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The role of microbial extracellular polymeric substances in psychrotolerance and geochemistry of subglacial environments

by

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ABSTRACT

Extracellular polymeric substances (EPS) were investigated to determine their potential role in microbial cryotolerance in the subglacial environment. The cell surface chemistry of *Hymenobacter aerophilus* cells, with and without EPS, was determined using potentiometric titrations and cadmium adsorption assays. The presence of EPS was associated with an increase in both variety and quantity of surface ligands available for environmental interactions and resulted in an increase in adsorbed cadmium. Survival of a freeze-thaw cycle with and without EPS was tested with *Flavobacterium* A97, a subglacial isolate, in the presence and absence of a variety of particulate substrata. Most probable number (MPN) and plate count data were unable to provide sufficient resolution to test the hypothesis that EPS production and the presence of a substratum would be associated with increased survival of a freeze-thaw cycle.

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1. INTRODUCTION

Since the early 1990s there has been an increase in interest in the microbiology of glacial systems. Formerly it was generally believed that living organisms were not present in or under glaciers and that weathering beneath glaciers was due solely to inorganic processes. However, the recent revelations that cultivable microbes can be isolated from glaciers (Skidmore et al. 2005), and the possibility of microbial activity in subglacial water (Sharp et al. 1999; Skidmore et al. 2000) has suggested that microbes play a role in chemical weathering and nutrient cycling in glacial systems.

The microbial ecology of cold ecosystems such as open oceans, sea ice, and permafrost has been well studied but there has been very little investigation into the ecology of glaciers beyond studies of heterotrophic microbial diversity. In particular, there is almost nothing known about microbial extracellular polymeric substances (EPS) in glacial systems, either from the viewpoint of microbial survival or carbon cycling.

1.1 Cold Environments

The Earth is not generally thought of as a cold place to live. Discussion of permanently cold environments evokes images of remote, desolate regions and ice scoured by wind. However, this image is not accurate. A large portion of the Earth's landmass consists of polar or alpine regions and 70% of the Earth's total surface is covered by oceans, with an average temperature of 4°C (Ponder et al.

2005). Life has adapted to these conditions and spread to all available niches. In particular, microbial life thrives in all of these regions.

The ocean is home to a large number of diverse microbes, from surface algae and cyanobacteria to bacteria and archaea living in deep sea sediments (Edwards et al. 2003; Jorgensen et al. 2010; Trias et al. 2012). In addition to open water, much of the polar oceans are covered by sea ice with a temperature range of 0 to -30°C and highly variable salinity, pH and nutrient concentrations (Mock and Thomas 2005). Sea ice microbiology has seen a recent increase in scientific interest, with a number of studies on microbial survival and growth in sea ice (e.g. Bakermans and Skidmore 2011; Collins et al. 2010; Junge et al. 2004; Junge et al. 2006; Koh et al. 2010).

Another major cold environment is permafrost. A substantial portion of the Northern hemisphere consists of permafrost and it covers approximately 20% of the Earth's surface (Ponder et al. 2005). Permafrost has received a lot of attention, in part due to its stability (Ponder et al. 2005). The primary characteristic of permafrost is that it is permanently below the freezing point of water, often located beneath a layer of unfrozen soil. As a result there is little biological activity within the permafrost, although recent studies have suggested that microbes in the permafrost may be active, albeit at low rates compared to microbes in temperate regions (Bakermans et al. 2003; Panikov and Sizova 2007). Thus, there is the possibility that ancient microbes (up to 10^6 years old) can be isolated from permafrost (Rivkina et al. 2005).

There has been a lot of research on marine and permafrost systems as well as many physiological and activity studies based on permafrost microbes (e.g. Bakermans et al. 2003; Ponder et al. 2005; Qiu et al. 2006). However, these systems are quite different from a glacial system. In particular, sea ice and glaciers have very different water chemistry. Sea ice is derived mostly from frozen sea water and thus has a high salt content, whereas glaciers consist of freshwater. A well studied system more similar to glaciers is frozen lakes, such as Lake Vostok, Antarctica.

