using the RDP classifier. Amongst the poorly resolved dominant members at T0 is an OTU representing 8.9% of the community, which can be classified with 100% confidence using the RDP classifier to the Cyanobacteria phylum, and has 99% sequence identity to an isolate *Mastigocladus laminosus* from the same phylum.

Deep sequencing of both Carb day 9 and 22 treatment communities revealed community structure similar to that of T0 (Figure 11 and Figure 12). The most significant response to this treatment is the enrichment of both the OP10 and cyanobacterial OTUs. The OP10 OTU represents 7.2% of the total enriched community after nine days, which is an increase from 4.4% at T0. The cyanobacterial OTU represents 15.6% of the total enriched community after 22 days which is an increase from 8.8% at T0. The Milli-Q, NO and Glu treatment communities showed the most significant responses during the experiment. Analyses of these three treatment communities show a dramatic shift from that

of T0, to one where 68-85% of each enriched community is represented by the same OTU. Using a representative sequence, this OTU was assigned with 89% confidence using the RDP classifier to the phylum Deinococcus-Thermus, and has 95% identity to the isolate Thermus filiformis. This OTU represented only 1.1% of the community at T0.

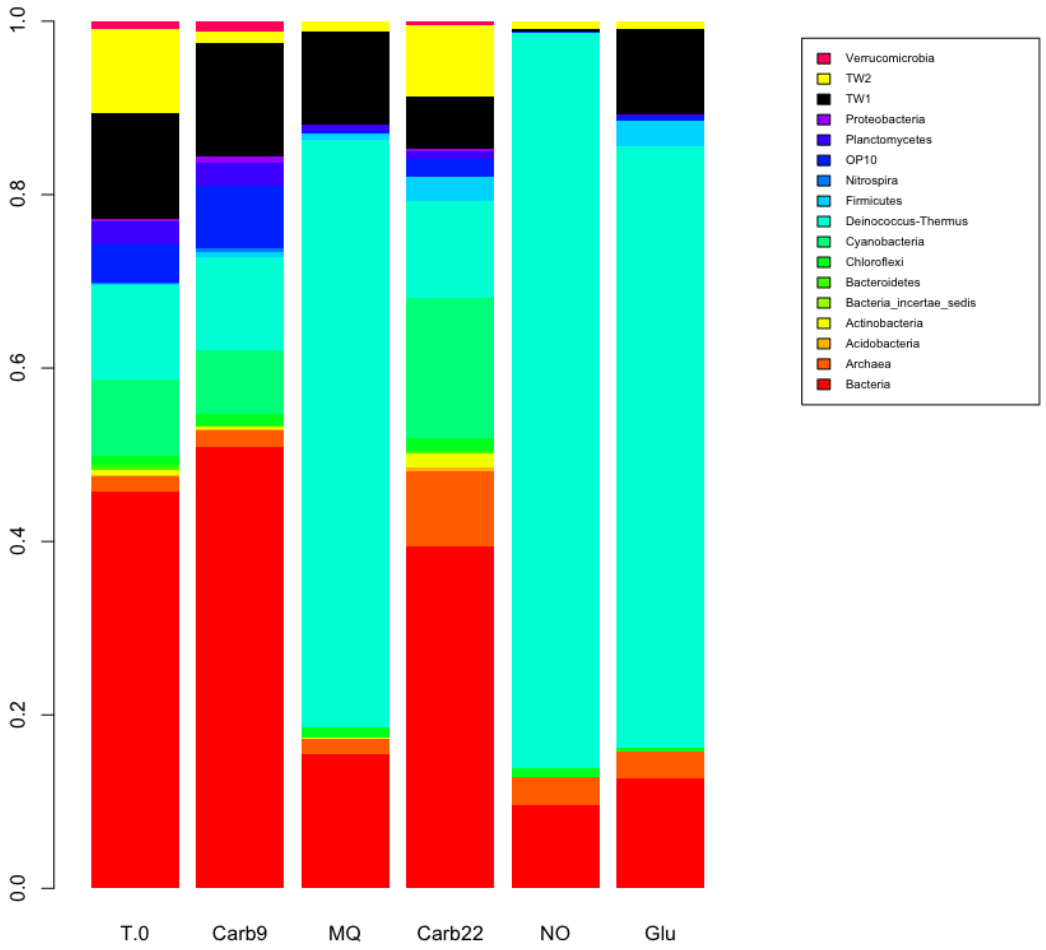

Figure 12. Community OTU distribution for T0 and enriched communities. All OTUs present in the graph have RDP classification of >80% with the exception of TW1 and TW2. TW1 represents the unresolved OTU that dominates T0 and carbonate communities. TW2 represents the unresolved OTU that dominates the carbonate community after two weeks. The area of each coloured block corresponds to the proportion of the community the OTU comprises. The red and orange coloured blocks at the bottom of each bar represent all OTUs that could not be classified with >80% confidence.

