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Diversity and Activity of Bacteria in Basal Ice Environments

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DIVERSITY AND ACTIVITY OF BACTERIA IN BASAL ICE ENVIRONMENTS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by
Shawn Doyle
B.S., Louisiana State University, 2008
May 2015
ACKNOWLEDGEMENTS

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ABSTRACT

Glacial ice currently occupies roughly 11% of Earth’s surface and contains approximately 70% of the planet’s freshwater. Once thought to be inhospitable due to the physiochemical challenges presented by freezing temperatures, the basal zones of glaciers and ice sheets have recently been identified as a potential habitat for psychrophilic microorganisms with the ability to mediate biogeochemical cycles on a global scale. Basal ice is found in the deepest layers of a glacier and has distinct chemical and physical characteristics as a result of its proximity to the glacier bed. Basal ice is generally the warmest ice found in a glacier and often contains entrained debris and sediment from the underlying subglacial substrate which may provide nutrients and redox couples for microorganisms immured in the basal ice matrix. ATP/ADP concentrations and ratios, enrichment culturing, 16S rRNA surveys, and cell counts were combined with nutrient, major ion, and gas chemistry analyses to evaluate the microbial assemblages immured in both sediment-rich and sediment-poor basal ice environments. The primary material for this study was a 4 m profile of basal ice collected from Taylor Glacier (Antarctica) but also included basal ice samples from the Matanuska Glacier (Alaska) and Støre Landgletscher Glacier (Greenland).

Microbial community abundance varied significantly between the different basal ice samples and was correlated with the presence of sediment in the ice. Sediment-rich banded basal ice from Taylor Glacier contained elevated concentrations of CO₂ (60,000 to 325,000 ppmv) occurring simultaneously with decreased O₂ concentrations (4 to 18% of total gas volume) suggesting the resident microbial assemblages may be respiring in situ and modifying the gas composition of the basal ice. Molecular surveys of 16S rDNA and rRNA sequences revealed species of the genus *Paenisporosarcina* to be numerically abundant and active members of the
microbial assemblages inhabiting these same basal ice horizons. Members of this genus were readily culturable from the basal ice samples and radiolabeled $[^3\text{H}]$-leucine and $[^3\text{H}]$-thymidine assays with these isolates revealed their ability to conduct macromolecular synthesis while frozen in basal ice melt-water at -15°C. These results support the hypothesis that basal ice environments are microbial habitats harboring bacteria with the physiological capacity to remain metabolically active and cycle elements within the cryosphere.
CHAPTER 1.
INTRODUCTION

Glacial Ice and the Cryosphere

From a biological perspective, the biosphere is cold with nearly 90% (by volume) of the deep ocean existing at temperatures <5°C and a large portion of the surface is perennially or seasonally frozen (i.e. the cryosphere). Approximately 70% of Earth’s freshwater reservoir is in the form of glacial ice (Shklomanov, 1993) and nearly 20% of all soils exist as permafrost (Priscu and Christner, 2004). Moreover, paleoclimatic studies of past glacial-interglacial cycles have gathered strong evidence that global ice cover has been significantly more extensive at certain points in the past including near total coverage during the so-called “snowball Earth” events of the Paleoproterozoic and Neoproterozoic periods (Kirschvink, 1992; Hoffman et al., 1998; Priscu and Christner, 2004). Despite the widespread occurrence and massive scale of permanently frozen ecosystems, estimated to cover approximately 3.7 \times 10^6 \text{ km}^2 of Earth’s surface (Williams, 2012), little is known about the survival, physiology, and ecology of microbial communities inhabiting these environments.

Glaciers are large masses of ice which flow under the influence of gravity. Found on every major continent except Australia, they form through the deposition and accumulation of snow over many years in locales where the temperature is cold enough that some snow persists through the summer seasons. At low latitudes, these are alpine regions where glaciers form in mountains and flow down valleys. In the polar regions (i.e. Greenland and Antarctica), glaciers form as expansive masses of ice up to \sim 4800 \text{ m} thick (Siegert, 2005) known as ice sheets which spread radially under their own weight to the continental margins. As accumulated snow is progressively buried, it is compressed and compacted by the overlying snowpack into a dense
layer of granular ice crystals known as firn (Paterson, 1994). Subsequently, after further compaction and densification, the transformation of firn into glacial ice occurs when the interconnecting air passages between ice crystal grains become sealed off as bubbles. This occurs at a density of approximately 830 kg m$^{-3}$ (Paterson, 1994). The rate and depth at which this transformation occurs is dependent on the air temperature, snowfall accumulation rate, snow moisture content, and the amount of meltwater produced on the glacier surface during the summer (Paterson, 1994). Generally, snow transforms to glacial ice most rapidly on glaciers in temperate regions where daily meltwater produced on the surface during the summer percolates into the underlying firn and subsequently refreezes, greatly increasing the firn density. For example, the firn-glacial ice transition in the upper Seward Glacier (Yukon, Canada) occurs at a depth of 13 m, which typically represents three to five years of snowfall deposition (Sharp, 1951). However, at the South Pole, where temperatures are perennially well below freezing and snowfall accumulation rates are low (~20 cm a$^{-1}$), the transition occurs at a depth of ~100 m and takes upwards of a thousand years (Kuivinen et al., 1982).

Despite the once held belief that ice is an environment inhospitable to life, there have been numerous reports on the recovery of viable microorganisms from permafrost (Steven et al., 2007), snow (Carpenter et al., 2000), glaciers (Doyle et al., 2013; Montross et al., 2014), and sea ice (Junge et al., 2011). Tentative estimates of the microbial biomass inside the Antarctic Ice Sheet (AIS) and Greenland Ice Sheet (GIS) alone are enormous (~4.38 x10$^{24}$ cells), representing roughly 3% of the estimated microbial biomass in all global surface freshwater environments combined (~1.31 x10$^{26}$ cells) (Priscu and Christner, 2004; Priscu et al., 2008). Nearly 10.2 Petagrams (Pg; 1 Pg = 10$^{15}$ g) of carbon, ~0.5% of the estimated world’s total soil organic carbon pool (Batjes, 2014), is contained within the AIS and GIS (Priscu et al., 2008).
Furthermore, ancient marine sediment deposits are thought to exist beneath the AIS, some estimated to be 14km thick, and may contain upwards of 21,000 Pg of organic carbon (Wadham et al., 2012). If accurate, the magnitude of this organic carbon reservoir would be second only to the entire oceanic pool, estimated at ~39,000 Pg (Batjes, 2014), and ten-fold greater than estimates for the entire northern circumpolar permafrost regions (Tarnocai et al., 2009).

Owing to the massive logistical effort required to access environments at the base of the AIS, there have been only two studies on the analysis of sediments and microorganisms from West Antarctica (Lanoil et al., 2009; Christner et al., 2014). As such, very little is known about the microbial turnover of carbon in Antarctic subglacial environments and the fate of organic carbon reservoirs beneath the ice sheet. Investigations of thawed basal ice sediments from John Evans glacier (Skidmore et al 2000), subglacial sediments from Robertson glacier (Boyd et al 2010), and subglacial melt-water discharged from the western margin of the GIS (Dieser et al 2014) have provided microbiological and biogeochemical evidence for the presence of viable assemblages of methanogens in subglacial environments. The production of biogenic methane beneath Earth’s ice sheets and its subsequent release during periods of ice sheet retreat and collapse has been hypothesized as a potential positive feedback mechanism for future global climate warming (Wadham et al., 2012). Microbial activities in subglacial regions of the world’s ice sheets may thus be an overlooked component of Earth’s climate oscillations over geologic timeframes.

**Basal Ice Environments**

A glacier can be divided into three distinct zones: supraglacial, englacial, and basal. The supraglacial zone contains all the material (e.g. snow, meltwater pools, firn, etc.) found above the firm-ice transition layer. Below the firm layer is the englacial zone, which constitutes the majority
of a glacier by volume and is characterized by relatively sediment-free ice formed by the
definition of snow from the glacial surface into ice. Any sediment particles found in englacial
dice originate from atmospheric deposition on the glacial surface (Hubbard et al., 2009). The
basal zone is found in the deepest layers of a glacier or ice sheet and contains ice whose
chemistry and physical structure is directly affected by its proximity to the glacier bed (Knight, 
1997). In areas where the temperature of the glacial bed is cold (<0 °C), sediments and liquid
water from subglacial sources are incorporated into the basal zone of the ice mass through two
distinct processes, regelation or glaciohydraulic supercooling (Cook et al., 2011). Regelation
occurs where the immense weight of overlying ice exerts sufficient pressure on the basal layers
to induce localized melting and refreezing around bedrock obstacles leading to the accretion and
accumulation of multiple layers of sediment-rich basal ice onto the bottom of an ice mass as it
flows over the terrain (Paterson, 1994). Basal ice layers formed through this process vary from a
few millimeters to tens centimeters in thickness. Glaciohydraulic supercooling occurs when
subglacial water is forced up the adverse slope of a subglacial trough causing a reduction in
hydraulic pressure. As a result, the pressure-melting temperature of the water increases causing it
to freeze onto the sole of the glacier, often with large amounts of incorporated sediment and silt
(Lawson et al., 1998). Basal ice formed through supercooling can be many meters thick and in
some cases where ice sheets flow over steep subglacial mountain ridges may constitute up to
50% of the ice sheet thickness (Bell et al., 2011). Basal ice can also form when the base of an ice
sheet traversing over a subglacial lake is below the pressure-melting point, causing lake-water to
accrete (freeze) to the underside of the ice sheet. The most extensively studied example of this is
the ~200 m of accretion ice formed above the central and southern regions of subglacial Lake
Vostok (Kapitsa et al., 1996; Jouzel et al., 1999; Bell et al., 2002; Royston-Bishop et al., 2005; Thoma et al., 2008).

The distribution, size, and concentration of debris in basal ice are controlled by the various combinations of subglacial processes which form and condition the basal zone (e.g. regelation, supercooling, crushing, glacial sliding, etc.). Particle sizes of entrained debris run the gamut from fine-grained clay and silt to coarse sand and gravel (Figure 1.1).

Figure 1.1. Close up view of a sample (approximate dimensions: 10 × 10 × 10 cm) of basal ice from Taylor Glacier, Antarctica with characteristic layers of incorporated silt, sand, and coarse gravel. The sample is lying on its side with the deepest horizon shown on the left.

Even large boulders are known to be entrained (via regelation) and transported by basal ice in a glacial phenomenon known as plucking. Likewise, debris concentrations within basal ice can range from relatively debris-free layers (<1% w/v sediment) to debris-rich layers composed almost entirely of sediment with only interstitial ice (Knight, 1997). Organic carbon (e.g. formate, acetate) and nutrients such as ferric and ferrous iron, ammonia, and sulfate have been documented in basal sediments (Skidmore et al., 2000; Wadham et al., 2004; Tung et al., 2006; Yde et al., 2010). When compared to englacial ice, sediment-rich basal ice contains higher
concentrations of organic carbon compounds and nutrients that active microbial populations would require to survive and persist. Basal ice debris may also be an important source of nutrients and energy for microorganisms inhabiting the subglacial environments which exist beneath Earth’s ice sheets. Over 350 subglacial lakes are known to exist beneath the AIS (Wright and Siegert, 2012). Efforts to access and investigate three of these lakes (i.e. Lakes Whillans, Ellsworth, and Vostok) are currently the focus of three major research drilling projects (Fricker et al., 2011; Siegert et al., 2012; Kotlyakov et al., 2013). In regions where the ice sheet is sufficiently thick for the basal ice to reach the pressure-melting point, basal ice melting and rain-out of debris from the overlying ice sheet is thought to be a large source of dissolved solutes, gases, water, and sediment to these lakes (Siegert, 2000; Siegert et al., 2000b). Microbial activity is a major catalyst of mineral weathering reactions in many natural environments (e.g. Nealson and Stahl, 1997; Ehrlich, 1998), however the microbiology of the basal ice zones of glaciers is poorly understood. Determining if basal ice environments are habitats possessing active populations of microorganisms will be important in understanding the downstream influence of basal ice on the chemistry and biology of subglacial lake ecosystems.

**Ice as a Microbial Habitat**

During freezing, impurities including solutes, microbes, particles and gasses are physically excluded from the ice crystal lattice and concentrated into micrometer-sized (and larger) aqueous channels of liquid water, known as ice veins, located at the interface between ice crystals (Price, 2000). As ice crystals grow, the ice veins between them narrow, further concentrating the excluded impurities. This process increases the ionic strength of the remaining unfrozen water and depresses its melting point until the system reaches equilibrium. At equilibrium, the ice veins have become too saline to freeze and no further ice crystal growth can
occur unless the temperature of the ice decreases further. As a result, the ionic strength of ice veins can be extremely high (Figure 1.2, B). For example, seawater has an ionic strength of approximately 0.72 M and a melting point of -1.86°C. When seawater is frozen at -10°C, the ionic strength of unfrozen water in the ice is four-fold the concentration in the bulk phase (3.2 M); at -20°C, it increases to nearly 5.4 M, ~7 times that of seawater (Mironenko, 1997; Appelo and Postma, 2005). Because ice veins are the direct product of melting point depression by dissolved solutions, ice vein ionic strength is a function of temperature and not affected by the initial solute concentrations. As such, ices of different chemical composition (e.g. sea ice versus basal ice) are predicted to have similar ionic strengths and water activity in their ice veins at a given temperature (Figure 1.2, B) even though the volume of unfrozen water and ice vein diameter can range drastically (Figure 1.2, A). Ice formed from dilute solutions (e.g. a freshwater lake) will have smaller ice veins than that formed from saltier solutions (e.g. seawater) because more water molecules are required in the ice phase to achieve an equivalent level of melting point depression. After freezing, the ice vein matrix can be further altered by recrystallization, an ice coarsening process wherein smaller ice crystals combine (i.e. recrystallize) into larger ice crystals which have less surface area per unit volume and are thus more thermodynamically favorable (Knight et al., 1995). Similar to a growing ice crystal during freezing, recrystallization also excludes impurities into the ice vein matrix and is thus the primary process by which ice veins are created and structured within glacial ice (formed by the firnification of snow as opposed to the cooling and freezing of an aqueous solution) (Mader, 1992a, 1992b).

The salts and acids commonly found dissolved in aqueous systems vary in the degree to which they depress the melting point of water. The specific concentration of a solute at which
Figure 1.2. (A) Bulk percentage estimates of unfrozen water inside various icy substrates. The inclusion of 1 M NaCl is for comparison. (B) Predicted ionic strength of the available unfrozen water. The solid black line represents the decline in water activity ($a_w$). Water chemistry data: Seawater, (Appelo and Postma, 2005); Antarctic Permafrost, (McLeod et al., 2008); Antarctic Glacial Ice, (Montross, 2012). Calculations were performed on dissolved major ions using FREZCHEM v. 11.2; (Mironenko, 1997). Adapted with permission from Doyle et al., (2012).

maximum melting point depression occurs is known as the eutectic point. For example, consider an ideal H$_2$O-NaCl system: the eutectic temperature is -21.2°C and a concentration of 23.2% (w/v). Between 0 °C and -21.2 °C, this solution exists as a mixture of ice and increasingly saline saltwater as described above. However, when lowered below its eutectic temperature, the NaCl
will precipitate as hydrohalite (NaCl·2H₂O) and almost all remaining water in the ice veins will freeze. The eutectics of different compounds can vary quite significantly in both their melting temperature and concentration. Indeed, acids tend to have the coldest eutectic points (e.g. H₂O-H₂SO₄ = -73.1 °C) leading to speculation that ice veins, especially in very cold systems, may be highly acidic with pH values of ~1 (Barletta et al., 2012). However, some salts such as magnesium perchlorate (Mg(ClO₄)₂), calcium perchlorate (Ca(ClO₄)₂), and potassium acetate (KCH₃CO₂) also have very low eutectics with melting points ≤ -60 °C (Table 1.1). On Earth, natural perchlorate salts are rare and found only in extremely arid deserts (e.g. the Atacama Desert), however their existence in Martian soil is well documented (Hecht et al., 2009; Catling et al., 2010) and would allow for the possibility of sporadically stable liquid brines in the shallow Martian subsurface (Chevrier et al., 2009; Marion et al., 2010; Gough et al., 2011). It should be noted that the eutectic thermodynamics of natural ice vein systems containing multiple solutes are more complex than the idealized binary cases described here.

### Table 1.1. Eutectic temperatures and concentrations for various salts (white) and acids (gray).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Eutectic Temperature (°C)</th>
<th>Eutectic Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>-21.0</td>
<td>4.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>-33.0</td>
<td>2.3</td>
</tr>
<tr>
<td>NaClO₄</td>
<td>-37.2</td>
<td>4.2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>-51.1</td>
<td>2.7</td>
</tr>
<tr>
<td>KCH₃CO₂</td>
<td>-60.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Ca(ClO₄)₂</td>
<td>-74.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Mg(ClO₄)₂</td>
<td>-67.2</td>
<td>2.0</td>
</tr>
<tr>
<td>HNO₃</td>
<td>-43.0</td>
<td>5.2</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>-73.1</td>
<td>3.7</td>
</tr>
<tr>
<td>HCl</td>
<td>-88.0</td>
<td>6.8</td>
</tr>
</tbody>
</table>
Microbial Activity in Ice

Beginning in the 1980s, Abyzov et al. (Abyzov et al., 1982; Abyzov, 1993; Abyzov et al., 1998; Abyzov et al., 2001) carried out a series of investigations on the microbiological contents of glacial ice cores recovered from the ice sheet at Vostok station. These pioneering studies were the first to demonstrate the presence of viable microorganisms within englacial ice from the AIS. Based on the numerous physiochemical challenges faced by microbial life when frozen (described below), it was assumed at the time that microorganisms in glacial ice survived in a state of dormancy, persisting in the absence of metabolic activity (Abyzov et al., 2006). However, this is good evidence that supports the notion that microorganisms are metabolically active within certain horizons of glacial ice. Analysis of the atmospheric gases entrapped in ice cores of englacial and basal ice from Antarctica (Siple Dome, Vostok), Greenland (North Greenland Ice Core Project, Greenland Ice Core Project) and Bolivia (Sajama ice cap) found anomalously high concentrations of N₂O, CO₂, and CH₄ that do not correspond to atmospheric values and have stable isotopic compositions consistent with a microbial origin. (Souchez et al., 1995(a); Sowers, 2001; Campen et al., 2003; Ahn et al., 2004; Flückiger et al., 2004; Tung et al., 2005; Souchez et al., 2006; Rohde et al., 2008; Miteva et al., 2009). Further, a variety of studies have demonstrated that cold-tolerant microorganisms isolated from frozen environments (e.g. permafrost, snow, sea ice, lake ice) can remain metabolically active at subzero temperatures in an ice matrix (Table 1.2). Based on these observations, it is reasonable to hypothesize that basal ice also harbors microorganisms with the capacity to remain metabolically active while frozen. However, no study to date has specifically examined basal ice with the idea that it is an active ecosystem and thus direct evidence of biogeochemical activity within basal ice is still lacking.
Table 1.2. Reports documenting evidence for subzero metabolic activity. Adapted from Doyle *et al.*, (2012).