Frozen lakes exist in both the Arctic and Antarctic, but the majority of microbiological work has focused on Antarctic lakes. Throughout Antarctica there are lakes that are permanently covered, either by a relatively thin 4- to 6- m layer of ice as in Lake Fryxell (Karr et al. 2005), or by glaciers (2-3 km) as in the case of Lake Vostok. Due to the layer of ice covering the lake, many of these lakes have not been exposed to the atmosphere for thousands of years (Mosier et al. 2007). Lake Vostok is one of the world's largest lakes, approximately the size of Lake Ontario (Kapitsa et al. 1996), and is estimated to have been isolated for over 15 million years (Christner et al. 2006). Not all frozen lakes are so isolated, however. During the summer air temperatures can remain above 0°C for several days or weeks and during this time the ice around the edge of the ice cover can melt, allowing inflow of glacial melt water (Mosier et al. 2007). There can also be some exchange of water between lakes due to subglacial water movement (Fricker et al. 2007; Mikucki et al. 2004; Wingham et al. 2006), although this is not well understood. Microbes are abundant in this environment and bacteria,

including sulfate reducing bacteria (SRB), have been found in freshwater lakes beneath ice in Antarctica (Paerl and Priscu 1998; Karr et al. 2005).

1.1.2 Glaciers

From a biological standpoint, glaciers are the least studied of these cold environments. Although there has been some investigation of the ecology of glaciers, most of it has focused on the diversity of microorganisms in glacial systems (e.g. Bhatia et al. 2006; Cheng and Foght 2007; Foght et al. 2004; Lanoil et al. 2009; Skidmore et al. 2000; Yde et al. 2010). Studies of microbial diversity are discussed in greater detail below. However, before discussing the ecology of glaciers it is first necessary to provide a brief summary of the geology of glaciers.

Glaciers exist in a variety of shapes, from cirque glaciers that occupy basins high in mountains, to ice sheets, which are glaciers that flow in all directions from a central dome (Hooke 2005). Temperature can also be used to classify glaciers as polar, polythermal or temperate. The temperature of the ice in polar glaciers is always below the melting point of water, except occasionally at the bed beneath the glacier, whereas a large volume of the ice near the base of a polythermal glacier is at the melting point, with surface ice below its melting point (Hooke 2005). In a temperate glacier nearly all of the ice is at the pressure melting point (Hodson et al. 2008). Clearly, the temperature of the ice is important when considering the life that may be present within. It is also important to remember that the melting point of water within a glacier is not necessarily 0°C because

increased pressure and chemical impurities in the water can lower the melting point.

Glaciers are formed as falling snow accumulates and pressure builds, resulting in a crust of snow called firn (Fig. 1) (Raymond 1980). Gradually, pressure compresses the snow into glacier ice with a density of about 0.85 g/cm^3 (Souchez and Lorrain 1991). This is a variable process because colder conditions result in a deeper firn–ice transition, lengthening the time required for firn to become ice (Souchez and Lorrain 1991). In general, ice forms in 100 to 200 years in polar regions, although at Vostok it takes approximately 4000 years due to the extreme cold (Souchez and Lorrain 1991). It is during this process that microbes can be incorporated into the bulk ice as they are deposited on the glacier along with snow and other particles in the wind.