3.4 **Discussion**

The results of this experiment showed a significant difference in diversity between nutrient treatments, which was revealed by DNA fingerprinting and supported by pyrosequencing. Initial ARISA diversity analysis revealed that all treatment communities have lower diversity than the T0 community. It was observed that Carbonate treatment samples maintained the highest community diversity. Although peak number per sample was lower in the tRFLP analysis, the findings validated the ARISA diversity rankings. Sequencing data revealed over 400 unique OTUs in the T0 community, compared to the 67 peaks identified in ARISA and 18 peaks identified in tRFLP. Although sequencing analysis revealed significantly higher diversity in all samples relative to both fingerprinting methods, the diversity rankings were in agreement.

Fingerprinting analysis revealed that the majority of nutrient treatments enrich for communities with reduced diversity and significantly different peak profiles to T0. From all treatments tested in this experiment, Carbonate is the only condition that maintained a community that could not be statistically differentiated from T0 in terms of both community diversity and structure. Sequencing analysis of representative samples provided in-depth information about how the members in the community responded to particular treatments. Figure 12 demonstrates that reduction in abundance of particular OTUs coincides with a significant change in community structure. For example, reduction in abundance of the OP10, TW, Cyanobacteria, and Chloroflexi OTUs in the non-Carb treatments, coincides with the increase in Deinococcus-Thermus OTU

abundance. This effect also results in significant community diversity reduction for the non-Carb treatments (Figure 11).

This study reveals three important findings about the Tramway Ridge microbial community. Firstly, a dramatic community shift occurred when reduction of OP10, TW2, Cyanobacteria, and Chloroflexi OTUs occurs. Because community composition changes so dramatically with reduction or loss of these organisms, it is hypothesised that these are keystone members of the community, and their presence is crucial in maintaining the original community structure. Secondly, carbonate is the only treatment in which loss of these 'keystone' members does not occur. Since several other carbon treatments (N-acetyl glucosamine, chitin, inositol, and inulin) failed to support the original community composition, it is hypothesised that at least one of these keystone members are obligate autotrophs, and rely on carbon fixation to maintain community equilibrium. Also, because Tramway ridge is oligotrophic in nature, and has been recorded to have very low organic carbon levels (Hansom and Gordon, 1998; Soo et al., 2009), carbon fixation is a likely source of carbon for this microbial community. Thirdly, because the enrichment experiment was incubated in the dark, it is hypothesised that carbon fixation occurred chemoautotrophically, instead of photoautotrophically (Madigan and Martinko, 2006).

What can be predicted about the potential role of the 'keystone' members, in the Tramway Ridge ecosystem, from the current knowledge of their physiologies? Candidate phylum OP10 is a poorly characterised bacterial group, comprised mainly of environmental 16S rRNA gene clone sequences, with the exception of three isolates (Tamaki et al., 2011). Each isolate is phylogenetically distinct and has been assigned to different novel genera (Lee et al., 2011; Stott et al., 2008;

Tamaki et al., 2011). To date all isolated representatives are documented as strict heterotrophs. The Cyanobacteria phylum is the largest, most diverse, and widely distributed group of photosynthetic organisms in the prokaryotic kingdom, however none have been identified as having a chemolithoautotrophic metabolism (Badger et al., 2002; Stanier and Cohenbazire, 1977). TW2 could not be phylogenetically resolved, and if this organism is responsible for carbon fixation by chemolithoautotrophy, it could justify the assignment of a new phylum level group. The closest match in the NCBI database was to an environmental clone sequence belonging to the Chloroflexi phylum, which has been recognized as a typical ubiquitous bacterial taxon containing a number of diverse environmental 16S rRNA gene clones, with a few of cultured representatives (Madigan and Martinko, 2006; Yamada and Sekiguchi, 2009). Cultured organisms within Subphylum III, the class Chloroflexi, show photoheterotrophic and/or chemolithoautotrophic growth, under mesophilic or moderately thermophilic conditions (White and Culver, 2012; Yamada and Sekiguchi, 2009). It is likely that either or both of the TW2 or Chloroflexi designated OTUs are responsible for chemolithoautotrophy in this community.