<table>
<thead>
<tr>
<th>Source</th>
<th>T (°C)</th>
<th>Activity Measurement</th>
<th>Technique(^a)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk community measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permafrost</td>
<td>-15</td>
<td>Respiration</td>
<td>[^{14}C]Glucose uptake</td>
<td>(Gilichinsky <em>et al.</em>, 2003)</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>Respiration</td>
<td>[^{14}C]Glucose and</td>
<td>(Steven <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[^{14}C]acetate mineralization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>CH4 production</td>
<td>[^{14}C]Bicarbonate and</td>
<td>(Rivkina <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[^{14}C]acetate utilization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>Lipid synthesis</td>
<td>[^{14}C]Acetate incorporation</td>
<td>(Rivkina <em>et al.</em>, 2000)</td>
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<td></td>
<td>-39</td>
<td>Respiration</td>
<td>[^{14}C]Glucose uptake</td>
<td>(Panikov <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Tundra</td>
<td>-30</td>
<td>N mineralization and</td>
<td>Measurement of nitrate and</td>
<td>(Schimel <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nitrification</td>
<td>ammonium</td>
<td></td>
</tr>
<tr>
<td>Snow</td>
<td>-17</td>
<td>Macromolecular</td>
<td>[^{3}H]Thymidine and</td>
<td>(Carpenter <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>synthesis</td>
<td>^{3}H^leucine incorporation</td>
<td></td>
</tr>
<tr>
<td>Sea Ice</td>
<td>-1</td>
<td>Protein synthesis</td>
<td>[^{3}H]Leucine incorporation</td>
<td>(Grossmann, 1994)</td>
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<tr>
<td></td>
<td>-1.5</td>
<td>DNA synthesis</td>
<td>[^{3}H]Thymidine</td>
<td>(Smith and Clement, 1990)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>incorporation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>Respiration</td>
<td>CTC reduction</td>
<td>(Junge <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Marine</td>
<td>-1.3</td>
<td>Protein synthesis</td>
<td>[^{14}C]Amino acid</td>
<td>(Ritzrau, 1997)</td>
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<td></td>
<td></td>
<td></td>
<td>incorporation</td>
<td></td>
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<td>Laboratory studies of environmental isolates</td>
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<tr>
<td>Permafrost</td>
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<td>Reproduction</td>
<td>Plate counts, optical density</td>
<td>(Bakermans <em>et al.</em>, 2003)</td>
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<td>DNA Repair</td>
<td>PFGE(^b)</td>
<td>(Dieser <em>et al.</em>, 2013)</td>
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<td>Metabolism</td>
<td>Resazurin reduction</td>
<td>(Jakosky <em>et al.</em>, 2003)</td>
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<td></td>
<td>-35</td>
<td>Metabolism</td>
<td>[^{14}C]CO(_2) uptake</td>
<td>(Panikov and Sizova, 2007)</td>
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<td>-80</td>
<td>Metabolism</td>
<td>Adenylate pool</td>
<td>(Amato and Christner, 2009)</td>
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<td>Sea Ice</td>
<td>-10</td>
<td>Motility</td>
<td>Microscopy</td>
<td>(Junge <em>et al.</em>, 2003)</td>
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<td></td>
<td>-12</td>
<td>Reproduction</td>
<td>Optical density</td>
<td>(Breezee <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td></td>
<td>-196</td>
<td>Protein synthesis</td>
<td>[^{3}H]Leucine incorporation</td>
<td>(Junge <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Subglacial Lake Ice</td>
<td>-15</td>
<td>Macromolecular</td>
<td>[^{3}H]Thymidine and</td>
<td>(Christner, 2002; Amato <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>synthesis</td>
<td>[^{3}H]Leucine incorporation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-32</td>
<td>N2O production</td>
<td>CF-IRMS</td>
<td>(Miteva <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td>Marine sediments</td>
<td>-1.7</td>
<td>Sulfate reduction</td>
<td>Reduction of [^{35}S] SO(_4)(^{2-})</td>
<td>(Knoblauch <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Metabolism inferred from biogenic gases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tundra</td>
<td>-5</td>
<td>N2O production</td>
<td>Gas chromatography</td>
<td>(Brooks <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td></td>
<td>-12</td>
<td>CO2 production</td>
<td>Infrared gas analyzer</td>
<td>(Mikan <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td></td>
<td>-10 to</td>
<td>Gas concentrations and</td>
<td>Gas chromatography and mass spectrometry</td>
<td>(Sowers, 2001; Campen *et al., 2003; Tung <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>-40</td>
<td>isotopic composition</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)CF-IRMS, continuous flow isotope ratio monitoring mass spectrometry; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride.

\(^b\)Pulsed field gel electrophoresis
Ice veins provide a liquid microenvironment which may serve as a habitat for microbial life under conditions where liquid water cannot exist in the bulk phase. However, microorganisms in the ice vein matrix microbes are confronted with a variety of physiochemical stresses that include low water activity, low pH, membrane damaging ice crystal formation, and reduced solute diffusion rates. Furthermore, they must also face various biochemical challenges associated with low temperatures including reduced enzymatic activity, protein denaturation, and decreased membrane fluidity and transport efficiency (D'Amico et al., 2006; Doyle et al., 2012). Although relatively little is known about what confers psychrophily, several adaptations have been identified which are believed to be important for life at cold temperatures. Examples include possession of catalytically efficient enzymes (Feller and Gerday, 2003), synthesis of specialized lipids which increase membrane flexibility (Los and Murata, 2004; Nichols et al., 2004), and the production of proteins (Raymond et al., 2008) and extracellular polymeric substances (Krembs et al., 2011) which affect ice crystal structure. What remains clear, however, is that the long-term survival of a microbial population inside ice is constrained by its capability to endure the genetic and cellular damage that would accumulate in the absence of a functional metabolism. This damage to cellular macromolecules can be caused by a variety of physical and chemical mechanisms, including natural background radiation (e.g., produced from the decay of $^{40}\text{K}$, $^{232}\text{Th}$ and $^{238}\text{U}$), L-amino acid racemization, and spontaneous reactions which oxidize, depurinate, and crosslink nucleic acids (Hansen et al., 2006). As such, microbial populations which exhibit metabolic dormancy while frozen are limited in the duration for which they can survive while frozen. Without an active metabolism to support cellular repair, the macromolecules of dormant microorganisms will gradually accumulate damage over time, reaching a time-dependent threshold beyond which they are longer viable. The recovery of viable
microorganisms from ancient ice (Abyzov et al., 1998; Christner et al., 2003; Bidle et al., 2007) and permafrost (Brinton et al., 2002; Steven et al., 2006; Johnson et al., 2007) estimated to be hundreds of thousands to millions of years old thus suggests that certain psychrophilic microorganisms remain metabolically active while frozen and maintain their cellular integrity under in situ conditions over extended timeframes. Indeed, a recent study by Dieser et al., (2013) demonstrated Psychrobacter arcticus 273-4, a bacterium originally isolated from ~25,000 year old Siberian permafrost, can repair seven to ten DNA double-strand breaks a year while frozen at -15 °C. This rate of DNA repair is >100,000-fold faster than the rate at which DNA double-strand breaks are predicted to occur as a result of background radiation in the permafrost. Hence, the long-term survival of metabolically active microorganisms in frozen environments may only be limited by the availability of suitable redox couples and nutrients. In this respect, sediment rich basal ice may be an unrecognized ecosystem with the capacity to harbor diverse communities of heterotrophic and lithotrophic microorganisms over extended time-frames.

**Objectives of This Study**

Although interest in microbiology of glacial ice has grown significantly in recent years, most investigations have actually focused on sediment and aquatic environments which exist beneath glaciers. Relatively little is known about microorganisms which are frozen in the basal ice which overlies and interacts with these subglacial environments. The primary objective of this study was to provide a better understanding of the distribution, composition, and metabolism of microbial assemblages found within basal ice. These data provide a basis for answering a fundamental yet still open question in cryospheric microbiology: Is ice a microbial habitat or archive? In other words, are microorganisms entrapped within the ice metabolically active and do they have the capacity to alter the chemical composition of their environment? The work
presented here focused primarily on basal ice from Taylor Glacier, Antarctica where the availability of physical and chemical data provided an opportunity to explore the relationship between microbial metabolic activity and the presence of basal sediment. This study is among the first to examine microbial assemblages from multiple types sediment-rich and sediment-poor basal ice from glaciers in Antarctica, Greenland, and Alaska. The results of this study help expand current understanding of the boundaries of life on Earth and may also provide valuable insight during future missions searching for microbial life in extraterrestrial frozen environments on Mars and Europa.
CHAPTER 2.
DIVERSITY OF BACTERIAL ASSEMBLAGES IN VARIOUS DEBRIS-RICH AND DEBRIS-POOR BASAL ICE CRYOFACIES

Introduction

Other than insights offered by some initial exploratory investigations of basal ice from Greenland and the Canadian High Arctic (Miteva et al., 2004; Skidmore et al., 2005; Yde et al., 2010) the composition and structure of microbial assemblages within basal ice environments, especially those beneath the AIS, remain largely unknown. Moreover, how microbial community membership varies between different types of basal ice has never been investigated. Indeed, the basal zones of glaciers often contain multiple distinct horizons of basal ice which can vary tremendously in their structure, layering, texture, thickness, and sediment content. These parameters, taken together, are used to classify basal ice into distinct categories known as cryofacies, eighteen of which have been classified (Hubbard et al., 2009). In this study, basal ice samples were collected from the Taylor Glacier (Antarctica), Støre Landgletscher Glacier (Greenland), and Matanuska Glacier (Alaska) from which four basal ice cryofacies were represented: (i) clean ice, containing no debris; (ii) banded ice, containing finely mixed layers of debris and ice; (iii) solid ice, containing frozen debris with only interstitial ice; and (iv) dispersed ice, heavily laden with scattered debris aggregates with no layering (Figure 2.1). Discerning how these cryofacies vary in their ability to harbor and support different populations of microorganisms will be valuable in determining how subglacial processes influence the microbiology of basal ice.

Sequencing of 16S rRNA genes was used to assess the composition of microbial assemblages frozen within the basal ice samples. In addition, an approach to identify metabolically active taxa in situ was used by reverse transcribing and sequencing 16S rRNA
molecules in parallel to the DNA-based approach. The results of these analyses were used to determine the community structure and potential metabolic functions of microbial communities immured in basal ice environments and further test the hypothesis that sediment-rich basal ice is a habitat for metabolically active microorganisms.

Figure 2.1. Examples of the four types of basal ice investigated in this study. (A) banded ice; Taylor Glacier; (B) clean ice; Taylor Glacier; (C) solid ice; Taylor Glacier; (D) dispersed ice; Matanuska Glacier.

Materials and Methods

Taylor Glacier basal ice sampling

During the austral summers of 2007 and 2009, two tunnels were excavated into the northern margin of Taylor Glacier (2007: 58C 434291 1371141, elevation 215 m; 2009: 58C 434436 1371161, elevation 211 m) to directly access a stratigraphic sequence of basal ice. Taylor Glacier is a 54 km outflow glacier of the East Antarctic Ice Sheet and is located at the western
end of Taylor Valley in the McMurdo Dry Valleys of Victoria Land, terminating on the western lobe of Lake Bonney. The tunnels were initiated on fresh ice aprons and extended 7-9 m in from the external glacier face. In 2007, a vertical shaft (~5 m) was constructed at the end of the tunnel (Figure 2.2, B), and a 4 m vertical profile of basal ice was sampled (Figure 2.2). Using the nomenclature of Hubbard et al., (2009), three distinct basal ice cryofacies were identified in the sample profile: clean ice, banded ice, and solid ice (Figure 2.3). The top of the profile sampled

Figure 2.2. (A) Beginning of tunnel construction during the 2009 austral summer at Taylor Glacier. Note the exposed horizon of sediment-laden basal ice directly below the tunnel entrance. (B) Vertical shaft used to sample a profile of basal ice from Taylor Glacier during the 2007 austral summer. (C) View from the end of the excavated tunnel adjacent to the location of the vertical shaft shown in panel B.
Figure 2.3. (A) Map and aerial photograph of Taylor Glacier, located in the McMurdo Dry Valleys of Victoria Land, Antarctica. The location of the 2007 and 2009 access tunnels are indicated. (B) Schematic of the 4 m deep basal ice profile sampled from Taylor Glacier in 2007. The top of the profile (designated 0 cm) was located in debris-poor clean ice, which was underlain by several layers of both debris-rich banded dispersed and laminated solid ice with a thick layer of basal solid ice as the lowermost unit. (C) Schematic of the debris-rich banded dispersed basal ice horizon sampled in 2009. Reprinted with permission from Doyle et al., (2013).

(i.e., the access tunnel floor) was designated as the zero depth, with depth designations representing distance below this reference point. Sample ice blocks measuring approximately 20 × 20 × 10 cm (length × height × width) were cut using electric chainsaws with carbide tipped chains and no lubricating fluids. During the 2009 season, another access tunnel was excavated to
directly intersect a layer of sediment-rich banded ice (Figure 2.3, C) and 27 large (40 × 30 × 15 cm; length × height × width) blocks containing banded ice were collected. All ice samples were shipped frozen to Louisiana State University or Montana State University and stored at -20°C.

During the 2009 field season, ~132 kg of banded ice from the Taylor Glacier was also melted and filter concentrated at the Crary Laboratory (McMurdo Station). The samples were preserved in RNAlater (Ambion) and shipped frozen to Louisiana State University. After decontamination, the ice was placed in sterilized polypropylene containers and allowed to melt at 4°C. Complete melting of the basal ice took place over a period of seven days, during which the meltwater was continuously collected and concentrated onto filters. In order to remove coarse sediment particles which would inhibit filtration of such a large sample volume, the sediment-meltwater slurry was pre-filtered consecutively through a series of five sterilized nylon monofilament filters of decreasing pore size (100, 75, 50, 25 and 10 μm) and then centrifuged at 700 ×g for 10 minutes at 4°C. The supernatant containing fine silt and clay sized particles (~90 L) was filtered at 4°C under a 20 cm Hg vacuum onto eleven 90 mm, 0.22 μm Supor-200 filters (Pall Corp). On each filter, 8.3 ± 1.9 L of supernatant was filtered in this manner. These filters (referenced below as CraryFilters) were then immersed in RNAlater (Ambion), frozen at -80°C, and shipped to Louisiana State University for storage and analysis.

**Basal ice sampling at Matanuska Glacier**

Matanuska Glacier is a 43km valley glacier located in south-central Alaska, approximately 140 km northeast of Anchorage (Figure 2.4). During July 2013, a horizon of debris-rich dispersed ice exposed near the terminus of the glacier was sampled using an electric chainsaw (06V 0459835 6849428, elevation 510m) (Figure 2.4). Ice samples from Matanuska Glacier were shipped frozen to Louisiana State University and stored at -20°C.
Figure 2.4. Map and photograph of the sampled horizon, highlighted in yellow, of dispersed basal ice located at the terminus of Matanuska Glacier, Alaska.

**Basal ice sampling at Støre Landgletscher Glacier**

In June 2011, basal ice samples were collected via chainsaw from the margin of the Støre Landgletscher Glacier, located at the north-western margin of the Greenland Ice Sheet (19X 520947 8494835, elevation 390 m) near Thule Air Base. The ice samples were placed in polyethylene bags and shipped frozen to the Polar Science Center at the University of Washington and stored at -20°C.

**Ice Sample Decontamination**

Prior to decontamination, the large ice blocks collected in the field were subsampled for analysis by dry-cutting; sediment-free ice samples were cut using a steel band saw (Delta 28-206) and sediment-containing ice was cut using a masonry saw equipped with a diamond blade.
(MK Diamond BX-4). The ice samples were handled with sterile stainless steel forceps and the outer contaminated surface was removed in a class 100 laminar flow hood that was housed within a -5 °C walk-in freezer. The decontamination procedure was modified from the protocol reported by (Christner et al., 2005) for sampling deep ice cores recovered using hydrocarbon-based drilling fluids. The outermost surface of debris-free ice samples was physically removed by scraping with an autoclaved microtome blade. The newly exposed ice surface was then washed with 0.22 μm filtered 95% ethanol that was equilibrated to -5°C. The initial microtome scraping step was omitted when processing sediment-rich ice samples due to the presence of coarse granules and stones embedded in the sediment-layers. Following the ethanol wash, samples were then rinsed with ice-cold, 0.22 μm filtered, twice-autoclaved deionized water until a minimum of five millimeters of the outer sample surface had been removed. Sterile forceps were used to hold the samples during washing and were exchanged frequently during the procedure to prevent carryover contamination. All samples were weighed before and after decontamination, which reduced the total mass of each samples by 15% to 25%. The decontaminated samples were placed in sterile containers and melted at 4 °C over 16 to 24h).

The effectiveness of the decontamination procedure was monitored by deliberately contaminating ice samples with a tracer solution. This tracer solution consisted of three components combined in equal volumes: (1) suspension (≥ 10^8 cells mL^-1) of *Escherichia coli* JM109 cells transformed with a pETBlue-2 vector (Novagen) containing the gene for alcohol dehydrogenase (ADH) from *Drosophila melanogaster*, (2) fluorescein (1000 ppm), and (3) sterile glycerol (to prevent immediate freezing of the tracer solution upon application). The removal of each tracer component was monitored after each step of the decontamination procedure (i.e. scraping, ethanol wash, H₂O wash). The fluorescein fluorophore component was
detected by quantifying blue-green fluorescence in 200 μL of rinse-water or melt-water using a BioTurner 20/20n Luminometer (P/N 2030-002) equipped with the blue fluorescence module (BioTurner 2030-041). The pETBlue-2 plasmid component was detected using a PCR with the following conditions: 1.0 unit of Taq DNA polymerase (5PRIME), 1× MasterTaq buffer, 1× TaqMaster PCR enhancer, 1.5 mM Mg(C₂H₃O₂)₂, 15 pmol of each primer (TF7: 5’-TAATACGACTCACTATAGGG -3’; pETBlue-DOWN: 5’-GTAAATTGCTAACGAGCAGTCA-3’), 200 μM deoxynucleotide triphosphates (dNTPs), and ~100 pg of template DNA. Thirty cycles of PCR were done with a 30 s denaturation step at 94 °C, 60 s annealing step at 55 °C and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. The E. coli component was recovered by spread plating of rinse-water and melt-water on agar-solidified LB media containing ampicillin (100 μg mL⁻¹) followed by incubation at 37 °C. Samples in which any component of the tracer solution was detected in the final melt-water were excluded from all analyses presented here.