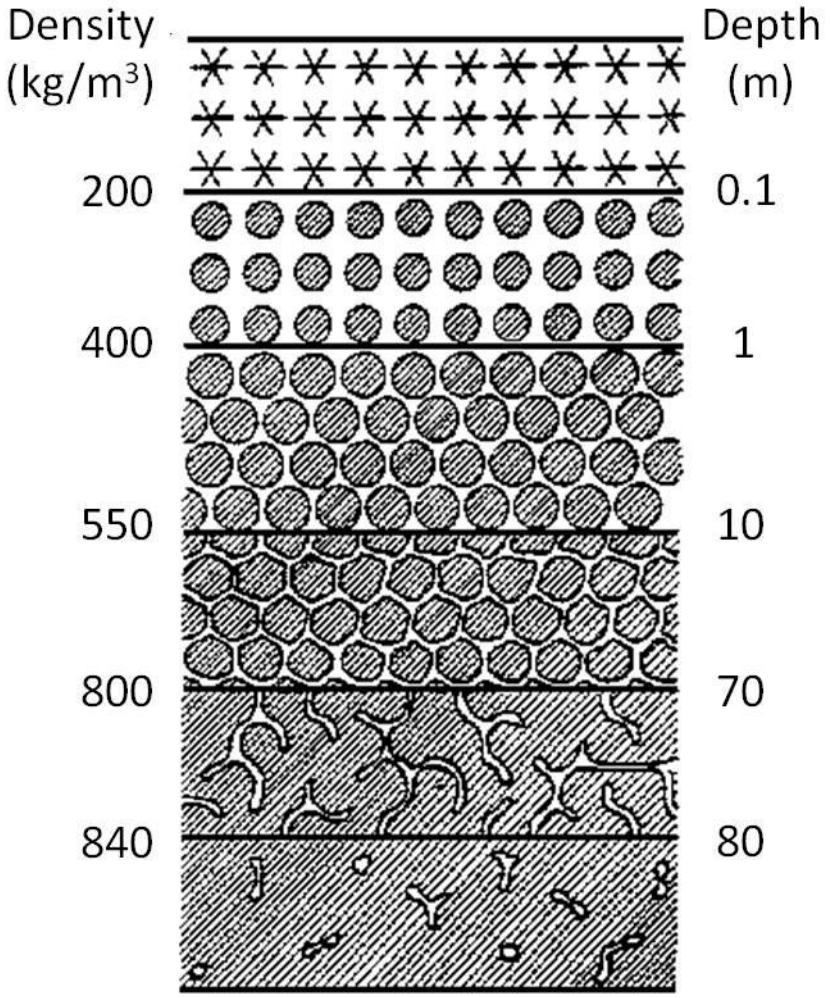

Figure 1-1. Schematic of firn and glacier ice. As snow falls, it covers the preexisting snow and as depth increases, pressure builds. This causes the snow to form large grains of ice that become denser and more compact with greater depth, eventually forming solid ice (Modified from Broecker (1995)).

Glaciers cannot accumulate mass indefinitely; some must eventually be lost due to ablation. The major cause of ablation is melting due to solar radiation increasing the surface temperature of the ice (Raymond 1980) although sublimation occurs as well (Souchez and Lorrain 1991). This process, mass balance, results in distinct zones forming on the glacier: the zone of accumulation and the zone of ablation (Fig. 2). Whether or not the glacier advances or retreats

depends on this balance between the two zones: if the balance shifts towards the zone of accumulation the glacier will advance, whereas a shift towards ablation will result in retreat (Pidwirny 2006).

Cryoconite holes are an excellent habitat for microbes on the glacier surface. They are relatively shallow holes in the surface ice of the ablation zone that tend to be filled with water in the summer and frozen in the winter (Wharton et al. 1985). Sediment and organic matter are deposited on the surface of glaciers by wind and snow, and, since they are darker than the reflective ice surrounding them, they absorb more solar energy than the ice. This results in melting of the ice and the formation of shallow holes (Wharton et al. 1985).

Although microbes can be found on the surfaces of glaciers (Sawstrom et al. 2002; Stibal et al. 2006) and in the snow cover (Amato et al. 2007; Liu Yongqin et al. 2006), those found within and beneath glaciers are most relevant to this thesis. As glaciers move over the ground they pick up sediment and biomass that becomes incorporated into the basal ice (the sediment rich bottom layer of a glacier, formed by freezing at the bed of a glacier). As a result, it is in the basal ice where the highest concentrations of microbes can be found (Sharp et al. 1999). Microbes can also be carried into a glacier (either basal ice or the clean ice above it) through surface melt water (Fig. 2). In the zone of accumulation the melting water moves down through the porous firn until it reaches the ice bed. At this point the water cannot penetrate the ice so it builds up to form a layer of water until it reaches a crevasse. The water is then able to flow down to the glacier bed

where it moves rapidly through subglacial channels. During seasons with less surface melting the water forms a distributed drainage system with a longer residence time beneath the glacier. If the water does not encounter a crevasse it may flow down slope into the ablation zone where it can drain off the end of the glacier or down another crevasse or moulin (a shaft through the ice) (Fountain and Walder 1998).