Overall, the results of this study have led to the hypothesis that the Tramway Ridge microbial community is driven by chemolithoautotrophic microorganisms. This hypothesis is based on preliminary work, and it is important to note that further investigations into this poorly understood microbial ecosystem are necessary before conclusions can be drawn. However, this study does provide insights into how this ecosystem functions, and our hypothesis is so far supported by a number of observations: (1) in the absence of carbonate, the community shifts to being dominated by obligate heterotrophs; (2) the microbial

ecosystem exists in subsurface soils which have limited, to no, light availability (Amend and Teske, 2005); (3) this system is nutrient poor, with low organic carbon content but a consistent carbon dioxide dominated gas supply created from constant volcanic activity; (4) two of the keystone members phylogenetically group with a bacterial phyla known for chemolithoautotrophic metabolism; (5) significant previous research shows that oligotrophic microbial ecosystems are commonly driven by chemolithoautotrophic organisms (Amend and Teske, 2005; Chen et al., 2009; Engel et al., 2004; Mills et al., 2010; Northup et al., 2011; Radford-Knoery et al., 2001; Swan et al., 2011; White and Culver, 2012). Future studies are necessary to test this new hypothesis, and to decipher the energy sources that drive chemolithoautotrophy on Mount Erebus.

The objective of this experiment was to identify the metabolic requirements of the Tramway Ridge microbial community, and understand how individual members contribute to community functioning. Applying both DNA fingerprinting and pyrosequencing to this cultivation based manipulation experiment has made it possible to investigate the metabolic requirements of the community in a way that previous cultivation dependent and independent work could not. The experiment was designed to not only identify what nutrients drive the microbial community, but also how the individual members respond to each treatment condition. The results have provided new insights into the metabolic requirements and putative roles of specific organisms, and how this drives the structure and functioning of the community.

3.5 References

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4 Summary

The objective of this project was to investigate the metabolic and physiological requirements of the dominant Tramway Ridge thermophilic microorganisms, in order to better characterise this community and validate assumptions generated from previous genetic analysis. Initial experiments included physical-chemical analysis, strategic cultivation, and community based phenotypic arrays. Physicochemical analysis of Tramway Ridge subsurface soil revealed an alkaline, oxygen deprived ecosystem. The objective of cultivation work was to identify the origin of novel physiological and metabolic capabilities detected by genetic analysis, and investigate their ecological significance and evolutionary relevance. The cultivation experiments were successful in the isolation of organisms previously uncultivable from Tramway Ridge soil, and further cultivation efforts hold promise to capture more representative members.

As a supplement to the targeted cultivation experiment, a range of ecologically relevant Biolog Phenotypic Microarrays were utilised to conduct community wide metabolic screening analyses. This experiment was incorporated into the project to provide additional information about the metabolic requirements of the microbial community. Several insights were gained from metabolic profiling using Biolog PM plates. The most exciting results involve pH and oxygen effects. pH plates showed that most respiration occurred at between pH 9 and 10, under sub-oxic conditions. Why the community responded positively to higher pH is intriguing and will require future work. The observation that this effect is most profound under suboxic conditions suggests a synergistic effect of suboxic

conditions and high pH. This finding certainly corroborates subsurface oxygen measurements and suggests a combined role of oxygen and pH in maintaining the community structure at Tramway Ridge. Future work will focus on elucidating the finer points of this control. These findings provide unprecedented nutritional and physiological information to guide more extensive cultivation efforts.

Results of the Biolog experiment also validated the putative metabolic potential discovered from the preliminary metagenomic data of the Tramway Ridge microbial community, data that was used to optimise cultivation experiments. We found high rates of microbial activity in the presence of compounds such as inulin, 2,3-Butanone, urea and several amino acids which differ substantially from the carbon and nitrogen sources commonly used for enrichment of geothermal soil isolates. These findings were unexpected and emphasised how unique and poorly understood the metabolic requirements of this microbial system actually are: for example, the extensive utilisation of amino acids, unusual peptide molecules, and complex polysaccharides for microaerophilic respiration.

The community based enrichment experiment was designed with the objective of detecting differences in community response to various nutrient treatment conditions. The experiment was designed to not only identify what nutrients drive the microbial community, but also how the individual members respond to each treatment condition. Applying both DNA fingerprinting and pyrosequencing to this cultivation based manipulation experiment has made it possible to investigate the metabolic requirements of the community in a way that previous cultivation dependent and independent work could not. Overall, the results of

this study have led to the hypothesis that the Tramway Ridge microbial community is driven by chemolithoautotrophic microorganisms. This hypothesis is based on preliminary work, and it is important to note that further investigations into this poorly understood microbial ecosystem are necessary before conclusions can be drawn. However, this study does provide insights into the metabolic requirements and putative roles of specific organisms, and how this drives the structure and functioning of the community.

This multifacted, metabolism driven approach incorporates both culture dependent and culture independent techniques in order to investigate the intriguing system in a way that is crucial to the understanding of this thermophilic community and the environment controlling it. Considering the detailed investigation of this apparently archaic and isolated microbial system, there is potential for this study to become an excellent model for future studies addressing the fundamental functioning and evolutionary processes associated with other thermophilic communities.