**Nucleic Acid Extraction and Quantification**

DNA was extracted from basal ice samples with low sediment content (i.e. Taylor Glacier clean ice and Støre Landgletscher Glacier banded ice) by filtering the meltwater onto 47mm, 0.22 μm pore size Supor filters (Pall Corp.) using a bench-top vacuum manifold (≤ 20 kPa). Genomic DNA was extracted from the filters using a MoBio PowerWater DNA Isolation kit as per the manufacturer’s instructions with the exception that the mechanical lysis step was performed on a BioSpec MiniBeadbeater-8 for 2 minutes at maximum speed. For the banded, solid, and dispersed basal ice samples (samples from Taylor Glacier and Matanuska Glacier), genomic DNA was extracted directly from 20g of sediment (wet weight) recovered after melting the ice using the MoBio PowerMax Soil DNA Isolation kit. The manufacturer’s instructions
were modified to increase yield by pooling two 10g extractions onto a single silica spin column. DNA from one of the CraryFilters containing material from banded ice sampled in Antarctica during the 2009 field season was extracted using the MoBio PowerMax soil DNA extraction kit with no modification to the manufacturer’s protocol.

For the extraction of RNA, meltwater from both sediment-rich and sediment-poor ice samples were filtered onto 90mm, 0.2 μm pore size Supor filters. Coarse sediment particles were previously removed using low speed centrifugation (700×g; 10 min, 4°C) and the supernatant containing fine clay and silt-like particles was concentrated on the filters. Clean ice samples required no centrifugation step and were directly filtered. With the exception of the CraryFilter samples, which were prepared in Antarctica and shipped to LSU, all filters were immediately processed for RNA extraction after filtration. The RNA extraction and isolation protocol was a modified version of the methodology described by (Dieser et al., 2014). The filters were cut into small pieces using a sterile scalpel and transferred into a 7mL bead beating tube containing 3 mL of TE buffer (1mM EDTA, 10mM Tris; pH 6.3) and lysozyme (15 mg mL⁻¹); the samples were mixed by vortexing for 30 minutes at room temperature. Following vortexing, ~1g of 0.1 mm glass beads were added and the mixture was homogenized in a BioSpec MiniBeadbeater-8 for 2 minutes at maximum shaking speed. After bead beating, the samples were immediately placed on ice. The crude extracts were transferred to sterile 50 mL tubes and amended with two volumes of a chilled (4°C) denaturing buffer (4 M guanidine thiocyanate, 50 mM Tris (pH 7.0), 10 mM EDTA (pH 8.3), 1% w/v N-lauroyl sarcosine, and 1% v/v β-mercaptoethanol). The insoluble material was pelleted via centrifugation (4,500×g for 5 min at 4°C) and the supernatant was collected. The pellet was washed with 3 mL of denaturing buffer, centrifuged, and the supernatants were pooled. The lysate was extracted with an equal volume of chilled
phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.6), followed by chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 2.5 volumes of ice-cold 100% ethanol, 0.1 volumes of 3 M sodium acetate (pH 6.0), 20 μg mL^{-1} (final concentration) linear acrylamide (Ambion), and incubated overnight at -20°C. After centrifugation (60 minutes at 17,000×g), the nucleic acid pellet was washed with 70% ethanol, centrifuged again, and suspended in 100 μL TE buffer. Genomic DNA was eliminated from the extracts by digestion with 4 U of TURBO DNase for one hour at 37°C using the TURBO DNA-free kit (Ambion) per the manufacturer’s instructions. The RNA extract was purified with MEGAclear clean up columns (Ambion) followed by a subsequent overnight ethanol precipitation (as described above). The RNA pellet was suspended in 50 μL TE buffer and stored at -80°C.

Extracted DNA and RNA concentrations were measured on a 20/20n Luminometer (Turner Biosystems) equipped with a blue fluorescence module (P/N 2030-041) using the Quant-iT Picogreen and Ribogreen assay kits respectively (Life Technologies) per the manufacturer’s instructions (Table 2.1).

Table 2.1. Extraction and quantification of DNA and RNA from various basal ice cryofacies. Basal ice samples which contained sediment are highlighted in gray.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Size for Extraction (g)</th>
<th>DNA Concentration (pg g^{-1} ice)</th>
<th>RNA Concentration (pg g^{-1} ice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG-Clean07</td>
<td>720.0</td>
<td>8500.0</td>
<td>4.7</td>
</tr>
<tr>
<td>TG-Clean09</td>
<td>1411.8</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>TG-Banded07</td>
<td>20.0</td>
<td>1913.7</td>
<td>16994.3</td>
</tr>
<tr>
<td>TG-Banded09</td>
<td>20.0</td>
<td>5582.0</td>
<td>16140.1</td>
</tr>
<tr>
<td>TG-Solid</td>
<td>20.0</td>
<td>3880.9</td>
<td>14366.2</td>
</tr>
<tr>
<td>Matanuska</td>
<td>20.0</td>
<td>2852.6</td>
<td>98860.2</td>
</tr>
</tbody>
</table>
**16S rRNA Library Preparation, Sequencing, and Curation**

Complementary DNA (cDNA) libraries of 16S rRNA molecules were reverse transcribed from ~1 ng of total RNA using SuperScript II reverse transcriptase and the 806R primer (5’-GGACTACVSGGGTATCTAAT-3’) following the manufacturer’s protocol. Negative controls as well reactions that omitted reverse transcriptase were included to monitor for contaminating RNA and DNA molecules, respectively.

The hyper-variable V4 region of the 16S rRNA gene was amplified from basal ice DNA extractions and cDNA libraries using the primers 515F and 806R that were synthesized with Golay barcodes and adapters required for Illumina dye sequencing on the MiSeq platform (Caporaso et al., 2012). The PCR reaction mixtures consisted of 2.5 U of AmpliTaq Gold DNA Polymerase LD (low DNA), 1 × GeneAmp PCR Gold Buffer, 2.5 mM MgCl₂, 10 pmol of each primer, 200 μM dNTPs, and ~100 pg of template DNA. After a 9 minute pre-incubation polymerase activation step at 95°C, 35 cycles of PCR were done under the following conditions: denaturation at 94°C for 1 minute, annealing at 50°C for 30 seconds, and elongation at 72°C for 30 seconds. A 10 minute extension at 72°C was the final step of the amplification reaction. For cDNA samples, the template concentration was ~600 pg of cDNA, the magnesium concentration was increased to 3.5 mM, and the number of PCR cycles was decreased to thirty.

PCR products were visualized using gel electrophoresis in a 1.5% agarose TAE (Tris-acetate-EDTA) gel that was stained with ethidium bromide. Amplicons of the predicted size (~350bp) were quantified using the Quant-iT PicoGreen dsDNA Kit (Invitrogen) on a 20/20n Luminometer (Turner BioSystems) equipped with a blue fluorescent module (P/N 2030-041) and pooled by mass (~15ng per amplicon). Additionally, although amplification of negative controls was not detectable via agarose gel electrophoresis, “amplicons” from two template-free blanks
were included in the pooled MiSeq library as recommended by (Salter et al., 2014) when sequencing samples from low-biomass environments. The pooled library was purified using a MoBio UltraClean PCR Cleanup Kit (#12500, MoBio Laboratories) and stored at -20°C. Paired-end sequencing (2 × 250 bp) was conducted on a MiSeq Desktop Sequencer (Illumina) at the Georgia Genomics Facility (Athens, GA, USA) and Selah Genomics (Greenville, SC, USA). A 20% spike-in of PhiX Control v3 was used as an internal control to increase library diversity and improve cluster generation and base-calling.

Sequence reads were processed and curated using a combination of MOTHUR v1.33.3 (Schloss et al., 2009) and SINA (SILVA Incremental Aligner) v1.2.11 (Pruesse et al., 2012). Paired-end reads were assembled and quality filtered to exclude sequences containing ambiguous bases or homopolymers runs longer than 8 bp. The sequences were aligned to the SILVA non-redundant 16S rRNA reference dataset (v.119) using SINA on LSU’s SuperMike-II HPC cluster and chimeras were identified and removed using the UCHIME algorithm (Edgar et al., 2011). To minimize errors introduced during sequencing, the aligned sequences were pre-clustered allowing 1-bp difference per 100 bp of sequence before calculating uncorrected pairwise distances (Kozich et al., 2013). Sequences were then clustered into operational taxonomic units (OTU) at 3% dissimilarity using the furthest neighbor algorithm. OTUs were classified using a naïve Bayesian classifier (Wang et al 2011) and the Ribosomal Database Project training set (Release 9). Representative sequences for each OTU, selected as the most abundant sequence within an OTU, were taxonomically classified with the Greengenes, SILVA, and NCBI databases. OTUs represented by a single sequence (i.e. singletons) or in which 1% or greater its member sequences were from either of the template-free blank libraries were removed from the dataset. Diversity, richness, coverage, and dissimilarity indices were calculated in MOTHUR.
Metabolically active OTUs were inferred by calculating the ratio of the relative abundance of sequences obtained in the 16S rRNA molecule (rRNA) and 16S rRNA gene (rDNA) data sets.

**Results**

A total of 4,482,737 paired-end contigs with an average read length of 269 bp were obtained from MiSeq sequencing. After screening and quality filtration, 2,955,583 sequence contigs with an average read length of 253 bp were used for downstream analysis. The culling of OTUs represented in the negative controls or containing only singletons removed 829,638 sequence reads from the overall 14-sample dataset (Table 2.2). In the rDNA libraries, this resulted in the removal of between 0.6% (Matanuska) and 29.5% (TG-Clean09) of the total sequence reads in a library. This was even more pronounced in the rRNA libraries where in some cases (i.e. TG-Solid ice) 99.9% of the sequence reads clustered with those obtained in negative control amplifications and were thus removed.

Table 2.2. Number of sequence reads in each 16S library before and after the removal of OTUs flagged as potential background contamination or containing only a single sequence read. Libraries prepared from basal ice samples which contained sediment are highlighted in gray.

<table>
<thead>
<tr>
<th>Library</th>
<th>rDNA Reads</th>
<th></th>
<th>rRNA Reads</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>% Remaining</td>
<td>Before</td>
</tr>
<tr>
<td>TG-Banded07</td>
<td>314255</td>
<td>232486</td>
<td>74.0</td>
<td>106213</td>
</tr>
<tr>
<td>TG-Banded09</td>
<td>321726</td>
<td>299843</td>
<td>93.2</td>
<td>179522</td>
</tr>
<tr>
<td>TG-Solid</td>
<td>286232</td>
<td>235472</td>
<td>82.3</td>
<td>157029</td>
</tr>
<tr>
<td>TG-Clean07</td>
<td>217781</td>
<td>173458</td>
<td>79.6</td>
<td>61260</td>
</tr>
<tr>
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<td>264590</td>
<td>186600</td>
<td>70.5</td>
<td>-</td>
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<td>211759</td>
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<tr>
<td>TG-CraryFilter2A</td>
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<td>-</td>
<td>204913</td>
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<tr>
<td>Matanuska</td>
<td>335587</td>
<td>333595</td>
<td>99.4</td>
<td>269909</td>
</tr>
<tr>
<td>SL Glacier</td>
<td>24807</td>
<td>24545</td>
<td>98.9</td>
<td>-</td>
</tr>
</tbody>
</table>

With the exception of the banded ice from Større Landgletscher Glacier, inverse Simpson and Shannon indices indicated that the total microbial diversity in all of the sampled basal ice cryofacies was low (Table 2.3).
Table 2.3. Summary of diversity and richness for 16S rRNA gene (rDNA) libraries at a genetic distance of 0.03. 95% confidence intervals are given in parentheses. Libraries prepared from basal ice samples which contained sediment are highlighted in gray.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of OTUs(^a)</th>
<th>Good's Coverage*</th>
<th>Inverse Simpson Index*</th>
<th>Shannon Index*</th>
<th>Chao1*</th>
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</thead>
<tbody>
<tr>
<td>Banded07</td>
<td>1232</td>
<td>0.997</td>
<td>4.58 (4.50, 4.67)</td>
<td>2.30 (2.28, 2.32)</td>
<td>387 (341, 465)</td>
</tr>
<tr>
<td>Banded09</td>
<td>826</td>
<td>0.999</td>
<td>2.54 (2.51, 2.57)</td>
<td>1.27 (1.25, 1.28)</td>
<td>179 (119, 318)</td>
</tr>
<tr>
<td>Solid</td>
<td>978</td>
<td>0.997</td>
<td>3.79 (3.73, 3.86)</td>
<td>2.12 (2.10, 2.14)</td>
<td>350 (316, 410)</td>
</tr>
<tr>
<td>Clean07</td>
<td>612</td>
<td>0.997</td>
<td>1.67 (1.65, 1.69)</td>
<td>1.10 (1.09, 1.12)</td>
<td>385 (282, 582)</td>
</tr>
<tr>
<td>Clean09</td>
<td>793</td>
<td>0.997</td>
<td>1.33 (1.32, 1.34)</td>
<td>0.69 (0.67, 0.71)</td>
<td>503 (321, 871)</td>
</tr>
<tr>
<td>CraryFilter2E</td>
<td>632</td>
<td>0.998</td>
<td>1.48 (1.46, 1.49)</td>
<td>0.74 (0.73, 0.76)</td>
<td>266 (186, 430)</td>
</tr>
<tr>
<td>Matanuska</td>
<td>1850</td>
<td>0.999</td>
<td>3.12 (3.09, 3.15)</td>
<td>1.45 (1.43, 1.46)</td>
<td>199 (139, 334)</td>
</tr>
<tr>
<td>SL Glacier</td>
<td>2208</td>
<td>0.766</td>
<td>37.76 (36.62, 38.97)</td>
<td>6.44 (6.41, 6.48)</td>
<td>41139 (38677, 43808)</td>
</tr>
</tbody>
</table>

\(^a\)After quality filtering and removal of chimeras, singletons, and background noise. *Calculations normalized to the smallest library by using a random subsample of 24,545 sequences.
Rarefaction curves (Figure 2.5) for all the rDNA libraries except that for SL Glacier banded ice approached saturation with OTUs at a 3% genetic distance cutoff. Good’s Coverage values for these libraries was also very high (>99%; Table 2.3), indicating the un-sampled diversity consisted almost exclusively of rare taxa. In contrast, the library for SL glacier appears to have incompletely captured a significant portion of the microbial diversity from this environment.

Figure 2.5. Rarefaction curves for MiSeq-derived 16S rRNA gene (rDNA) sequence OTUs defined at a genetic distance of 0.03 for various basal ice samples.

Overall, the composition of microbial assemblages in samples from the three different glaciers sampled varied substantially. The Firmicutes were the most abundant phylum present (32.5% of all sequence reads); however, their dominance was exclusive to basal ice from Taylor Glacier and they were rare members in both the dispersed ice from Matanuska Glacier (0.13%) and banded ice from Støre Landgletscher Glacier (0.03%). The next most abundant group
observed were those that could not be classified to any bacterial phylum, consisting of 14.1% of all sequence reads, the vast majority of which were collected from the TG-Banded09 and Matanuska basal ice samples.

**Taylor Glacier Basal Ice: Clean Ice**

Both clean ice rDNA libraries displayed very low diversity (Inverse Simpson’s Indices ≤ 1.67) and were heavily dominated by members of the Firmicutes, representing 73.7% (2007) and 61.6% (2009) of all sequence reads. Over 70% (2007) and 98% (2009) of the Firmicutes reads belonged to the genus *Tumebacillus*, and other genera abundant included *Chryseobacterium*, *Marinobacter*, *Paenisporosarcina*, and *Acinetobacter*.

Owing to the extremely low biomass present in clean ice (~10^2 cells/g; Chapter 3), RNA molecules were difficult to recover from the clean ice and required extractions from over 8 kg of 2007 clean ice (Table 2.1) to recover an amplifiable amount of template. A comparable size of clean ice (>5 kg) was not available from the 2009 samples. Attempts to extract RNA from smaller ~1 kg samples of clean ice which could be trimmed off the ends of a 2009 banded ice sample (for example, see Figure 2.3, C) were not successful. Moreover, although 16S rRNA was successfully reverse-transcribed, amplified, and sequenced, 99.2% of the sequence reads in the rRNA library clustered with those obtained in negative control amplifications. The remaining 481 reads were not considered adequate to draw any conclusions regarding metabolic activity.

**Taylor Glacier Basal Ice: Banded Ice**

Similar to clean ice, the banded ice also displayed a fairly low diversity of OTUs (Inverse Simpson’s Indices ≤ 4.58). Although the Firmicutes were the most abundant phylum in the Banded07 library (29.0%), the Actinobacteria (27.5%) and Bacteroidetes (17.8%) were also abundant. In contrast, the Banded09 library was predominantly populated by unclassifiable
bacterial taxa (38.9%) with comparable quantities of the Bacteroidetes (21.3%) and Actinobacteria (17.7%). The most abundant OTU (18.3%) in the 2007 banded ice could not be classified beyond the phylum Firmicutes. BLAST analysis of a representative sequence from this OTU against the NCBI database did not identify any closely related cultured representative (all alignments ≤ 83% identity) but did reveal a 99% identity to an uncultured bacterium from ice-entrained brine from Lake Vida, a sub-ice hypersaline lake in the McMurdo Dry Valleys. Similarly, the most abundant OTUs in the 2009 banded ice were also unclassifiable but were related to uncultured bacteria detected previously in cold and/or salty environments.

After curation, 30,618 sequence reads remained in the TG-Banded07 rRNA library, representing 76 OTUs. Based on the ratio of the relative abundance of reads in the RNA library to the DNA library, two genera of Firmicutes, *Paenisporosarcina* and *Clostridium*, appeared to stand out with ratio values >10-fold higher than any other OTU (Table 2.4, A). Other taxa identified with large rRNA:rDNA sequence ratios were *Virgibacillus*, *Paenibacillus*, and *Desulfosporosinus*. In contrast, most of the TG-Banded09 rRNA library (99.5%) clustered with the template-free negative controls. In an effort to avoid falsely identifying some members as metabolically active, the remaining 0.5% of the reads was not considered sufficient to draw any conclusions regarding *in situ* metabolic status.