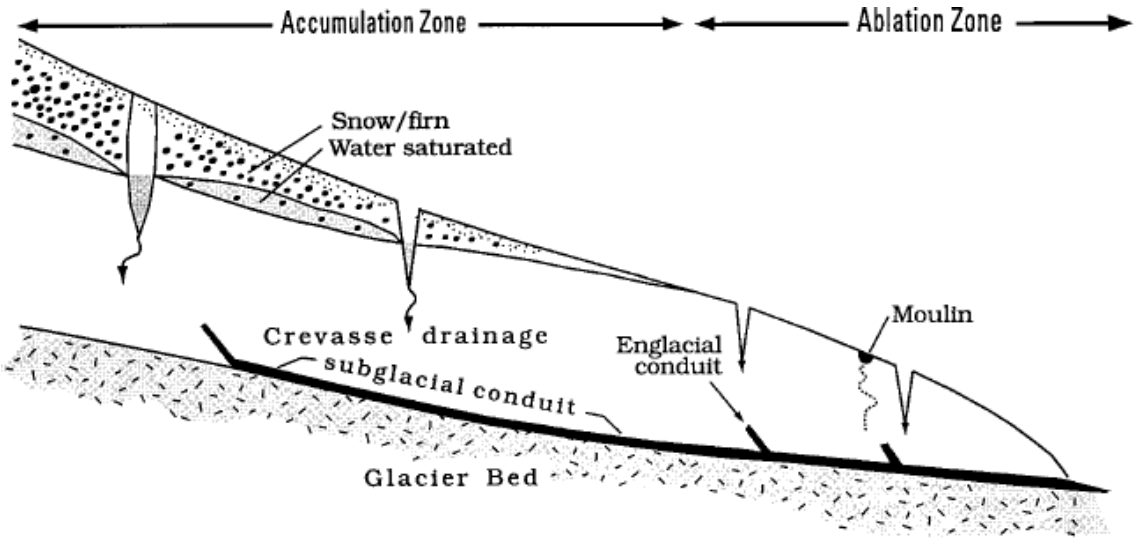

Figure 1-2: Schematic of mass balance and melt water drainage on a temperate glacier. Deposition of snow results in the build up of firn in the Accumulation Zone. As the pressure increases with depth the firn turns to ice. As the ice flows downslope it enters the Ablation Zone, where mass is lost due to melting and sublimation. Water in the Accumulation Zone moves down through firn, where it pools on the ice and either drains down through crevasses or flows down to the Ablation Zone. Water in the Ablation Zone can run off the end of the glacier or drain through crevasses and moulins (vertical shafts) (Fountain and Walder 1998 © American Geophysical Union 1998).

1.3 Psychrophiles and Psychrotolerant Microbes

Cold environments are prevalent throughout the earth and, as a result, many microbes have evolved to take advantage of this. There are two categories for

microbes living in cold environments: psychrophiles and psychrotolerant microbes. Psychrophiles require cold conditions to survive and have a maximum growth temperature of ≤ 20°C and an optimal growth temperature of ≤ 15°C (Morita 1975). As the name implies, psychrotolerant microbes do not require cold conditions, but are able to grow and reproduce in cold environments. There is not a precise definition for psychrotolerant microbes, as there is for psychrophiles, but it is generally considered that a microbe is psychrotolerant if its optimum growth temperature is above 15°C yet it can still grow at 0°C (Morita 1975). Psychrotolerant microbes comprise the majority of the bacteria in subglacial environments described to date (Cheng and Foght 2007; Foght et al. 2004; Gaidos et al. 2009; Xiang et al. 2005) although many psychrophiles have been discovered as well (e.g. Edwards et al. 2003; Gaidos et al. 2009; Rodrigues et al. 2006). The preponderance of psychrotolerant microbes, relative to psychrophiles, in an environment that is permanently below 15°C may seem counterintuitive, but is probably the result of large numbers of allochthonous microbes incorporated into the ice through snow and wind deposition.