The CraryFilter2E (rDNA) library had the lowest diversity of all the banded ice samples (Inverse Simpson’s Index = 1.48, Table 2.3) and clustered more closely with the clean basal ice samples than those prepared directly from banded ice (Figure 2.6). *Acinetobacter* was the most abundant genera identified and represented 47.9% of all sequence reads, followed by members of *Bacillus* (17.8%) and *Paenisporosarcina* (15.6%). After curation, 143,137 sequence reads remained in the TG-CraryFilter2A (rRNA) library and clustered into 298 OTUs. Several OTUs
<table>
<thead>
<tr>
<th>OTU#</th>
<th>rDNA Reads</th>
<th>RelAbund %</th>
<th>rDNA Reads</th>
<th>RelAbund %</th>
<th>rRNA/rDNA Ratio</th>
<th>MOTHUR</th>
<th>SILVA</th>
<th>Greengenes</th>
<th>NCBI BLASTN Accession</th>
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<tbody>
<tr>
<td>Otu01819</td>
<td>957</td>
<td>0.4116</td>
<td>21096</td>
<td>68.9</td>
<td>167.4</td>
<td>unclassified Bacilli</td>
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<td>Paenibacillus sp. MB05</td>
<td>NR_044222.1</td>
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<td>Otu01378</td>
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<td>Desulfospirillum</td>
<td>Desulfospirillum barensis</td>
<td>NR_05999.2</td>
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<td>Otu01144</td>
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<td>0.2594</td>
<td>894</td>
<td>2.9</td>
<td>11.3</td>
<td>Desulfosporosinus unclassified Chloroflexi</td>
<td>Desulfosporosinus barensis</td>
<td>Desulfosporosinus barensis</td>
<td>NR_10421.2</td>
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<tr>
<td>Otu00727</td>
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<td>3241</td>
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<td>10.9</td>
<td>Vigilibacillus</td>
<td>Virgilibacillus</td>
<td>Virgilibacillus sp.</td>
<td>NR_11610.6</td>
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<td>Otu07206</td>
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<td>63</td>
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<td>7.6</td>
<td>unclassified Firmicutes</td>
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<td>Paenibacillus</td>
<td>NR_116426.3</td>
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<td>Otu00536</td>
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<td>213</td>
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<td>Paenibacillus</td>
<td>Paenibacillus</td>
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<td>Otu01185</td>
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<td>155</td>
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<td>unclassified Chloroflexi</td>
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<td>139</td>
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<td>Caldicoprobacter</td>
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<td>Otu02220</td>
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<td>4.2751</td>
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<td>3.0</td>
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<td>Demepaque</td>
<td>Demepaque</td>
<td>Demepaque</td>
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</tbody>
</table>

Table 2.4. Identification of OTUs inferred to be metabolically active within rDNA Reads.

<table>
<thead>
<tr>
<th>OTU#</th>
<th>rDNA Reads</th>
<th>RelAbund %</th>
<th>rDNA Reads</th>
<th>RelAbund %</th>
<th>rDNA/DNA Ratio</th>
<th>MOTHUR</th>
<th>SILVA</th>
<th>Greengenes</th>
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<td>0.0005</td>
<td>3073</td>
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<td>Paenibacillus sp. MB05</td>
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<tr>
<td>Otu00927</td>
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<td>0.0005</td>
<td>1028</td>
<td>0.7</td>
<td>150.5</td>
<td>unclassified Bacilli</td>
<td>Bacillus</td>
<td>Bacillus</td>
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<tr>
<td>Otu01542</td>
<td>2</td>
<td>0.0010</td>
<td>977</td>
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<td>unclassified Bacilli</td>
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<tr>
<td>Otu01129</td>
<td>8</td>
<td>0.0038</td>
<td>1071</td>
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<td>Bacillus</td>
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<td>unclassified Planococcales</td>
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<td>Otu00001</td>
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<td>Bacillus</td>
<td>Bacillus</td>
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</tr>
</tbody>
</table>

(B) Banded basal ice from Taylor Glacier sampled in bulk during the 2009 field season (CrazyFilter).

<table>
<thead>
<tr>
<th>OTU#</th>
<th>rDNA Reads</th>
<th>RelAbund %</th>
<th>rDNA Reads</th>
<th>RelAbund %</th>
<th>rDNA/rDNA Ratio</th>
<th>MOTHUR</th>
<th>SILVA</th>
<th>Greengenes</th>
<th>NCBI BLASTN Accession</th>
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<tbody>
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<td>0.001</td>
<td>3222</td>
<td>1.3</td>
<td>2107.8</td>
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<td>Desulfosporosinus</td>
<td>Desulfosporosinus</td>
<td>Desulfosporosinus</td>
</tr>
<tr>
<td>Otu00549</td>
<td>950</td>
<td>0.285</td>
<td>115870</td>
<td>45.3</td>
<td>159.1</td>
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<td>Desulfosporosinus</td>
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<td>1540</td>
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<td>unclassified Actinobacteria</td>
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<td>Brevispirillum antarcticum</td>
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<td>Syntrophus</td>
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<td>1783</td>
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<td>unclassified Bacteria</td>
<td>unclassified</td>
<td>unclassified</td>
<td>Uncultured</td>
</tr>
</tbody>
</table>
Figure 2.6. Cluster analysis of the microbial phylogenetic structure of microbial assemblages within various basal ice cryofacies using a UPGMA dendrogram based on Bray-Curtis dissimilarity (top) and the relative abundance of bacterial phyla in the samples (bottom). The Proteobacteria were split into classes for greater detail. Phyla that were represented by <1% of the sequence reads were grouped into the Other category.
related to the genus *Paenisporosarsina* and/or *Sporosarsina* also had very high rRNA:rDNA read ratios (49.0 to 136685.0; Table 2.4, B).

**Taylor Glacier Basal Ice: Solid Ice**

The microbial assemblage contained in Taylor Glacier solid ice is similar that of the TG-Banded07 sample, with both assemblages exhibiting a very similar distribution of represented phyla (Figure 2.4). The solid ice assemblage is slightly less diverse (*i.e.* Inverse-Simpson/Shannon indices) than the Banded07 assemblage but was not significantly different in estimated richness (Chao1) (Table 2.3). Likewise, the most abundant OTU in the solid ice was the same as that for the 2007 banded ice: an unclassifiable genus closely related to an uncultured bacterium from Lake Vida, Antarctica (Accession No. GQ167317). However, in contrast to the banded ice, rRNA transcripts in the solid ice could not be reliably detected (85 out of 157,029 sequence reads passed curation and background control), suggesting the abundance of rRNA molecules in cells from the solid ice was very low.

**Matanuska Basal Ice: Dispersed Ice**

The composition of the microbial assemblage entrapped within Matanuska Glacier’s dispersed basal ice was distinct from that of any of Taylor Glacier’s sampled cryofacies, with 84.0% of the OTUs identified in the Matanuska sample being unique to these samples. Moreover, the assemblage was dominated (51.4%) by OTUs which appear to be unrelated to any currently known bacterial phyla. The most abundant classifiable OTUs belong to the Betaproteobacteria and are mainly affiliated with the genera *Albidiferax* (5.9%), *Thiobacillus* (4.4%), *Methylophilus* (1.5%), and *Desulfocapsa* (0.3%). Among these, *Albidiferax* and *Desulfocapsa*, were highly abundant in the rRNA library: of the 255,786 sequence reads in the curated rRNA library, 45.3% and 11.6% belonged to *Desulfocapsa* and *Albidiferax*, respectively.
Additionally, many of the unclassifiable OTUs also possessed large rRNA:rDNA ratios (Table 2.4, C).

**Støre Landgletscher Glacier Basal Ice: Banded Ice**

The Støre Landgletscher banded ice rDNA library had the fewest number of sequence reads (24,545; Table 2.2) but the greatest observed richness (2,208 OTUs; Table 2.3). The rarefaction curve for SL Glacier did not approach saturation, indicating that a substantial portion of the microbial diversity was not represented in the sequencing data (Figure 2.5).

Compositionally, the microbial community within the SL Glacier sample was distinct from both Taylor Glacier and Matanuska Glacier and largely consisted of members belonging to the Bacterioidetes (41.1%) or Betaproteobacteria (36.7%). Abundantly represented bacterial genera include *Hymenobacter* (34.9%), *Polaromonas* (11.4%), *Cryobacterium* (3.4%), and *Metylophilus* (1.9%).

**Discussion**

**Basal Ice Microbial Assemblage Composition**

The composition and structure of microbial assemblages in basal ice environments is likely to be influenced by both the bedrock lithology and the various basal ice formation processes (e.g. regelation, glaciohydraulic supercooling, glacial sliding, etc.) which condition the sediment and chemical composition of the basal ice facies. Clean basal ice facies differ from other basal ice facies in that impurities are derived from the atmosphere and become incorporated into the ice matrix during the firnification of snowfall. As a result, the surface-derived clean ice is a source of atmospheric gasses (e.g. N\textsubscript{2}, O\textsubscript{2}) and snowfall-deposited microorganisms to the basal zones of glaciers. In contrast, banded, solid, and dispersed basal ice facies contain significant quantities of sediment that may provide a source of organic carbon and
other energy sources (e.g. NH$_4^+$; Miteva et al., 2007) or electron acceptors (e.g., NO$_3^-$, Fe$^{3+}$, SO$_4^{2-}$) to microorganisms in the ice. Other than a nutrient source, subglacial sediments may also provide an inoculum of microorganisms from the subglacial substrate into the basal zones of glaciers during glacial advance. Supporting these hypotheses, Bray-Curtis dissimilarity analysis revealed a clear difference between basal ice facies which were sediment-free (i.e. Clean07 and Clean09) and those which were sediment-rich (i.e. Banded07, Banded09, and Solid) (Figure 2.6). The clustering of the CraryFilter-2E sample with the clean ice libraries, however, did not follow this trend. This inconsistency may however be a result of sample preparation as large sediment particles were removed in order to filter-concentrate large amount of melted basal ice (~132kg). As such, microorganisms that were attached to such sediment particles would be underrepresented in these samples.

Molecular analysis of 16S rRNA genes (rDNA) amplified from Taylor Glacier’s basal ice revealed a low overall diversity of microbial assemblages that were dominated by a few abundant OTUs. In general, the low observed species richness is consistent with previous studies of basal ice from the Bench Glacier (Alaska), the John Evans Glacier (Nunavut, Canada), and Russell Glacier (West Greenland) (Skidmore et al., 2005; Cheng and Foght, 2007; Yde et al., 2010). However, sediment-rich basal ices from these glaciers differed from Taylor Glacier in that they were largely dominated by members of the Betaproteobacteria (constituting between 25 to 68% of their clone libraries) and Bacteroidetes (1.1% to 24.8%). Based on the phyla composition, the assemblages within Taylor Glacier’s banded and solid ice facies were more similar to those found in permanent ground ice and permafrost in the Canadian Arctic, where the microbial assemblages were dominated by members of the Firmicutes (64% and 59%, respectively; (Steven et al., 2008; Lacelle et al., 2011). One possible explanation for this
inconsistency is that mineralogical differences between the bedrock materials of these glaciers bias the microbial communities which are immured in the basal ice. For example, (Mitchell et al., 2013) demonstrated that the availability of pyrite strongly influenced the composition and abundance of the bacterial community inhabiting subglacial sediments beneath Roberston Glacier (Alberta, Canada). Alternatively, this disparity may be due to differences in the temperature regimes of these glaciers. The Bench Glacier, John Evans Glacier, and Russell Glacier are all temperate or polythermal glaciers meaning their basal ice zones are entirely or partially, respectively, at the melting point. In contrast, Taylor Glacier is categorized as a cold glacier: the entire ice mass is below the melting point, similar to permafrost or permanent ground ice. Indeed, temperature loggers deployed in the basal ice zone of Taylor Glacier during the 2007 season indicated an ice temperature of -15 °C (Doyle et al., 2013). It is possible that the low temperature of Taylor Glacier basal ice in may be selective for microorganisms tolerant to the stresses encountered at subzero temperatures (e.g. increased salinity). This may also explain the increased abundance of spore-forming taxa (i.e. Firmicutes) and decreased species richness relative to the other glaciers examined. Indeed, one of the most abundant OTUs (up to 22% relative abundance) recovered from the banded and solid basal ice (OTU24; unclassified Firmicutes) is most closely related to bacteria recovered from hypersaline environments. (Mitchell et al., 2013)

One consideration for the decreased richness observed at Taylor Glacier (612 to 1232 OTUs; Table 2.3) compared to other sites (Matanuska, 1850 OTUs; SL Glacier, 2208 OTUs) may be related to the age of the ice. Assuming some portion of the microorganisms entrapped in basal ice environments cannot survive for extended timeframes while frozen (discussed in Chapter 1), species richness should decrease overtime through attrition. Previous analyses of the
stable isotopic composition of melt-water (δD and δ¹⁸O) from Taylor Glacier surface ice (i.e. englacial ice) has estimated these ices are between 11,500 and 65,000 years old (Aciego et al 2007). However, more recent radiometric ⁸¹Kr dating efforts have since dated Taylor Glacier surface ice near the glacial terminus to be approximately 123,500 years old (Buizert et al 2014). Glacial ice age increases with depth from the surface in a stratigraphic fashion and as such these age estimates presumably represent a lower limit for the age of the basal ice samples collected in this study. However, there are no established methods for precisely dating basal ice as the chronology of the basal ice zone is heavily distorted by the various folding and deformation processes which occur at the base of the glacier. As a result, the age of Taylor Glacier’s basal ice zone is difficult to ascertain but is probably on the order of several tens of thousands years old. Matanuska Glacier basal ice on the other hand contains trace amounts of anthropogenic tritium produced by atmospheric thermonuclear weapon tests in the 1950s and 1960s and is likely no more than 50 years old (Strasser et al., 1996). Estimates for the age of the basal ice samples from the Bench, John Evans, Russell (Skidmore et al., 2005; Cheng and Foght, 2007; Yde et al., 2010), and Støre Landgletscher Glacier (this study) have not been published. However, the Bench Glacier and John Evans Glacier are both alpine glaciers believed to have formed no more than a few thousand years ago (Calkin, 1988; Blake, 1989; Calkin et al., 2001; Willerslev et al., 2007; Barclay et al., 2009). On the other hand, the Russell Glacier and Støre Landgletscher Glacier both outflow from the Greenland Ice Sheet and are thus likely older than alpine glaciers formed during the late Holocene. However, due to higher precipitation and accumulation rates in Greenland (~30 cm year⁻¹, twice that of Antarctica) coupled with increased summer melting (Rignot and Thomas, 2002), mass turnover of the Greenland Ice Sheet is comparatively more rapid than that of the East Antarctic Ice Sheet (from which Taylor Glacier flows). As a
result, basal ice from the Russell Glacier and Støre Landgletscher Glacier is probably younger than that from Taylor Glacier.

As expected, the microbial community within the Matanuska ice was different from those of Taylor Glacier reflecting the stark differences between these glaciers. Matanuska is a temperate glacier possessing basal ice which is significantly warmer (~0°C) and younger (~50 years old) than that of the cold-based Taylor Glacier (-15°C; estimated to be ≥10,000 years old). Moreover, the basal zone of the Matanuska Glacier receives seasonal inputs of surface-derived meltwater to its basal layers (Lawson and Kulla, 1978; Lawson, 1979). Matanuska Glacier basal ice was dominated by sequences unrelated to any currently known bacterial phyla, indicating many of the taxa detected in the ice are novel. The genera which could be identified (i.e. *Sideroxydans, Albidiferax, Desulfocapsa, Thiobacillus*) all have cultured representatives capable of using iron and/or sulfur species as electron donors/acceptors.

The lack of archaea in the basal ice sequence data from Taylor Glacier is puzzling in at least two respects. First, many studies of subglacial water and sediment (Boyd et al 2010, Hamilton et al 2013, Dieser et al 2014) have reported the presence of active communities of methanogens *in situ*. Presumably these phylotypes should also be present in sediment-rich basal ice facies as they are predominantly formed from the entrainment of subglacial material.

However, all of the ice samples sampled in this study appear to contain oxygen concentrations of at least 4% (gas chemistry analyses presented in Chapter 3) and thus may be inhospitable to oxygen-sensitive taxa such as methanogens. Secondly, the liquid water available within an ice matrix is highly saline (Figure 1.2, Chapter 1) due to the partitioning of solutes into ice veins during freezing. Thus, the complete absence of any phylotypes related to halophilic archaeal taxa (*e.g. Halobacteriaceae*) is surprising as they would presumably be well adapted to the highly
The saline nature of ice veins. Indeed, although not a frozen environment, psychrotolerant haloarchaea nearly completely dominate the microbial biomass of Deep Lake, a hypersaline (210 to 280 g L\(^{-1}\) salts) meromictic lake located in the Vestfold Hills of Antarctica which reaches temperatures as low as -20 °C during the winter (Franzmann et al., 1988; Bowman et al., 2000). The Ribosomal Database Project (RDP) probe match analyzer indicates the primers used in this study (515F and 806R) would successfully amplify 97.0% of archaeal sequences in their database and other environmental studies have been successful in detecting archaea with this primer combination (e.g. Christner et al 2014; Subglacial Lake Whillans), so it is unlikely their absence is a result of molecular incompatibility. Furthermore, previous investigations have either failed to detect archaeal 16S rRNA genes in glacial ice (Christner et al., 2001; Christner et al., 2003; Sheridan et al., 2003; Skidmore et al., 2005; Simon et al., 2009; Yde et al., 2010) or recovered only a few sequences (≤1%; (Miteva et al., 2009). Tung et al., (2005) claim to have detected methanogenic archaea in the Greenland Ice Sheet Project 2 (GISP2) ice core based on blue-green autofluorescence of the F420 coenzyme, but no molecular data were obtained to confirm these observations. It remains unclear why archaea are exceedingly rare in glacial ice but may be due to their inability to tolerate the stresses of freezing over extended time-frames. For example, although archaea are abundant in polar seawater (Murray et al., 1999) and first-year sea ice (Collins et al., 2010) during the winter, efforts to detect them within multi-year sea ice have been inconsistent; often failing to detect them at all or finding they represent only a small fraction of the total sea ice microbial community (Junge et al., 2004; Cowie et al., 2011; Bowman et al., 2012; Bramucci et al., 2013).
**Basal Ice Metabolic Activity**

16S rRNA molecules are only synthesized by metabolically active cells and degrade relatively rapidly once produced (Hirsch et al., 2010), and thus have been highlighted as a means to help identify the functioning members of a microbial community (Janssen, 2006). For example, both (Hamilton et al., 2013) and (Dieser et al., 2014) have employed this technique previously to identify metabolically active members of natural microbial communities in subglacial sediments and water, respectively. Based on the abundance of reads in an rRNA library compared to an rDNA library, several OTUs in basal ice samples from this study were inferred to potentially be metabolically active in situ. In both the Banded07 and CraryFilter samples, the ratio of sequences affiliated with each OTU in the 16S rRNA and rDNA libraries was highest for OTU01819, which affiliated with the genus *Paenisporosarcina* (Table 2.4). Members of the *Paenisporosarcina* have been documented in a variety of permanently cold environments, including permafrost (Steven et al., 2007; Steven et al., 2008), arctic saline springs (Perreault et al., 2008), alpine glaciers (Reddy et al., 2013) and the McMurdo Dry Valleys (Reddy et al., 2003). cDNA sequences in the Banded07 rRNA library also revealed several members of the order Clostridiales (e.g. *Clostridum, Desulfosporosinus*) as active members. The co-presence of active aerobic (*i.e. Paenisporosarcina*) and anaerobic taxa (*i.e. Clostridum, Desulfosporosinus*) in this facies suggests banded basal ice facies contain a complex conglomeration of aerobic and anaerobic microhabitats such as those found in some soils (Peters and Conrad, 1995; Stotzky, 1997). This observation is consistent with those of (Hamilton et al., 2013) who found subglacial sediments collected beneath the basal ice layer of Robertson Glacier (Alberta, Canada) harbored strictly anaerobic methanogens (*i.e. Methanomicrobiales*)
concurrently with aerobic bacteria (*i.e.* *Sideroxydans* sp., *Sulfurihydrogenibium* sp. and *Planococcus* sp.).

Based on the rDNA libraries, the solid ice and banded ice facies contained very similar microbial communities (Figure 2.6). However, evidence for metabolically active species was found only in the banded ice. One explanation for this puzzling discrepancy may be due to differences in the gas content of solid and banded basal ice. Although the sedimentary material present in both facies originates primarily from the same subglacial material, the total gas volume contained in these two types of basal ice is considerably different. Samyn *et al.*, (2005) reported that the banded ice facies in Taylor Glacier contained a total gas volume similar to that of the meteoric englacial ice. In contrast, solid basal ice (reported as massive ice in (Samyn *et al.*, 2005)) contains saturating sediment concentrations close to 50% by volume and is essentially gas bubble free (Figure 2.1, C). Hence, banded ice may represent a unique habitat within Taylor Glacier wherein subglacial sediments containing potential nutrients and electron donors are entrained into ice which still contains atmospheric gases such as oxygen. This would explain why OTUs related to the strictly aerobic *Paenisporosarsina*, although present in all three basal ice facies (clean, banded, and solid), were only found to be active in the banded ice. Likewise, some anaerobic taxa (*e.g.* *Clostridium*) appeared to be slightly enriched in the solid ice rDNA libraries, representing 7% of the solid ice assemblage (cf. 4% in the banded ice); however, based on cDNA sequencing, they did not appear to be active.

In Matanuska’s dispersed basal ice, both *Albidiferax* and *Desulfocapsa* related OTUs were overwhelming abundant in the rRNA library suggesting such metabolisms may be advantageous *in situ*; however based on the relatively young age of the Matanuska Glacier’s dispersed basal ice (≥50 years), it is also possible these taxa were preserved in a way that
reflected their activity in the subglacial environment prior to entrainment into the basal ice. Interestingly, phylotypes related to the *Desulfocapsa* have also been identified in the outflow of iron-rich hyper-saline water from the terminus of Taylor Glacier, colloquially known as Blood Falls for its bright red coloration. The subglacial outflow from Blood Falls is hypothesized to originate from a subglacial basin of ancient, anoxic, marine brine wherein a microbial assemblage catalytically cycles sulfur to facilitate the oxidation of organic matter with ferric iron (Fe$^{3+}$) as the terminal electron acceptor (Mikucki *et al.*, 2009). Significant populations of iron oxidizing (e.g. *Gallionella, Thiobacillus*) and reducing (e.g., *Rhodoferax*) bacteria have also been reported in basal zones of the Bench Glacier (Skidmore *et al.*, 2005), the John Evans Glacier (Cheng and Foght, 2007), the Kamb Ice Stream (Lanoil *et al.*, 2009), the Russell Glacier (Yde *et al.*, 2010), and the Robertson Glacier (Hamilton *et al.*, 2013). Additionally, (Tung *et al.*, 2006) reported the potential presence of iron reducing bacteria at the bottom of the GISP2 (Greenland Ice Sheet Project 2) ice core and further speculated that electron shuttling along mineral grain edges would allow a population of iron reducers to persist in debris-rich basal ice under the Greenland ice sheet for a million years. Nevertheless, it is important to remember that phylogenetic data allow a prediction of physiology that can only be verified by direct metabolic measurements in situ. As such, these data serve as a hypothesis generator for future work aimed at determining the metabolic strategies of subglacial microbial communities.

**Conclusions**

This first detailed assessment of the phylogenetic composition, diversity, and metabolic status of microbial assemblages within multiple basal ice environments reveals substantial variability not only between different glaciers but also between the distinct basal ice cryofacies which can be found within a single glacier. This study also provides supporting evidence for the
hypothesis that sediment-rich basal ice cryofacies support metabolically active microbial communities. Moreover, the results of this study suggest banded ice facies, which are composed of a mixture of ice containing material from both the subglacial (i.e. sediments) and supraglacial (i.e. oxygen) environments, may be a unique habitat for aerobic, heterotrophic microorganisms in the basal zones of glaciers. Finally, the comparison made here between sediment-rich basal ice facies from multiple glaciers has opened new questions regarding the differences between cold-based and warm-based (e.g. temperate and polythermal) glaciers and how this may play a large role in basal ice microbial community composition and structure.
CHAPTER 3.
ABUNDANCE AND ACTIVITY OF MICROORGANISMS IN DEBRIS-CONTAINING BASAL ICE

Introduction

Glacier and ice sheet movement incorporates substrate from the bed, producing thick layers of sediment-rich ice in the basal zone. Due to the extremely oligotrophic nature of the overlying englacial ice (e.g. Taylor Glacier englacial ice: DOC = 0.2 to 1.0 mg L\(^{-1}\), dissolved nitrogen \(\approx 0.05\) mg L\(^{-1}\); Montross, 2012), the sediments and solutes from the subglacial material are a source of organic carbon, minerals, and nutrients (e.g. NH\(_4^+\), SO\(_4^{2-}\), Fe\(^{3+}\)) to the basal ice (Tung et al., 2006). Viable microorganisms have been recovered from basal ices from a variety of glaciers [e.g. John Evans Glacier (Skidmore et al., 2000), Fox Glacier and Franz Josef Glacier (Foght et al., 2004)] and multiple reports have demonstrated metabolic activity by cold-adapted microorganisms at subzero temperatures (Chapter 1; Table 1.2). The discovery of anomalously high concentrations of CO\(_2\), N\(_2\)O, and CH\(_4\) in basal ice environments have been attributed to biogeochemical processes occurring within the basal ice matrix (Campen et al., 2003). However, direct evidence of in situ microbial activity in basal ice is currently lacking. This study assessed microbial cell abundance, gas concentration and isotopic composition, bulk DNA/RNA ratios, and adenylate concentrations in various debris-rich and debris-poor basal ice cryofacies to investigate the physiological status of microorganisms in the basal ice.

Materials and Methods

Ice Sampling

Basal ice samples from Taylor Glacier and Matanuska Glacier were decontaminated as described in Chapter 2. When possible, parallel samples from the same basal ice samples used
for nucleic acid extraction and 16S rRNA library preparation (i.e. TG-Clean, TG-Banded, TG-Solid, and Matanuska) were used (Figure 3.1).

Figure 3.1. Visual representation of the sources of basal ice samples used in this study. Red dashed-lines for the TG-Banded07 and TG-Banded09 samples denote the layer used for 16S rDNA and rRNA sequencing in Chapter 2. Details of the 2007 and 2009 sample profile collection are detailed in Chapter 2.
Microbial Cell Density

For enumeration, microbial cells attached to sediment particles were liberated from the solid phase with a modification of the method described by (Trevors and Cook, 1992). Nine milliliters of the sediment-melt water slurry was amended with 1 mL of a 1% (w/v) solution of Na₄P₂O₇ (pH 7.0), shaken at 200 rpm for 1 hour at 4°C and allowed to settle for 30 min. The supernatant was collected, and the cells within were fixed with sodium borate-buffered formalin (5% final concentration), stained with 2× SYBR Gold (Invitrogen) and filtered onto black polycarbonate 0.22 um pore filters (GE Water & Process Technologies). Identical samples that did not contain the formalin fixative were also prepared, stained with Baclight (Invitrogen) and filtered within 6 h after melting. The filters were mounted on glass slides with a glass coverslip using two drops of antifade solution and stored in the dark at 4 °C until counted. The antifade solution consisted of 90 mM p-phenylenediamine and 45% glycerol in phosphate buffered saline and was filtered through a 0.45 uM filter. Fifty random fields (field of view, 41,500 um²) were counted using an Olympus BX51 epifluorescence microscope and a FITC filter cube (excitation from 455 to 500 nm and emission from 510 to 560 nm). Cell density estimates were calculated based on the average number of cells per field and normalized per gram of ice.

Taylor Glacier Basal Ice Gas Analysis

All gas concentration and stable isotope analyses were performed by Scott Montross at Montana State University as described in (Montross, 2012). Due to low gas content, no gas data are available from the solid basal ice cryofacies.

Adenylate Extraction

Immediately after melting, the samples were filtered through 0.22 μm pore size polyethersulfone (Supor; Pall) filters using a bench-top vacuum manifold (≤ 20 kPa). Clean ice
samples were filtered through a 25 mm diameter filter while sediment-laden samples were processed through either a 47mm or 90mm diameter filter, depending on total sediment content. The filters were cut into small pieces using a sterile scalpel and placed into a centrifuge tube (25 mm filter: 1.5 mL; 47 mm: 15 mL; 90 mm: 50 ml). Five milliliters (1 mL for 25 mm filters) of boiling Tris-acetate buffer (TAB; 50mM Tris base adjusted to pH 7.75 with acetic acid) was added to the centrifuge tubes and vortexed vigorously for 10 seconds. The tubes were then incubated in a boiling water bath for 5 minutes and immediately placed on ice for at least 30 minutes. Extracted samples were centrifuged for 5 minutes at 4500×g and the supernatant was harvested for measurement of ATP and ADP concentrations.

Measurement of ATP and ADP Concentration by Luminescence

The concentration of ATP and ADP were measured in the extractant using a modification of the firefly luciferase-luciferin assay described by Amato and Christner, (2009). For the measurement of ATP, 10 μL of supernatant from each sample was added to an equal volume of triethanolamine buffer (TEOA buffer; 200 mM triethanolamine pH 7.6, 2 mM MgCl₂, 240 mM KCl) and incubated at 37°C for 10 minutes. To correct for adenylate adsorption to and luciferase inhibition by the sediment in the sample extracts, two samples were prepared in parallel for each measurement. Following incubation at 37°C, 10 uL of TAB supplemented with 10 μM ATP was added to one of the samples to serve as an internal standard for each measurement. ADP was measured by enzymatic phosphorylation to ATP following the procedures described above except that TEOA buffer containing 4.5 mM phosphoenolpyruvate and 5 U/mL pyruvate kinase (MP Biomedicals) was used. All samples were kept on ice during preparation but were equilibrated to room temperature (22°C) prior to the addition of luciferase and measurement of luminescence.
Weighted linear regression was used to generate calibration curves for each experiment using concentrations of ATP (in TAB) from 10 μM to 1 pM. The luciferase-luciferin cocktail was prepared fresh for each series of measurements and consisted of 100 U ml⁻¹ luciferase and 0.140 mM d-luciferin, which were suspended in molecular biology grade water (Quality Biological Inc.). Luminescence was quantified with a 20/20n luminometer (Promega) using auto-injection of 100 μL of the luciferase-luciferin cocktail. Relative luminescence units (RLU) were integrated for three seconds immediately after injection. Sample RLU values were corrected for adsorption and inhibition using the following formula:

$$\frac{1}{\left(\frac{RLU_{sample+std} - RLU_{sample}}{RLU_{blank+std}}\right)} \times RLU_{sample} = RLU_{corrected}$$

The corrected RLU values were used together with the standard calibration curve to determine the ATP concentration in the extractant. Final ATP and ADP concentrations were corrected for dilution and normalized to the mass of the ice sampled. All values reported are the means of replicate samples (n = 3 to 5). The limit of detection was determined following Armbruster and Pry, (2008).

**Sediment Content**

Subsamples of 50-100g of ice were taken from each basal ice horizon and dried overnight at 120°C. The sediment content (%w) of the ice was determined by measuring the dry weight of sediment per gram of basal ice.

**Results**

**Cell Concentration and Viability within Horizons of the Basal Ice**

Direct counting using epifluorescent microscopy was used to quantify cell density in sampled horizons of the basal ice (Figure 3.2). The clean ice horizons from the 2007 profile
Figure 3.2. Analyses of the microbial cell density (A), concentrations of O$_2$ and CO$_2$ (B), sediment content (C), ammonium concentration (D), and DOC (dissolved organic carbon) concentration (E) throughout a vertical profile of banded basal ice (F). Error bars represent the standard error of the direct cell counts. The horizontal dotted lines in panel F denote the horizon used for 16S rDNA and rRNA analysis in Chapter 2 (TG-Banded07). Adapted from Doyle et al., (2013) and Montross et al., (2014).
(depth 60 cm to 80 cm) were selected as representative samples of the clean ice and contained the lowest total cell concentrations of the entire basal ice profile. SYBR Gold and BacLight staining (Life Technologies) revealed counts ranging from 2.6 ±0.2 to 4.9 ±0.4 ×10^2 cells g^{-1} ice (n = 4). A one-way ANOVA was conducted to test for differences in cell concentrations in the clean ice replicates, and the data did not differ significantly within the 60-80 cm block [F(3,196) = 1.23, p = 0.30]. In banded ice from the 2007 profile (depth 220 cm to 240 cm), total cell concentrations were approximately one to two orders of magnitude higher than the clean ice (~3.8 ×10^2 cells g^{-1} ice). The concentration of cells estimated by SYBR Gold staining ranged from 1.8 ±0.1 ×10^3 cells g^{-1} ice to 1.8 ±0.4 ×10^4 cells g^{-1} ice, which was not significantly different (α = 0.05) from values obtained by BacLight staining (2.4 ±0.1 ×10^3 cells g^{-1} ice to 1.6 ±0.3 ×10^4 cells g^{-1} ice; Figure 3.2, A). Cell concentration was positively correlated with sediment content [r(14) = 0.60, p < 0.05] and the concentration of CO₂ [r(14) = 0.35, p < 0.10; Figure 3.2].

In debris rich ice containing ≥4% sediment (depth 230 to 237.5 cm; Figure 3.2, C), sediment content was positively correlated with CO₂ [r(6) = 0.72, p < 0.025] and negatively correlated with the O₂ [r(6) = -0.79, p < 0.01] (Montross, 2012; Montross et al., 2014). The concentration of SYBR Gold-stained cells in the Taylor Glacier solid ice (6.7 ±1.0 ×10^3 cells g^{-1} ice) were intermediate to values in the clean ice and banded ice at. An overall view of cell concentration and viability data is summarized in Table 3.1.

Table 3.1. Summary of microbial abundance and viability in Taylor Glacier basal ice based on SYBR Gold and BacLight staining. Basal ice cryofacies which contain sediment are highlighted in gray. N.D. = not determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Profile Depth</th>
<th>Cells g^{-1} ice (×10^3)</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG-Clean07</td>
<td>60-80 cm</td>
<td>0.3 - 0.5</td>
<td>78 ±5%</td>
</tr>
<tr>
<td>TG-Banded07</td>
<td>220-240 cm</td>
<td>1.8 - 18.2</td>
<td>73 ±9%</td>
</tr>
<tr>
<td>TG-Solid</td>
<td>380-400 cm</td>
<td>6.7</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
BacLight staining of clean and banded ice horizons indicated that cell viability in these ices was similar ($\alpha = 0.05$) at $73 \pm 9\%$ and $78 \pm 5\%$, respectively. The autofluorescence of sediment particles with the TRITC filter combination (excitation: 532 to 554 nm; emission: 570 to 613 nm) made cells stained with propidium iodide (“dead” component of BacLight) difficult to resolve in solid ice. Hence, viability data from these samples are not available.

**Basal Ice Gas Chemistry**

In the clean ice cryofacies, O$_2$ concentrations were approximately 20% and similar to atmospheric values. The isotopic composition of O$_2$ ($\delta^{18}$O-O$_2$) in the clean ice ranged between 0.4 and 1.2‰ (Montross, 2012), comparable to values measured in ice cores from the GIS and AIS (Sowers *et al.*, 1989). CO$_2$ concentrations in the clean ice ranged from 280 to 360 ppmv while $\delta^{13}$C-CO$_2$ values were between -11.9 to -12.5‰ (Montross, 2012).

In the banded ice cryofacies, bulk O$_2$ concentrations were near 20% in the sediment-free horizons but decreased approximately five-fold to 4% in the horizons containing ≥28.6% sediment (c. 235 cm depth in the 2007 field season profile). In these sediment-rich sections of the banded ice, $\delta^{18}$O-O$_2$ values were also markedly enriched to between 3.0 to 115.0‰. Likewise, CO$_2$ concentrations here were very high, up to 320,000 ppmv (Figure 3.2, B), and contained $\delta^{13}$C-CO$_2$ values ranging from -15.7 to -24.4‰ (Montross, 2012), indicative of CO$_2$ derived from oxidation of organic matter.

**ATP and ADP Quantification**

In the horizons of banded ice containing no sediment, the concentration of ATP ranged from 0.04 pg g$^{-1}$ ice to 0.27 pg g$^{-1}$ ice and ADP was at a concentration below the limit of detection (3 nM). However, in the banded ice horizons containing ≥3% sediment, ATP varied between 0.24 pg g$^{-1}$ ice to 2.80 pg g$^{-1}$ ice while ADP was approximately two orders of magnitude
higher, ranging between 238.73 pg g\(^{-1}\) ice and 556.68 pg g\(^{-1}\) ice (Figure 3.3). A Tukey range test was used in conjunction with one-way ANOVA to determine which banded ice horizons contained significantly different (\(\alpha=0.05\)) adenylate concentrations. ATP measurements in Taylor Glacier clean ice were very low but above the detection limit (~2 pM), yielding an average concentration of 20 ±13 fg g\(^{-1}\) ice, similar to values for ATP in the sediment-free sections of the banded ice (Table 3.2). However, ADP measurements in the clean ice were not

Table 3.2. Concentration of ATP and ADP within several horizons of basal ice from Taylor Glacier and Matanuska Glacier. Horizons containing sediment are highlighted in gray. b.d. = below detection, N/A = not available.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Profile Depth (cm)</th>
<th>ATP (pg g(^{-1}) ice)</th>
<th>ADP (pg g(^{-1}) ice)</th>
<th>Sample Size (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG-Clean07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.04</td>
<td>b.d.</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>0.03</td>
<td>b.d.</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>b.d.</td>
<td>b.d.</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>0.01</td>
<td>b.d.</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>b.d.</td>
<td>b.d.</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>0.01</td>
<td>b.d.</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0.02</td>
<td>b.d.</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>155</td>
<td>0.01</td>
<td>b.d.</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>b.d.</td>
<td>b.d.</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>165</td>
<td>b.d.</td>
<td>b.d.</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>0.01</td>
<td>b.d.</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>b.d.</td>
<td>b.d.</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>TG-Banded09</td>
<td>2</td>
<td>0.04</td>
<td>b.d.</td>
<td>636</td>
</tr>
<tr>
<td>6</td>
<td>0.08</td>
<td>b.d.</td>
<td>632</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.90</td>
<td>498.28</td>
<td>758</td>
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<td>b.d.</td>
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53
significantly different from blank controls. Both ATP and ADP concentrations in the solid ice were below detection. The Matanuska dispersed basal ice contained 0.13 ±0.02 pg g⁻¹ ice ATP and 147.76 ±4.99 pg g⁻¹ ice ADP (Table 3.2).