Psychrophiles have developed a number of adaptations to help them survive in permanently cold environments. One of the least studied strategies for survival in cold environments is the production of extracellular polymeric substances (EPS), which will be discussed in greater detail below. Differential expression and adaptation of proteins and enzymes to the cold are important survival strategies. *Exiguobacterium sibiricum*, a bacterium isolated from Siberian permafrost (Vishnivetskaya et al. 2000), contains at least 39 cold acclimation proteins

preferentially or uniquely expressed at 4°C, compared to 25°C (Qiu et al. 2006). Unsurprisingly, this includes proteins described as cold shock proteins in *E. coli*, however heat shock proteins were also overexpressed at 4°C, suggesting that protein folding is problematic at low temperatures (Qiu et al. 2006).

Most biochemical reactions are slowed with a decrease in temperature. To compensate for this, cold-adapted proteins follow two main strategies: increasing both the specific activity (k_{cat}) and the dissociation constant (K_m), which results in an increased reaction rate but decreased substrate affinity, or improving the k_{cat}/K_m ratio, which increases both the rate of the reaction and the substrate specificity (Georlette et al. 2004). An increase in k_{cat} is largely a result of efficient lowering of activation energy for the reaction (Georlette et al. 2004). However, even these cold-adapted enzymes are most efficient at temperatures near the maximum growth range of psychrophiles, if not at temperatures as high as mesophilic enzymes. For instance, an extracellular aminopeptidase from *Colwellia psychrerythrea* was able to function at -1°C but had an optimal k_{cat}/K_m ratio at 19°C (Huston et al. 2004). However, this is lower than the usual optimum for extracellular proteases, between 30 and 60°C (Huston et al. 2004).

Another problem that microbes must overcome in order to grow at low temperatures is a decrease in membrane fluidity. Rigidity of the lipid membrane can result in a loss of permeability to ions (Morgan-Kiss et al. 2006). Increasing the proportion of polyunsaturated fatty acids in the membrane is the primary method for maintaining membrane fluidity at low temperatures (Morgan-Kiss et

al. 2006). *Exiguobacterium* sp. *255-15* and *Psychrobacter* sp. *273-4* both show an increase in the percentage of unsaturated fatty acids in their membranes at 4°C, relative to 24°C. However, this is followed by a decrease in unsaturated fatty acids at -2.5°C, which is not understood (Ponder et al. 2005).

One of the most interesting questions raised by the discovery of viable microbes in ice is whether the microbes are metabolically active within the ice, or if they are simply dormant. It is well known that some microbes are able to grow at sub-zero temperatures in liquid medium or brine (Bakermans et al. 2003); (Breezee et al. 2004) and *Colwellia psychrerythrea* is even motile at -10°C (Junge et al. 2003), but there are few studies examining microbial activity in solid ice. It has been calculated that, in theory, metabolism can occur at least down to -40°C (Price and Sowers 2004). Junge et al. (2004) have indirectly detected actively respiring cells in sea ice incubated within the ice sheet, and have also detected the incorporation of leucine into protein at temperatures between -1 and -20°C under laboratory conditions (Junge et al. 2006).

It is generally accepted that liquid water is a necessity for life. Veins of liquid water form in the spaces where three ice crystals meet, forming a network throughout the ice (Fig. 3) and it has recently been suggested that microbes could potentially live within these liquid veins (Price 2000). The question then becomes: do bacteria move to these veins as ice freezes or are they embedded in the crystals? Mader et al. (2006) have shown that almost 100% of polystyrene beads 1 µm in diameter (used as model cells), and approximately 80% with a

diameter of 1.9 µm, are excluded to the veins as water freezes (Fig. 3A). This is similar to the size of most microbes and, indeed, vegetative cells of *Clostridium vincentii* were also shown to be excluded to veins (Fig. 3B) (Mader et al. 2006). Although the above experiment was performed under laboratory conditions, exclusion of bacteria to veins has been observed in sea ice (Junge et al. 2004).