Figure 3.3. Analyses of the concentrations of ATP (A), ADP (B), and sediment content (C) throughout a vertical profile of banded basal ice collected in 2009. Error bars represent 95% confidence intervals for the ATP and ADP measurements. Means with different letters are significantly different (Tukey’s HSD, p < 0.05).
Discussion

Sediment-rich and sediment-free basal ices differ markedly in their physical structure and geochemical composition (Figures 3.1 and 3.2). Compared to sediment-free clean ice, the sediment rich banded and solid cryofacies in Taylor Glacier contained between 10- and 100-fold more microbial cells. Sharp et al., (1999) reported similar observations in basal ices from Glacier de Tsanfleuron and Haut Glacier (both in Switzerland) wherein microbial population sizes, although approximately an order of magnitude larger than those observed in this study, increased concurrently with sediment concentration. The positive correlation between cell concentration and sediment content suggests that cells were preferentially attached to the sediments, which may also explain the process by which these cells became entrained in the banded basal ice. Alternatively, it is possible that available carbon and nutrients from the sediments can fuel microbial metabolism and reproduction within the banded ice. The latter hypothesis is plausible given that certain bacteria have demonstrated cell division to temperatures of -15 °C in brine (Mykytczuk et al., 2013) and various investigations have shown microbial activity at similar subzero temperature in ice (Christner, 2002; Amato et al., 2010; Dieser et al., 2013; Doyle et al., 2013). Although no investigation to date has observed cellular division inside ice it may be that growth and reproduction inside ice simply occurs at a very slow rate which cannot be easily measured. For example, Amato et al., (2010) estimated the generation time for Psychrobacter cryohalolentis frozen at -15 °C was approximately 94 years while (Price and Sowers, 2004) estimated that a microbial cell frozen at -40 °C would require $10^7$ years to turnover its cellular carbon.

While the growth of microorganisms within an ice matrix remains an open question, the data gathered in this study lend support to the hypothesis that microorganisms within sediment-
rich basal ices are metabolically active. The banded ice cryofacies in Taylor Glacier contained high concentrations of CO$_2$ (between 60,000 to 325,000 ppmv) that occurred concomitantly with depleted concentrations of O$_2$ between 4 and 18% of the total gas volume (Montross, 2012; Montross et al., 2014). The negative correlation between the concentration of CO$_2$ and O$_2$ together with their isotopic composition [depleted δ$^{13}$C-CO$_2$, enriched δ$^{18}$O-O$_2$; (Montross, 2012)] is consistent with their production or consumption during aerobic respiration. Similar high CO$_2$ concentrations have been observed in another McMurdo Dry Valleys outlet glacier (~220,000 ppmv, Seuss Glacier; Lorrain et al., 1999; Sleewaegen et al., 2003) and silty basal ice (135,000 ppmv) from the Greenland Ice-core Project (GRIP) in central Greenland (Souchez et al., 1995(a); Souchez et al., 1995(b)). Gas analyses in this study also appeared to provide evidence for limited microbial CO$_2$ production in Taylor Glacier’s clean ice as well: CO$_2$ concentrations in the clean ice were lower than current atmospheric values (~395 ppmv), but higher than the pre-industrial values (260 to 280 ppmv) preserved in the Taylor Dome Ice Core, the inland region of the East Antarctic Ice Sheet which provides ice to Taylor Glacier (Indermühle et al., 1999). Furthermore, δ$^{13}$C-CO$_2$ values in Taylor Glacier clean basal ice were approximately 5‰ lower than those also recorded in englacial ice from Taylor Dome (Indermühle et al., 1999). Explanations for the high CO$_2$ concentrations in basal ices have suggested production via carbonate mineral dissolution [e.g. (Smith et al., 1997)] or the preferential concentration and depletion of CO$_2$ and O$_2$, respectively, during localized micro melt-refreeze events (cryo-concentration) due to differences in solubility (Samyn et al., 2005). However, neither possibility adequately explains the results given that the isotopic composition of carbon from carbonate dissolution would be near 0‰ (Anderson and Arthur, 1983) and a cryo-concentration mechanism can only account for a maximum CO$_2$ concentration of up to
20,000 ppmv (the solubility of CO$_2$ at 0 °C). Based on these considerations, microbial respiration of organic carbon is the most plausible explanation for the concentration and isotope compositions of CO$_2$ and O$_2$ in Taylor Glacier’s basal ice.

Reflecting the abundance of cells (Table 3.1), the quantity of extractable DNA was significantly higher in banded and solid ice samples. The concentration of extractable RNA, however, was only marginally higher in the sediment-rich basal ice samples (Table 2.1; Chapter 2). As a result, the bulk RNA:DNA ratios, a parameter that may serve as an indicator of metabolic activity (Kerkhof and Ward, 1993; Fabiano et al., 1995), did not correlate well with the microbiological data. For example, the lowest cellular and ATP concentrations were in the clean ice, which contained the highest ratio of RNA to DNA (~0.15). In contrast, the sediment-rich cryofacies (i.e. banded, solid, and dispersed) all had RNA:DNA ratios (≤0.001) that were substantially lower than those measured in deep sea marine sediments (~0.004 to 0.11) (Dell’Anno et al., 1998). However, the interpretation of the RNA/DNA ratios measured in this study is likely compromised by the probable likelihood that, due to the extreme nature of the ice vein environment, dead or metabolically dormant cells make up a substantial portion of the microbial assemblages within these basal ice samples. Indeed, (Jeffrey et al., 1996) found no evidence to support the reliability of RNA/DNA ratios as an accurate proxy for metabolic activity at the population level in environmental samples.

ATP and ADP concentrations in Taylor Glacier banded ice appear to be related to the presence of sediment entrained within the ice (Figure 3.3). While the concentration of CO$_2$ and O$_2$ correlated with sediment content ($r$(6) = 0.72 and -0.79, respectively), the highest ATP concentrations were found at intermediate sediment concentrations between 5 and 20%. Indeed, banded ice samples with the highest sediment content (i.e. >25%) had ATP concentrations more
comparable to sediment-free samples, and in the solid ice (≥55% sediment), the ATP concentration was below the limit of detection despite having a comparable population of microbial cells (Tables 3.1 and 3.2). Although steps were taken to account for inhibition and adsorption by sediment particles it is possible that the reliability of the firefly luciferase-luciferin assay is compromised in samples with extremely high sediment concentrations such as the solid ice. On the other hand, these observations reflect the 16S rDNA and rRNA analyses reported in Chapter 2: cDNA sequencing found no evidence of metabolic activity in the clean ice and solid ice cryofacies. Only the 16S rRNA libraries from the banded ice and dispersed basal ice cryofacies, both of which contain sediment concentrations intermediate to that of clean ice and solid ice, were found to harbor metabolically active taxa. Nevertheless, the lack of apparent metabolic activity in the solid ice cryofacies remains puzzling. The solid ice and banded ice both contain entrained sediment from presumably the same subglacial origin, and based on the 16S rDNA sequencing, also harbor very similar microbial assemblages (Chapter 2). OTUs related to both obligate aerobes (e.g. *Paenisporosarcina*) and obligate anaerobes (e.g. *Clostridium*) occur in both the banded and solid ice cryofacies. However, unlike the banded ice, the solid basal is composed almost entirely of sediment with only interstitial ice and contains a very small total gas volume (≤0.03 cm³ g⁻¹ ice). If for the sake of argument only aerobic respiration is considered, one possible explanation for the lack of evidence of metabolic activity in the solid ice is the decreased availability of oxygen in the solid ice: At low sediment concentrations (such as in the clean ice) there is not enough organic carbon available to support meaningful rates of aerobic respiration; at high sediment concentrations organic carbon is more plentiful but oxygen becomes limiting. Nevertheless, this hypothesis does not explain why outside of a slight enrichment of a *Clostridium*-related OTU in the solid ice rDNA library (Chapter 2), limited
evidence for metabolically active anaerobes could be found in the solid ice. Further work is needed to investigate the physiology and metabolism of anaerobic microorganisms under frozen conditions.

Taken together, these data support the hypothesis that sediment-rich basal ice is a habitat capable of supporting metabolically active microbial communities. However, the data also suggests that habitability of the sediment-rich basal ice is more complex than the availability of minerals and nutrients from the glacial subsurface. While the entrained debris and sediments are assumed to be the primary source of organic carbon and nutrients in basal ice, the availability of atmospheric gasses (e.g. O₂) may also need to be considered.
CHAPTER 4.
MACROMOLECULAR SYNTHESIS BY BACTERIA AT SUBZERO TEMPERATURES

Introduction

Microorganisms entrapped in ice endure a plethora of physiochemical and biochemical challenges (Chapter 1) which has led to some speculation that microorganisms are likely in a dormant state while frozen and thus any microbial activity in glacial environments is primarily relegated to liquid water in the subglacial and supraglacial zones (Hodson et al., 2008). The discovery of anomalous gas concentrations in the basal ice zones of glaciers, which appear to be the product of microbial activity (reviewed in Chapter 1; see also: Figure 3.1, Chapter 3), support the hypothesis that sediment-rich basal ice may serve as a microbial habitat. Basal ice is generally the warmest ice in a glacier, and by the nature of its formation, often contains significant quantities of sediment that can serve as a source of nutrients and energy for microorganisms. RNA-based approaches in Chapter 2 identified that the sediment-rich facies of basal ice from Taylor Glacier and Matanuska Glacier may contain metabolically active microbial assemblages. Despite recent advances in our understanding of the distribution and composition of microbial assemblages in icy environments, very little is known about the physiology and activity of microorganisms which persist in these environments. Several laboratories have successfully isolated viable microorganisms from basal ice sampled from glaciers in Switzerland (Sharp et al., 1999), New Zealand (Foght et al., 2004), Canada (Skidmore et al., 2000), Greenland (Miteva et al., 2004; Yde et al., 2010), and Antarctica (Doyle et al., 2013).

This study sought to isolate and characterize microorganisms from banded basal ice from Taylor Glacier, which appears to harbor metabolically active microbial communities that have modified the gas chemistry of the basal ice (Chapters 2 and 3). Serendipitously, many isolates of
the genera *Paenisporosarcina*, the same genera as the most active OTUs identified in Chapter 2 (Table 2.4), were recovered during cultivation efforts. Experiments were conducted to quantify the rates of DNA and protein synthesis by one of these isolates, *Paenisporosarcina* sp. TG14, when frozen within the basal ice melt-water from which it was recovered. Additionally, the genome of this isolate was sequenced and analyzed to determine if it possessed any characteristics or adaptations which might explain the observed abundance of this genus in Taylor Glacier basal ice. One mechanism expected to increase the survival of an ice-associated microbe was the presence of a functional ice-binding protein (IBP) in TG14. IBPs protect cells against freeze damage caused by ice recrystallization, a coarsening process whereby larger ice crystals grow at the expense of smaller ones (Achberger et al., 2011; Brown et al., 2012).

Experiments were also conducted to explore how conditions in the ice vein environment affected the metabolic capacity of microorganisms. As discussed in Chapter 1, freezing concentrates solutes, nutrients, and cells into veins of liquid water between ice crystals, providing a liquid environment for microorganisms inside the ice. Although freezing is a major stress for cells, it is possible that freeze tolerant microorganisms in oligotrophic environments (e.g. glacial ice) may actually gain benefit from the concentration of nutrients which occurred during ice crystal formation. As such, experiments were performed to test this hypothesis and discern differences in metabolic activity at subzero temperatures under frozen and liquid conditions.

**Materials and Methods**

**Isolate Culturing and Characterization**

Meltwater from the 2007 banded ice (sample profile depth 195 cm to 200 cm; Figure 2.1, B) was vortexed for 1 min to produce a slurry, and 100 μL was spread plated on R2A (Difco),
10% R2A, 1% R2A, marine agar 2216 (Difco) and M9 minimal salts media (supplemented with 20 mM glucose, acetate or pyruvate) in triplicate. The media were subsequently incubated in the dark at 4, 10, 22 and 37°C and inspected daily for 60, 30, 15 and 7 days, respectively. Uninoculated plates served as controls and were similarly incubated. Additional isolates from ice samples collected at Taylor Glacier in 1999 were made available by colleagues and also examined for this study; the tunnel location and ice properties are detailed in Samyn et al., (2008). Once in pure culture, each isolate’s growth at 5, 15 and 22 °C was measured via optical density (620 nm) in marine broth 2216 (Difco) to ascertain the approximate temperature for optimal growth.

To ascertain the fraction of culturable microbes persisting as vegetative cells inside the ice, basal ice melt-water was pasteurized by heating it to 80°C for 10 minutes followed by spread plating on marine agar 2216 (Difco) in triplicate. The cultures were incubated aerobically at 22°C for one week and the number of colony-forming units (CFU) was quantified and compared to control samples that were not heated.

Salt tolerance was examined in select isolates by culturing in marine broth 2216 (Difco) supplemented with up to 10% (w/v) NaCl (intervals of 2% NaCl). All cultures were grown at 10 °C and their optical densities at 620 nm were monitored over a two week period using a NanoDrop spectrophotometer.

**Phylogenetic Analysis of Isolates**

Based on variations in colony morphology, colony pigmentation, and optimal growth temperature, twenty-five isolates were selected for 16S rRNA gene sequencing. An UltraClean Microbial DNA Isolate Kit (MoBio Laboratories) was used to extract genomic DNA and the 16S rRNA gene was amplified by the PCR using primers 27F (5’- AGAGTTTGATCCTGCTGAGC-
3') and 1492R (5'-GGTACCTTGTTACGACTT-3') (Lane, 1991). The PCR mixtures (50 μL) contained ~300 ng of template, 1× Taq buffer containing 1.5 mM Mg$^{2+}$ (5 PRIME), 1× TaqMaster PCR enhancer (5 PRIME), 200 μM of each dNTP (Promega), 15 pmol of each primer, and 1.0 U of Taq DNA Polymerase (5 PRIME). The cycling conditions for the PCRs consisted of an initial denaturation at 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C for 1 min, annealing at 50.8 °C for 1 min, extension at 72 °C for 2 minutes, and a final extension at 72 °C for 10 minutes. The ~1,500 bp PCR products were purified via ethanol precipitation and sequenced using BigDye Terminator (v.3.1; Invitrogen) on an ABI 3130XL Genetic Analyzer (Applied Biosystems). Taxonomic assignments were made using the EzTaxon database (Kim et al., 2012).

Bacterial DNA and Protein Synthesis at Subzero Temperatures

DNA and protein synthesis under frozen conditions at -15 °C was measured using a modification of the procedure described by Christer (2002). Cultures (50 mL) of Paenisporosarcina sp. TG14 were grown aerobically with shaking (200 rpm) at 15 °C in marine broth 2216 (Difco). After approximately 72 h, cells from the mid-exponential phase of growth were harvested by centrifugation at 4,500 × g for 10 min at 15 °C. The cell pellet obtained was washed with 50 mL of Taylor Glacier melt-water, centrifuged, and suspended to a final concentration of 3.1 ×10$^6$ CFU mL$^{-1}$. This melt-water was generated directly from clean ice (2007 profile depth: ~100 cm) and banded ice (2007 profile depth: ~300 cm) samples (Chapter 2, Figure 2.3, B). In order to reduce quenching of the luminescent signal during liquid scintillation counting, coarse sediment particles were removed from the basal ice melt-waters by settling for ~18 h.
Duplicate aliquots (500 μL) of the *Paenisporosarcina* sp. TG14 melt-water suspensions were amended with 20 nM (final concentration) of either $[^3\text{H}]$-leucine ($\text{L-leucine}[4,5-^3\text{H}]; 84 \text{Ci mmol}^{-1}$ in ethanol:water 2:98; MP Biomedical) or $[^3\text{H}]$-thymidine (thymidine [Methyl-$^3\text{H}$]; 64 Ci mmol$^{-1}$ in sterile water; MP Biomedical). Half an hour prior to the $[^3\text{H}]$-leucine and $[^3\text{H}]$-thymidine amendments, killed control samples were prepared by amending a portion of the cell suspension with ice-cold trichloroacetic acid (TCA) to a final concentration of 7% (w/v). All samples and killed controls were prepared in triplicate. The sample aliquots were kept on ice and were rapidly frozen by incubation at -80 °C immediately after the addition of $[^3\text{H}]$-leucine or $[^3\text{H}]$-thymidine. After 16 h at -80 °C, the samples were transferred to a -15°C freezer (Revco ULT350-3-A32), which was designated as experimental time point zero. The temperature of the freezer was logged every 10 minutes during the experimental time-course with an external HOBO U12 (Onset) temperature data logger and averaged -15.0 °C ± 0.5 °C. At designated experimental time-points, the frozen aliquots were transferred to a 4 °C incubator and immediately overlain with 100 μL of ice-cold 50% TCA to a final concentration of 7% (w/v). After 30 min at 4 °C, the acid-insoluble macromolecules were pelleted by centrifugation at 17,000 × g for 15 min, washed with 1 mL of 5% TCA, and centrifuged. The rinsed pellet was then washed with 1 mL of 70% ethanol, centrifuged, and the supernatant removed. The pelleted material was suspended in 1 mL of Cytoscint scintillation cocktail (Fisher, cat. no. BP458-4) and the number of CPM (counts per minute) was measured with a Beckman LS 6000IC scintillation counter. CPM measurements were converted to disintegrations per minute (DPM) using efficiencies determined with acetone-quenched standards of $[^3\text{H}]$-toluene (American Radiolabeled Chemicals, cat# ARC182). Incorporation rate measurements were converted to grams of substrate carbon incorporated per gram of cell carbon per day (gC gC$^{-1}$ day$^{-1}$) based on
an estimated mean biomass of 65 fg C cell\(^{-1}\) (Hoehler and Jorgensen, 2013). Non-linear regression with residual plot analyses was used to model the data.

Measurement of DNA and protein synthesis by *Paenisporosarcina* sp. TG14 cells frozen in ice or suspended in brine at -5°C was carried out as described above with modifications only to the media. Instead of Taylor Glacier melt-water, incubations were performed in 1% marine broth 2216 (Difco) with and without the addition of 10% (w/v) NaCl (used for freeze point depression).

**Genome Sequencing and Identification of IBP gene**

Genomic DNA was isolated from *Paenisporosarcina* sp. TG14 cells using a MasterPure DNA extraction kit (Epicentre) and sequenced on an Illumina HiSeq 2000 at the Korea Polar Research Institute (Koh et al. 2014). Genomic assembly and contig curation was performed as described by (Koh et al., 2012). IBP-like sequences were identified in the contigs using BLAST with query sequences from known IBPs (e.g. IBP from sea ice bacterium *Colwellia* sp. SLW05; Accession#: DQ788793). The theoretical pI and molecular mass was predicted with the ExPASy pI tool (http://web.expasy.org/compute_pi) and the signal peptide was predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP).

**RNA Extraction, Purification, and Analysis**

Expression of *Paenisporosarcina* sp. TG14’s hypothetical IBP as a function of growth temperature was analyzed via a modified version of the methodology described by Achberger et al., (2011). Aliquots (500 μL) of mid-log phase *Paenisporosarcina* sp. TG14 cells that were grown at 5, 10, 15, and 25 °C were removed from culture tubes, immediately preserved in two volumes of RNAprotect (Qiagen), and stored at -80 °C. The preserved samples were subsequently thawed on ice and lysed by the addition of 15 mg mL\(^{-1}\) lyzosyme and 20 mg mL\(^{-1}\)
proteinase K in 200 μL of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The tubes were mixed by vortexing for 10 seconds and then incubated for 10 min at 25 °C. The bulk RNA in the lysate was purified with the RNeasy Mini Kit (Qiagen) and eluted into 50 μL of RNase-free water. Genomic DNA was digested with TURBO DNase (Ambion) and its removal was confirmed using PCR.

The mRNA for the IBP gene and 16S rRNA molecules were reverse transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen) with reverse primers TG14-IBP-1323R (IBP; detailed below) and 758R (16S rRNA; detailed below). The optional RNaseOUT reagent (40 U μL⁻¹) was included in all reverse transcription reactions per the manufacturer’s instructions. The IBP mRNA molecule sequence was amplified from the cDNA by PCR with forward and reverse primers TG14-IBP-31F (5’-TCACTTTTATTAGTTGTAAGCATGGT-3’) and TG14-IBP-1323R (5’-GGTGATACTTGGATCATCTGTCA-3’), designed with the aid of Primer3 (Rozen and Skaletsky, 1998). Likewise, primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 758R (5’-CTACCAGGGTATCTATCC-3’) were used to amplify a ~800bp region of 16S rRNA molecules. The cDNA products were amplified for 30 cycles using the following conditions: 96 °C for 0.5 min, 55°C for 0.5 min, and 72 °C for 1.5 min, with a terminal elongation at 72 °C for 10 min. The amplified DNA products were examined after electrophoresis through a 1.5% (w/v) agarose gel that was stained with 1 μg mL⁻¹ ethidium bromide.

**Recrystallization Inhibition Assay**

Cultures (50 mL) of *Paenisporosarcina* sp. TG14 were grown aerobically (200 rpm) at 4°C in Marine Broth 2216 (Difco). The cells were pelleted from mid-exponential phase cultures by centrifugation (10 min, 17,000 × g) and extracellular proteins (≥ 30-kDa) were recovered
from 15 mL of the supernatant using an Amicon Ultra-15 (30-kDa nominal molecular weight
cutoff) centrifugal filter (4,000 × g; 20 min). After centrifugation, the concentrated protein (~200
μL) was washed with 14.8 mL of Tris-sucrose buffer (50 mM Tris, 30% (w/v) sucrose; pH 7.5)
and concentrated again with another Amicon Ultra-15 centrifugal filter (4,000 × g; 20 min).
Concentrated extracellular proteins prepared from a bacterium known (isolate 3519-10;
Flavobacteriaceae family) to secrete an ice-binding protein that inhibits ice recrystallization were
prepared in parallel and used as a positive control (Raymond et al., 2008). Likewise,
concentrated extracellular proteins prepared from mid-exponential phase *Escherichia coli*
cultures (200 rpm; 37 °C; Difco Tryptic Soy Broth) as well as blank Tris-sucrose buffer
containing no cells were used as negative controls.

Ice recrystallization inhibition (RI) activity in the prepared concentrates was assayed
using a modification of the method described by Smallwood *et al.*, (1999) using a Linkam
LTS350 cryostage (Linkam Scientific Instruments) mounted on an Olympus BX51 microscope.
A 2 μL droplet of the extracellular protein concentrate was layered between two coverslips and
placed on the cryostage. Small ice crystals were created by lowering the temperature at a rate of
30 °C/min till the samples were at -80 °C. After one minute at -80 °C, the cryostage was warmed
to -6 °C and the growth of the ice crystals was monitored microscopically for one hour. A
counting grid was digitally superimposed over micrographs of the ice crystals and used to
calculate the average number of ice crystals per 100 μm². The counting grid was calibrated using
a 1-mm SPI PS8 micrometer.
Results

Isolate Culturing and Characterization

Growth was observed at all incubation temperatures tested except 37°C and on all media except M9 acetate. Marine media (Difco 2216) inoculated with banded ice-meltwater (2007 profile depth: 195-200 cm) and incubated at 22°C yielded the highest average colony counts (9.7 ± 1.5 ×10⁶ CFU mL⁻¹), indicating that ~0.7% of the cells counted via SYBR Gold and Baclight staining (Chapter 3; Figure 3.1, A) were culturable under the conditions tested. Due to the abundance of taxa related to endospore-forming bacteria (Chapter 2), parallel melt-water samples were pasteurized (80°C, 10 min) to determine if the isolates cultured originated from vegetative cells or endospores. Marine agar 2216 (Difco) was chosen for this assay because it regularly yielded the highest CFU mL⁻¹ from basal ice samples. Pasteurization of the melt water reduced the recovery of culturable cells by 96%, suggesting that endospores were not the origin for the majority of CFUs. All of the isolates recovered were psychrotolerant and capable of growth at 4°C but had optimal growth at temperatures between 15°C and 22°C. Of the eleven isolates tested for halotolerance, all grew the fastest in marine broth (Difco 2216) that contained no additional NaCl. For each isolate, the growth rate decreased with increasing NaCl and the maximum halotolerance displayed by each isolate varied between 5.9% and 11.9% (w/v) NaCl (Table 4.1).

Phylogenetic Analysis of Isolates

Of the 30 phylogenetically unique isolates recovered in this study, 28 were most closely related to species of *Paenisporosarcina*, a genus in the phylum Firmicutes. There were two other isolates that were members of the Firmicutes but classified within the genera *Bacillus* and *Paraliobacillus* (Table 4.1). Five additional isolates obtained from basal ice samples collected in
Table 4.1. Phenotypic description of basal ice isolates and their phylogenetic relationships to cultured bacteria. N.D. = not determined. Reprinted with permission from Doyle et al., (2013).

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<th>Closest Relative</th>
<th>% Identity</th>
<th>Sequence Length (bp)</th>
<th>Isolation Media</th>
<th>Isolation temp</th>
<th>Optimal temp (± 5 °C)</th>
<th>Halotolerance (% NaCl)</th>
<th>Description</th>
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<td>15 °C</td>
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<td>1363</td>
<td>marine</td>
<td>10 °C</td>
<td>15 °C</td>
<td>~9.9% (w/v)</td>
<td>tan, circular, convex</td>
</tr>
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<td>1366</td>
<td>M9 glucose</td>
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<td>~7.9% (w/v)</td>
<td>offwhite, shiny, convex</td>
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<td>22 °C</td>
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<td>offwhite, circular, shiny</td>
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<td>N.D.</td>
<td>cream-yellow, shiny, convex</td>
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<td>~7.9% (w/v)</td>
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<td>TG3</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1375</td>
<td>R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>~5.9% (w/v)</td>
<td>offwhite center, mucoid</td>
</tr>
<tr>
<td>TG6</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1382</td>
<td>1% R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>~5.9% (w/v)</td>
<td>white-yellow, mucoid</td>
</tr>
<tr>
<td>TG7</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1387</td>
<td>1% R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>offwhite, mucoid</td>
</tr>
<tr>
<td>TG18</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1349</td>
<td>R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>offwhite, mucoid</td>
</tr>
<tr>
<td>TG11</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1373</td>
<td>R2A</td>
<td>22 °C</td>
<td>15 °C</td>
<td>N.D.</td>
<td>cream yellow, circular</td>
</tr>
<tr>
<td>TG2</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1382</td>
<td>R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>cream yellow, mucoid</td>
</tr>
<tr>
<td>TG15</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1376</td>
<td>R2A</td>
<td>10 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>yellow, mucoid</td>
</tr>
<tr>
<td>TG17</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1377</td>
<td>R2A</td>
<td>10 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>white-yellow, convex, mucoid</td>
</tr>
<tr>
<td>TG26</td>
<td>Paenisporosarcina macmurdensis CMS21w</td>
<td>99</td>
<td>1369</td>
<td>marine</td>
<td>10 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>offwhite, mucoid</td>
</tr>
<tr>
<td>TG19</td>
<td>Paenisporosarcina indica</td>
<td>99</td>
<td>1369</td>
<td>marine</td>
<td>22 °C</td>
<td>22 °C</td>
<td>~7.9% (w/v)</td>
<td>dark brown, convex</td>
</tr>
<tr>
<td>TG9</td>
<td>Paenisporosarcina indica</td>
<td>99</td>
<td>1372</td>
<td>R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>~7.9% (w/v)</td>
<td>dull yellow, translucent, flat, mucoid</td>
</tr>
<tr>
<td>TG20</td>
<td>Paenisporosarcina indica</td>
<td>99</td>
<td>1386</td>
<td>marine</td>
<td>22 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>cream-yellow, convex</td>
</tr>
<tr>
<td>TG39</td>
<td>Paenisporosarcina indica</td>
<td>99</td>
<td>1370</td>
<td>marine</td>
<td>4 °C</td>
<td>22 °C</td>
<td>N.D.</td>
<td>white, shiny, convex</td>
</tr>
<tr>
<td>TG10</td>
<td>Paenisporosarcina quisquiliarum SK 55</td>
<td>99</td>
<td>1383</td>
<td>R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>~5.9% (w/v)</td>
<td>offwhite, shiny, convex</td>
</tr>
<tr>
<td>TG8</td>
<td>Bacillus humi LMG18435</td>
<td>97</td>
<td>1367</td>
<td>1% R2A</td>
<td>22 °C</td>
<td>22 °C</td>
<td>~5.9% (w/v)</td>
<td>tan with brown center, circular</td>
</tr>
<tr>
<td>TG4</td>
<td>Paraliobacillus quinghaiensis YIMC158</td>
<td>99</td>
<td>1418</td>
<td>R2A</td>
<td>22 °C</td>
<td>15 °C</td>
<td>~11.9% (w/v)</td>
<td>yellow, convex, rough</td>
</tr>
</tbody>
</table>
1999 (Samyn et al., 2008) were also identified as species of Paenisporosarcina. The 16S rRNA gene sequences from the Paenisporosarcina-related isolates were highly similar (≥97% identity) to sequences recovered in the 16S rRNA gene MiSeq libraries (Chapter 2; Table 2.4).

**DNA and Protein Synthesis of Isolated Bacteria at -15°C**

Of all the isolates tested, Paenisporosarcina sp. TG14 had the shortest generation time (3.0 hours generation\(^{-1}\) at 15 °C) and was selected for subzero incorporation assays to explore its ability to remain metabolically active under conditions similar to those found in the Taylor Glacier basal ice. Regardless of which melt-water was used for the assay (i.e. banded ice or clean ice), Paenisporosarcina sp. TG14 cells remained active at -15 °C and incorporated \[^3\text{H}\]-leucine and \[^3\text{H}\]-thymidine into acid-insoluble macromolecules while frozen (Figure 4.1). A rectangular hyperbole (i.e., one site saturation curve) was determined as a best fit for the data.

Figure 4.1. \[^3\text{H}\]-Leucine and \[^3\text{H}\]-thymidine incorporation into trichloroacetic acid (TCA) precipitable material at −15 °C by frozen cell suspensions of Paenisporosarcina sp. TG14 in clean ice melt water (white circles) and banded dispersed ice melt water (black triangles). The initial cell concentration of the TG14 cell suspensions was 3.1 × 10\(^6\) colony-forming units (CFU) mL\(^{-1}\). Error bars are the standard error of triplicate samples. Incorporation is reported as molecules of substrate incorporated per CFU on the left axis and grams of substrate carbon incorporated per gram of cell carbon on the right-hand axis. Where significant (\(p < 0.05\)), the best-fit regression curves (hyperbolic) are plotted as dashed lines. Reprinted with permission from Doyle et al., (2013).
When frozen in the clean ice melt-water, *Paenisporosarcina* sp. TG14 cells incorporated an average of $1.5 \pm 0.2 \times 10^4$ and $4.0 \pm 0.2 \times 10^3$ molecules CFU$^{-1}$ of $[^3]$H-leucine and $[^3]$H-thymidine, respectively, over the 70 day time-course. Incorporation of both radiolabeled substrates was continuous and followed a hyperbolic trend ($R^2 > 0.94; p < 0.0001$) throughout the entire incubation. The fastest incorporation rates for both substrates occurred during the first 90 h of the incubation at $2.5 \pm 0.9 \times 10^3$ molecules of $[^3]$H-leucine CFU$^{-1}$ day$^{-1}$ and $2.7 \pm 1.0 \times 10^2$ molecules of $[^3]$H-thymidine cell$^{-1}$ day$^{-1}$.

When frozen in banded ice melt-water, *Paenisporosarcina* sp. TG14 cells incorporated an average of $8.1 \pm 1.1 \times 10^3$ and $1.6 \pm 0.3 \times 10^3$ molecules CFU$^{-1}$ of $[^3]$H-leucine and $[^3]$H-thymidine, respectively, over the 70 day incubation. Similar to the clean ice, $[^3]$H-thymidine incorporation in the banded ice incubations appeared to be partially hyperbolic ($R^2 = 0.41; p = 0.0455$; Figure 4.1) and occurred most rapidly in the first 90 hours ($2.7 \pm 1.0 \times 10^2$ molecules cell$^{-1}$ day$^{-1}$). Likewise, $[^3]$H-leucine incorporation in the banded ice incubations was also most rapid in the first 90 hours ($2.5 \pm 0.9 \times 10^3$ molecules CFU$^{-1}$ day$^{-1}$), but due to high variance in the data, a significant regression model ($\alpha < 0.05$) could not be determined.

Overall, the maximum rates $[^3]$H-leucine and $[^3]$H-thymidine incorporation were not significantly different between the two meltwater samples ($[^3]$H-leucine: $p > 0.38$; $[^3]$H-thymidine: $p > 0.79$). However, after 70 days, the clean ice incubations did incorporate a significantly larger amount of $[^3]$H-leucine ($p = 0.049$) and $[^3]$H-thymidine ($p = 0.003$) than the banded ice incubations.

**DNA and Protein Synthesis of Isolated Bacteria in Ice and Brine at -5°C**

During frozen incubation over 80 d at -5°C in 1% marine broth, TG14 incorporated an average of $6.6 \pm 0.3 \times 10^3$ and $3.6 \pm 0.1 \times 10^3$ molecules CFU$^{-1}$ of $[^3]$H-leucine and $[^3]$H-thymidine,
respectively (Figure 4.2). TG14 cells incubated in brine incorporated an average of $3.5 \pm 0.1 \times 10^3$ and $2.8 \pm 0.1 \times 10^2$ molecules CFU$^{-1}$ of [$^3$H]-leucine and [$^3$H]-thymidine, respectively. For both ice and brine incubations, incorporation of [$^3$H]-leucine and [$^3$H]-thymidine was continuous and hyperbolic ($R^2 > 0.82; p \leq 0.0005$). The maximum rates of [$^3$H]-leucine and [$^3$H]-thymidine incorporation the frozen incubations occurred during the first three weeks at $1.7 \times 10^2$ molecules CFU$^{-1}$ day$^{-1}$ and $1.1 \times 10^2$ molecules CFU$^{-1}$ day$^{-1}$, respectively. For the brine incubations, the maximum rates of [$^3$H]-leucine and [$^3$H]-thymidine incorporation occurred over the first 6 days at $1.3 \times 10^2$ molecules CFU$^{-1}$ day$^{-1}$ and $1.3 \times 10^1$ molecules CFU$^{-1}$ day$^{-1}$, respectively. The maximum rates of [$^3$H]-leucine and [$^3$H]-thymidine incorporation between frozen and brine incubations were significantly different ($p = 0.012$ and 0.0002, respectively).

![Figure 4.2. [$^3$H]-Leucine and [$^3$H]-thymidine incorporation into trichloroacetic acid (TCA) precipitable material at −5 °C by cell suspensions of *Paenisporosarcina* sp. TG14 in ice (white circles) and brine (black triangles). The initial cell concentration of the TG14 cell suspensions was $3.21 \times 10^6$ colony-forming units (CFU) mL$^{-1}$. Error bars are the standard error of triplicate samples. Incorporation is reported as molecules of substrate incorporated per CFU on the left axis and grams of substrate carbon incorporated per gram of cell carbon on the right-hand axis. Where significant ($p < 0.05$), the best-fit regression curves (hyperbolic) are plotted as dashed lines.](image-url)
Genome Sequencing and Identification of IBP gene

The draft genome of *Paenisporosarcina* sp. TG14 (~3,826,160 bp) containing 135 contigs ($N_{50} = 60,912$ bp) was deposited in the NCBI database under the accession number PRJNA199691 (Koh *et al.*, 2012). The search for IBP-like sequences with BLAST revealed that *Paenisporosarcina* sp. TG14 possessed an open reading frame for a hypothetical 47k-Da protein with a secretory peptide and a putative conserved domain (DUF3494; NCBI) which showed high identity (47.5% to 58.6%) with several putative IBPs (Figure 4.3, Figure 4.4).

**Figure 4.3.** Predicted amino acid sequence of *Paenisporosarcina* sp. TG14’s hypothetical ice-binding protein. Underlined sequence is a predicted secretory peptide; shading indicates a putative conserved domain (DUF3494; NCBI) which showed significant identity with other ice-binding proteins.

IBP Gene Expression

Reverse transcription and PCR amplification detected mRNA for *Paenisporosarcina* sp. TG14’s ice-binding protein at all growth temperatures tested between 5 °C and 25 °C (Figure 4.5), indicating that the IBP gene was constitutively expressed during logarithmic growth. Likewise, the expression of 16S rRNA was also detected under all conditions tested. No amplicons were detected when the reverse transcription step was omitted, confirming that amplifiable DNA carryover was removed.
Figure 4.4. Multiple alignment of amino acid sequences of *Paenisporosarcina* sp. TG14’s hypothetical ice-binding protein along with those from putative IBPs. Only the conserved domain is shown. Alignment was constructed using ClustalW. The letters in the first column designate the source organism: Pae, *Paenisporosarcina* sp. TG14; Rho, *Rhodoferax ferrireducens*; Fer, *Ferroplasma acidarmanus*; She, *Shewanella denitrificans*; Cyt, *Cytophaga hutchinsonii*; Pol, *Polaribacter irgensii* 23-p; Shf, *Shewanella frigidimarina*; Psy, *Psychromonas ingrahami*. Equivalent residues, based on physico-chemical properties, are boxed. Conserved residues are shaded. Percent identities between the sequences are at the end.