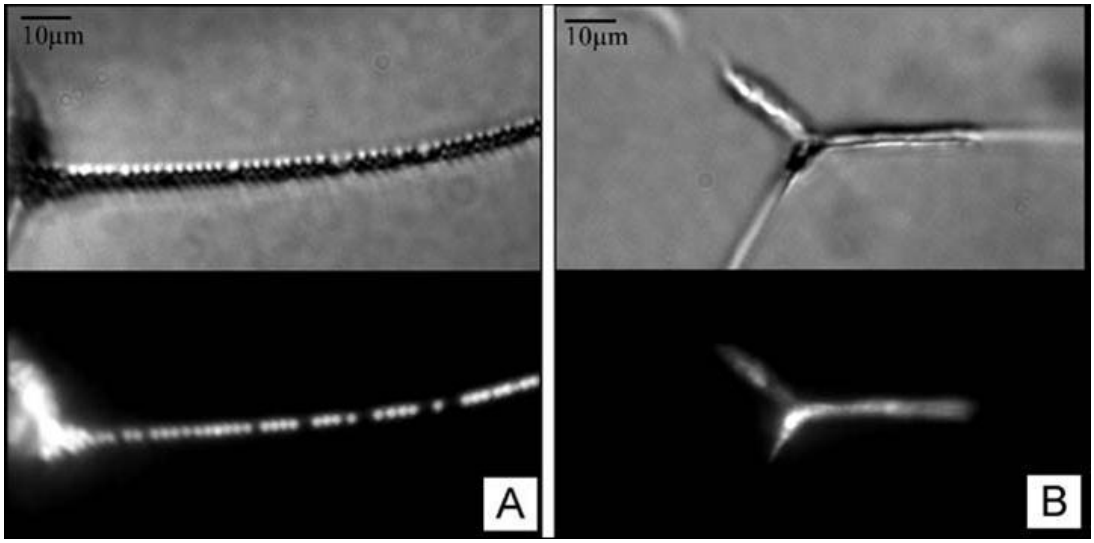

Figure 1-3: Light (top) and fluorescence (bottom) microscopy of fluorescent polystyrene beads (A) and acridine orange stained *Clostridium vincentii* (B) that have been excluded to the veins between ice crystals (Mader et al. 2006 © Geological Society of America 2006).

Even though microbes can be found in the veins they still require a source of nutrients. The dilute ice above the basal ice in glaciers is considered to be low in nutrients. However, although that may be the case for bulk ice, it does not appear to be so in the veins. As water freezes, not only insoluble impurities are excluded to the veins, but soluble impurities also concentrate in the veins (Price 2000). Based on typical bulk glacial chemical concentrations (of formate, acetate, oxalate

and nitrate, for example), Mader et al. (2006) calculated that the vein solution would be concentrated by a factor of 10^4-10^5, resulting in concentrations similar to those used in rich microbial growth media. However, because ionic strength increases as temperature decreases, the salt tolerance of an organism will affect its ability to metabolize within the ice veins (Bakermans and Skidmore 2011). Taken together, all of the above evidence suggests that it is certainly possible, and even likely, that microbial metabolism can occur within ice.

1.4 Microbial Diversity

1.4.1 Approaches to Studying Microbial Diversity in Cold Environments

To understand a biological system, it is necessary to know what organisms are present and in what numbers they are present. There are two possible approaches to this problem: culture or molecular methods. I will begin by outlining what has been learned about cold-tolerant microbes, especially from glaciers, with enrichment and cultivation, followed by the results from molecular investigations.