Figure 4.5. Reverse transcription and PCR analysis of gene expression in *Paenisporosarcina* sp. TG14. The 802-bp bands in lanes 1-4 represent the presence of 16S rRNA; the 1,293-bp bands in lanes 9-12 are fragments of IBP transcripts. The template for each lane was RNA from mid-log cultures of *Paenisporosarcina* sp. TG14 grown at 5, 10, 15, and 25 °C, respectively. Lanes 5-8 and 13-16 are identical samples to those in lanes 1-4 and 13-16, respectively, wherein the reverse transcription step was omitted. L is a 2kb DNA Ladder.
IBP Recrystallization Inhibition

As shown in Figure 4.6 (panels A and B), ice recrystallization occurred in the control samples after one hour of incubation at -6 °C. In contrast, the samples prepared from Paenisporosarcina sp. TG14 and Vostok ice core bacterium 3519-10 displayed significant RI activity (Figure 4.6, C and D), containing approximately four-fold and three-fold more crystals per 100 μm² than the Tris-sucrose buffer and E. coli samples, respectively (Figure 4.7). Additionally, the Paenisporosarcina sp. TG14 sample uniquely appeared to modify ice crystal morphology, producing distinct angular ice crystals (Figure 4.6, D) similar to previous IBP activity investigations (Lee et al., 2010) and indicative of a protein binding to ice crystal planes. This effect is also partially visible for the Vostok ice core bacterium 3519-10 sample (Figure 4.6, C, bottom). Conversely, ice crystals in the negative controls were rounded or ovoid in shape.

Figure 4.6. Ice recrystallization inhibition (RI) assay with frozen drops of concentrated extracellular proteins (≥30-kDa) suspended in Tris-sucrose buffer. (A) Tris-sucrose buffer only; (B) Extracellular proteins of Escherichia coli; (C) Extracellular proteins of Vostok ice core bacterium 3519-10; (D) Extracellular proteins of Paenisporosarcina sp. TG14. Top micrograph panels were taken after immediately after warming the sample to -6 °C. Bottom micrograph panels were taken after incubation at -6 °C for one hour. Panel dimensions: 200 μm × 200 μm.
Figure 4.7. Number of ice crystals per 100 μm² for each ice sample. Error bars represent one standard deviation. Columns with different letters are significantly different (p < 0.05).

Discussion

Isolate Culturing and Characterization

Twenty-eight psychrotolerant bacterial isolates with 16S rRNA gene sequences >97% identical to the *Paenisporosarcina*-related OTUs identified in Chapter 2 were obtained using standard culturing methods. Using both molecular and culture-dependent methodologies, species of *Paenisporosarcina* have now been observed in basal ice samples from three separate basal ice zone tunnels constructed along the north margin of the Taylor Glacier (~0.5 km apart). Further, members of this genus have also been observed in a wide range of other permanently cold or frozen environments including permafrost (Steven *et al.*, 2007; Steven *et al.*, 2008), arctic saline springs (Perreault *et al.*, 2008), alpine glaciers (Reddy *et al.*, 2013), the Greenland Ice Sheet (Miteva *et al.*, 2004), and the McMurdo Dry Valleys (Reddy *et al.*, 2003), suggesting these
bacteria may possess adaptations that enhance their survival in icy environments. Alternatively, observations of *Paenisporosarcina* sp. in a variety of frozen environments could be attributed to their ability to differentiate into a durable endospore, enabling them to persist for extended timeframes in ice. However, the observed 96% reduction in total CFUs after pasteurizing Taylor Glacier melt-water implies the majority of the isolates obtained in this study did not originate from endospores.

One such adaptation may be the production of ice-binding proteins which offer some protection against damage caused by the growth of large membrane-damaging ice crystals. Genomic sequencing of *Paenisporosarcina* sp. TG14 revealed this bacterium possesses an open reading frame related to putative IBPs (Koh *et al.*, 2012). Ice-binding proteins bind to the prism face of ice crystals and inhibit ice recrystallization, significantly altering the morphology of the liquid vein network within polycrystalline ice and its evolution over time (Brown *et al.*, 2012). This activity has been shown to confer a distinct survival advantage to cells during freeze-thaw cycling (Kang and Raymond, 2004; Walker *et al.*, 2006; Achberger *et al.*, 2011). As such, while there is certainly more to cold adaptation than just the production of ice-binding proteins, the ability for *Paenisporosarcina* sp. TG14 to control the structure of its ice vein habitat provides one possible explanation for its abundance and activity in the banded ice horizons of Taylor Glacier (Chapter 2). The expression of the IBP gene by *Paenisporosarcina* sp. TG14 did not appear to be affected by growth temperature, mirroring a similar observation of constitutive IBP gene expression by Vostok ice core bacterium 3519-10 (Achberger *et al.*, 2011). However, considering the energetic cost of protein synthesis and export, it remains enigmatic why IBP expression is not down-regulated in these organisms at temperatures significantly above 0 °C, where ice formation is not a threat. One possibility is that due to the decreased ability to
synthesize and excrete proteins after being immured in ice (discussed below), it is necessary to
constitutively produce IBPs so that protection against ice recrystallization is still available during
a sudden freeze event (e.g. ‘freeze-on’ of subglacial water during glaciohydraulic supercooling;
Chapter 1). Alternatively, these genes may be regulated by a temperature-independent
mechanism which has not yet been elucidated (e.g. osmotic stress). For example, Krell et al
(2008) discovered that salt-stress induced the production of four hypothetical IBPs in the
psychrophilic diatom *Fragilariopsis cylindrus*.

In addition to ice recrystallization, microorganisms found in ice veins are subjected to
considerable osmotic stress due to the high concentration of solutes present in the liquid fraction
of the ice matrix. For example, the ionic strength of an ice vein at -15 °C is estimated to be
approximately 4.5 M (Chapter 1; Figure 1.2, B). All of the *Paenisporosarcina*-related isolates
from this study exhibited moderate halotolerance and were able to tolerate salt concentrations up
to between 6% and 12% (w/v). These levels of halotolerance would not be sufficient to sustain
growth *in situ* where the estimated salt concentrations in the ice veins approach an ionic strength
of 4.5 M (Chapter 1; Figure 1.2, B), equivalent to a 26.9% (w/v) solution of NaCl. However, the
cells may still be able to sustain a low rate of maintenance-oriented metabolic activity at salt
concentrations beyond those at which growth is possible. Alternatively, it may be that the
isolates recovered in this study exhibit an increased halotolerance while frozen *in situ* compared
to the values measured *in vitro*. Indeed, Leblanc *et al.*, (2003) observed that growing *Shewanella
putrefaciens* at decreasing temperatures induced an increased resistance to osmotic stress.

**Macromolecular Synthesis While Frozen**

The incorporation assays performed at -15 °C were designed in a manner to investigate
bacterial metabolism under conditions that simulated those within the Taylor Glacier basal ice
environment as closely as possible. Under these conditions, the highest rates of metabolism occurred initially and were followed by reduced rates as incubation time increased. While frozen at -15 °C, *Paenisporosarcina* sp. TG14 was able to incorporate a maximum of 1,500 to 2,500 molecules of $[^3]$H-leucine and 200 to 300 molecules of $[^3]$H-thymidine per day. Based on a mean protein length of 267 amino acids containing around 7.3% leucine residues (Amato *et al.*, 2010), these rates would allow TG14 to synthesize roughly four proteins every hour and replicate its 3.83 Mb genome approximately once every two decades. These metabolic rates (0.81 to $2.1 \times 10^{-7}$ gC gC$^{-1}$ hour$^{-1}$) are comparable to those predicted by Price and Sowers (2004) to be sufficient to support maintenance metabolism at -15°C but are approximately two orders of magnitude below that expected for reproductive growth.

Dissolved organic carbon, inorganic nitrogen, and inorganic phosphorus concentrations in horizons of the banded ice melt-water used in the -15 °C incubation experiments were 63 mg L$^{-1}$, 0.68 mg L$^{-1}$, and 5.5 μg L$^{-1}$, respectively (measured from banded ice recovered from profile depth 230–235 cm; *i.e.* Banded07). These values were approximately a hundred times higher than those measured in the clean ice (Montross, 2012). Unexpectedly, this did not significantly increase the rate of macromolecular synthesis by *Paenisporosarcina* sp. TG14 while it was frozen at -15°C (Figure 4.1). In fact, it was the clean ice incubations which incorporated the most $[^3]$H-leucine and $[^3]$H-thymidine over the entire 70 day incubation. One potential explanation for this observation is that endogenous sources of leucine or thymidine were available in the banded ice meltwater and were preferentially used over the radiolabeled substrates by the TG14 cells. Alternatively, although efforts were taken to limit this potential issue, the reduced incorporation rates measured in the banded ice melt-water incubations may be due to scintillation interference from fine clay particulates not found in the sediment-free clean
ice. There also remains a third possible explanation for these data wherein the varying rates of radiolabeled substrate usage by the TG14 cells may have been a result of differences in the ice vein matrix between the clean ice and banded ice incubations. As described in chapter 1, the diameter of the ice veins (and subsequently the unfrozen water volume contained within them) inside polycrystalline ice formed from a dilute solution (i.e. clean ice) would be smaller than those in ice formed from a more concentrated solution (i.e. banded ice). Hence, the volume of liquid water inside clean ice is significantly smaller than that of banded ice simply due to differences in bulk solute concentration \([ I = 0.1 \text{ and } 160 \text{ mM, respectively; (Montross, 2012)}\]. As a result, after freezing the experimental aliquots, the final concentration of \(^3\text{H}\)-leucine or \(^3\text{H}\)-thymidine in the clean ice incubations was significantly higher than those prepared from banded ice. This raises the interesting possibility that freezing, despite all the challenges it poses, may provide some benefit in extremely oligotrophic environments by concentrating dilute nutrients into the ice veins and thus making them more readily available for microorganisms.

The results of the ice and brine incorporation experiments remarkably support this possibility: *Paenisporosarcina* sp. TG14 cells incorporated significantly more \(^3\text{H}\)-leucine and \(^3\text{H}\)-thymidine at -5°C while frozen compared with unfrozen brine incubations (Figure 4.2). The decreased metabolic activity in the brine incubations should not be a result of differences in osmotic stress because cells in both the frozen and brine incubations experienced equivalent salinities. Due to solute concentration during freezing at -5°C, the ice veins present in the frozen incubations are predicted to have an ionic strength of ~1.7M, which is equivalent to a concentration of 10% (w/v) NaCl. Another intriguing possibility is that damage incurred during freezing induced the up-regulation of cellular repair processes in the frozen incubations, thereby increasing rates of \(^3\text{H}\)-leucine and \(^3\text{H}\)-thymidine incorporation. This may also explain why
rates of macromolecular synthesis by frozen cells in this study, both at -5 and -15 °C, appeared to decrease over time: After any freeze-induced or ice crystallization damage is repaired and physiological adaptations to the frozen environment are made (e.g. high solute concentration), frozen *Paenisporosarcina* sp. TG14 cells transition to a metabolic strategy focused not on growth but on the long-term maintenance of cellular and genomic integrity.

**Conclusions**

The results of this study lend support to the hypothesis that sediment-rich basal ice is a microbial habitat harboring metabolically active microbial assemblages. Several of the *Paenisporosarcina* isolates recovered from the banded ice cryofacies of Taylor Glacier where DNA- and RNA- based 16S rRNA analyses (Chapter 2) indicated they were numerically abundant and metabolically active. Species of this genus appear to have characteristics that would promote survival in ice including psychrotolerance, halotolerance, and a spore-based survival stage. *Paenisporosarcina* sp. TG14 also clearly demonstrated an ability to synthesize macromolecules while frozen at -15 °C in meltwater from both clean and banded basal ice. Similarly, Bakermans and Skidmore (2011) demonstrated another isolate of *Paenisporosarcina* respired acetate down to -33 °C in M9 minimal media. However, in both cases, it remained unclear if this activity would continue over an extended time-frame or if the cells exhibited a burst of rapid macromolecular synthesis upon freezing to repair freeze-induced damage and prepare for long-term dormancy. *Paenisporosarcina* sp. TG14 also secretes an IBP with significant RI activity and thus may be able to control the local structure of the ice vein environment within Taylor Glacier’s banded basal ice cryofacies. IBPs with RI activity are known to protect bacteria from ice recrystallization, even those which did not secrete the IBP themselves. For example, Achberger *et al.*, (2011) demonstrated that an IBP (NCBI Accession:
ACD76102) from Flavobacteriaceae bacterium 3519-10 (NCBI Taxon ID: 531844), an Antarctic basal ice isolate, increased the freeze-thaw survival of *Escherichia coli* 34-fold. Hence, *Paenisporosarcina*-related bacteria may represent keystone species in Taylor Glacier’s basal ice zone which commensally facilitates the survival of other microorganisms immured in the basal ice. Whether such a relationship actually occurs *in situ* and if it is a widespread phenomenon or not in other glaciers remains unknown but provides a working hypothesis for future ecological studies of basal ice and other frozen environments.
CHAPTER 5.
FINAL REMARKS

Summary

Glacial ecosystems are among the least explored frontiers of Earth’s biosphere. While it is recognized that habitats for microbial life exist on the surface and beneath glaciers, current understanding of the phylogenetic and functional diversity of the microorganisms frozen inside these ice masses remains largely in its infancy. The research presented in this dissertation sought to help elucidate the role microorganisms play in the cycling of carbon and other elements in the basal zones of glaciers. Specifically this project focused on the basal ice environment, where the ice is warmest and entrained subglacial sediments were hypothesized to provide a range of potential nutrient and energy substrates (e.g. organic carbon) for microorganisms. Collectively, the results presented in this dissertation demonstrate that sediment-rich basal ice can serve as a habitat capable of harboring active microbial communities that mineralize organic carbon and modify the gas composition of the ice.

Chapter 2 represents the first coordinated effort to characterize and compare the composition and structure of microbial assemblages inhabiting different types of basal ice, providing valuable insight into the microbial communities inhabiting sediment-rich and sediment-poor basal ice cryofacies. Molecular analyses of 16S rRNA gene sequences showed Firmicutes-related microorganisms were abundant in Taylor Glacier basal ice. Similar analyses of basal ice from Matanuska Glacier revealed a bacterial community composed primarily by members unrelated to any currently known bacterial phyla. The scarcity of archaea in all of the basal ice samples remains puzzling as methanogens have been detected in subglacial water (Dieser et al., 2014) and large populations of ammonia-oxidizing archaea were recently detected in subglacial Lake Whillians (Christner et al., 2014).
Analyses of basal ice cryofacies from Taylor Glacier showed that debris-rich basal ice contained larger concentrations of microbial cells that occurred concomitantly with elevated concentrations of CO$_2$ and depleted concentrations of O$_2$. An isotopic analyses of these gases indicated that microbial respiration was the most probable explanation for the gas signatures in the basal ice (Chapter 3). Measurements of ATP and ADP concentrations in these horizons indicated microorganisms inhabiting banded basal ice of intermediate sediment content (5 to 20%) were the most active, possibly due to the co-availability of atmospheric oxygen and subglacial sediments.

Isolates of *Paenisporosarcina* were readily cultivable from the banded ice and appear to have characteristics that would promote survival in ice (e.g., psychrotolerance, halotolerance, metabolic activity at -15°C, antifreeze protein production, and a spore-based survival stage). Members of this genus have also frequently been isolated from similar icy environments (e.g. permafrost); however, current understanding of the exact mechanisms and adaptations which allow *Paenisporosarcina* sp. to thrive in cold and frozen environments remains incomplete.

Interestingly, activity assays with *Paenisporosarcina* sp. TG-14 in ice and brine suggest habitability of frozen environments may be more favorable than previously recognized: freeze concentration of nutrients into ice veins may enable frozen microbial populations to persist in environments where nutrients are scarce in the bulk liquid phase (Chapter 4).

The observations presented here highlight that given the right conditions and a suitable microbial inoculum, sediment-rich basal ice is a habitat which supports biogeochemical cycling in the cryosphere. The demonstration of microbial metabolic function within ice also suggests that frozen worlds, such as Mars, Europa or Enceladus, could harbor cryogenic habitats suitable for microbial life (Priscu and Hand, 2012).
Future Directions in Microbiological Studies of Glaciers

Although physiochemical models have provided an initial framework so far, one of the least understood areas in glacial and subglacial microbiology is the exact nature of the ice vein environment. Analyzing the chemical composition of ice veins is notoriously difficult even with current techniques. For example, due to the low eutectic temperatures of acids (e.g. \(-73.1^\circ\text{C}\) for \(\text{H}_2\text{SO}_4\), see Chapter 1), ice veins should be very acidic (pH \(-1\)) (Fukazawa et al., 1998; Price, 2000; Barletta and Roe, 2011; Barletta et al., 2012; Dani et al., 2012). Paradoxically, there are no reports of acidophilic microorganism ever being isolated from glacial ice. Ohno et al., (2005) argued that sulfate ions measured in ice veins using micro-Raman spectroscopy were incorrectly assumed to exist as an acid \textit{in situ} and instead exist as salts. However, the eutectics of sulfate salts are relatively warm [e.g., \(-1.1^\circ\text{C}\) for \(\text{Na}_2\text{SO}_4\); \(-3.9^\circ\text{C}\) for \(\text{MgSO}_4\); \(>0.05^\circ\text{C}\) for \(\text{CaSO}_4\) (Hall and Sherrill, 1928)], meaning they would likely precipitate as hydrated solid inclusions, potentially leading to ice vein collapse. These difficulties are confounded by the fact that it is currently unclear how ice veins differ (if at all) between ice formed from the firnification of snow (\textit{i.e.} glacial ice) versus the cooling and subsequent freezing of a liquid solution (\textit{e.g.} laboratory studies and some forms of basal ice).

Sediment-rich basal ice exists in alpine glaciers and is widespread across the Antarctic and Greenland ice sheets, likely representing a significant source of organic matter, nutrients, and microorganisms to subglacial lakes and downstream marine environments (Statham et al., 2008; Hood et al., 2009). Geochemical transformations catalyzed by microorganisms in basal ice may be an important aspect of element cycling (\textit{e.g.} carbon or nitrogen) in these environments. Elucidating further detail on the specific metabolic strategies of these microbial communities is a current research need and will provide a wider perspective on the pools and fluxes of carbon in
glacial ecosystems. Continued expansion of molecular tools (e.g. metagenomics and/or metatranscriptomics) combined with high-throughput culturing efforts will significantly expand our ability to investigate the ecology of these frozen environments and help refine the known limits of the biosphere. Additionally, as anthropogenic climate change accelerates the retreat and wastage of Earth’s glaciers, investigations of these environments (especially AIS and GIS) will be provide a critically important framework to our understanding of how the structure and function of microbial communities upon, within, and beneath glaciers respond to a rapidly changing ecosystem.
REFERENCES


Nealson, K. H. and D. A. Stahl (1997). "Microorganisms and Biogeochemical Cycles: What Can We Learn from Layered Microbial Communities?" Reviews in Mineralogy and Geochemistry 35: 5-34.


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