Many bacteria have been isolated from cold environments, such as permafrost (Vishnivetskaya et al. 2000), the deep sea (Edwards et al. 2003), sea ice (Nichols et al. 2005a), and permanently frozen lakes (Karr et al. 2005). There have been fewer attempts to cultivate microbes from glaciers, which tend to have lower concentrations of cells. The search for viable microbes in glaciers has focused mainly on the subglacial environment, such as basal ice and meltwater, although there have also been a number of studies of glacier surfaces (e.g. Amato et al.

2007). Microbes have been successfully cultivated from both polar (Miteva and Brenchley 2005; Sheridan et al. 2003; Skidmore et al. 2000) and alpine glaciers (Foght et al. 2004) but the majority of diversity studies now use molecular methods for culture-independent identification. Some microbes require specific growth conditions and micronutrients to grow and it is not possible to accommodate all of these requirements in a laboratory setting. This results in only a small proportion of the species present in a sample growing during cultivation. Enrichment is useful for encouraging the growth of a desired type of microbe, but by its very design puts a heavy bias on the observed species and is thus not sufficient for general surveys of diversity. In spite of these drawbacks, cultivation is useful because it allows the confirmation the metabolic potential within a sample.

Molecular screening methods are very useful for detecting a broader range of microbes. Using polymerase chain reaction (PCR) of the 16S rRNA gene, which is very conserved, it is possible to detect organisms without having to culture them. Of course, there are some drawbacks to molecular techniques. The major problem, especially in extreme environments like glaciers, is that it is not possible to determine if the sequences you have amplified are from active microbes, or merely microbes that are pulled into the basal ice along with sediment, or have been introduced into the environment as firn is compacted into ice. This problem can be compounded when using high throughput sequencing methods that can generate tens of thousands of sequences. Samples from multiple time points, varied environments, or with different treatments can be compared using rapid

methods of screening diversity, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993; Webster et al. 2004) or terminal restriction fragment length polymorphism (T-RFLP) (Bhatia et al. 2006). It is also possible to generate phylogenetic trees using amplified ribosomal DNA restriction analysis (ARDRA) (Heyndrickx et al. 1996; Vaneechoutte et al. 1992).

1.4.2 Microbial Diversity of Glacial Environments

On glacier surfaces, microbes are most abundant in cryoconite holes, where photosynthetic cyanobacteria dominate (Stibal et al. 2006). However, in the subglacial environment there is no light, and often little oxygen (Tranter et al. 2005). Indeed, anaerobic bacteria and archaea have been found beneath arctic glaciers, in addition to aerobes (Sheridan et al. 2003; Skidmore et al. 2000). It is not possible to describe the microbial community structure of all glaciers here because, as with other types of environments, the conditions between glaciers, and even within a glacier, can vary greatly. Skidmore et al. (2005) compared the subglacial microbial communities of Bench Glacier, Alaska, and John Evans Glacier, Nunavut and found pronounced differences in the groups of bacteria present in the communities. The microbial community of Bench Glacier included bacteria related to genera involved in iron and/or sulfur cycling, such as *Gallionella* and *Thiobacillus*, whereas John Evans Glacier showed no evidence of these microbes (Skidmore et al. 2005). It is possible that the community composition of each glacier is influenced by the differences in bedrock beneath

the glaciers (Skidmore et al. 2005). Even when studying only one glacier there are differences in bacterial communities depending on what part of the glacier is being examined, for example, subglacial, supraglacial, and proglacial communities (Bhatia et al. 2006). There can also be differences in the microbes present at different depths in the same ice core, potentially reflecting deposition history and/or nutrient availability (Xiang et al. 2005).

Trends can be observed in the groups of bacteria found in various glacial environments. Thus far, members of the *Proteobacteria*, especially α-, β-, and γ-*Proteobacteria,* have been found at almost all sites (Battin et al. 2001; Cheng and Foght 2007; Foght et al. 2004; Priscu et al. 1999; Sheridan et al. 2003; Skidmore et al. 2005; Xiang et al. 2005). It is also common to observe members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (now classified within the phylum *Bacteroidetes*), although not usually in such abundance as the *Proteobacteria* (Battin et al. 2001; Foght et al. 2004; Sheridan et al. 2003; Simon et al. 2009a; Skidmore et al. 2005; Xiang et al. 2005; Yde et al. 2010). Interestingly, high throughput sequencing and clone libraries from anoxic bottom waters from a subglacial volcanic lake showed higher numbers of *Clostridia* (~5900 reads, 141 clones) than *Bacteroidetes* (~760 reads, 24 clones) or *Proteobacteria* (~650 reads, 97 clones), however the trend was reversed when samples were observed using Fluorescent In Situ Hybridization (FISH) (10%, 27% and 64%, respectively) (Gaidos et al. 2009). Unfortunately, the molecular techniques used in many of these studies are based on sequencing of 16S rRNA genes and can only be used to infer metabolic potential. Metagenomics offers an

alternative approach by utilizing all of the DNA available in a sample, including functional genes, to determine the diversity and metabolic potential of a glacial microbial community (Simon et al. 2009a). Metagenomic analysis of temperate glacial ice revealed a community dominated by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, based on 16S rRNA genes, as well as functional genes indicating autotrophy, nitrate reduction and psychrophilic adaptations (Simon et al. 2009a). Unfortunately, to date, there are no other publications utilizing this technique to analyze microbial communities in glacial environments, although cold-environment metagenomes have been used to probe for novel genes (Latha et al. 2009; Simon et al. 2009b; Zhang Yuhong et al. 2009).

1.4.3 Autotrophic microbes

Most studies of microbes in glaciers have focused on diversity and cultivation (e.g. Bhatia et al. 2006; Cheng and Foght 2007; Foght et al. 2004; Skidmore et al. 2000), however little is known about the community structure of subglacial microbial communities. To date only heterotrophic bacteria have been isolated from subglacial environments, excluding subglacial lakes, but molecular techniques have identified bacteria related to *Gallionella ferruginia* and *Thiobacillus* spp., which are autotrophic, in Bench Glacier, Alaska (Skidmore et al. 2005). Methanogenesis has also been demonstrated from enrichments of subglacial sediment, demonstrating the potential for autotrophy (Boyd et al. 2010). Studies of microbial communities in subglacial lakes have provided definitive evidence of autotrophic potential. Although it has not been shown *in situ*, incorporation of $H^{14}CO_3$ by microbes in water and ice samples, from a

subglacial volcanic crater lake, was demonstrated at near *in situ* conditions (Gaidos et al. 2004). Clone libraries of subglacial brine from Blood Falls, Antarctica, were dominated by the autotrophic sulfur oxidizer *Thiomicrospira arctica* (99% sequence similarity based on 1420 bp of the 16S rRNA gene) (Mikucki and Priscu 2007). *Shewanella frigidimarina* (99% sequence similarity based on 1400 bp) was cultivated from "autotrophic enrichments" of the brine (Mikucki and Priscu 2007), but inspection of the cited recipe reveals the presence of fixed carbon in the medium (Boone et al. 1989), so it is not a true autotrophic enrichment.

1.5 Nutrient Cycles

Elements essential to life include nitrogen, sulfur and carbon. However, these elements can exist in numerous oxidation states, some of which are not in a state accessible to all microorganisms (Kroneck 2005), whether due to insolubility (Fe^{3+} at pH~7) or complexation in an inorganic compound (CO_2, FeS_2, N_2). Nutrient cycling is the process through which the elements change oxidation states and become available to new groups of organisms. Many of these conversions can occur spontaneously but depending on the environmental conditions, the reaction may proceed very slowly. In these situations the reactions may be catalyzed by microorganisms (Lowenstam 1981). Many of these reactions occur during metabolism by an organism, with the waste products becoming available to other organisms (Kroneck 2005).

Three major elements are N, S, and C. However, because each of these cycles warrants its own comprehensive review I will focus mostly on biologically mediated cycling in icy environments, especially with respect to geochemical cycles. Where possible I will discuss what is known of the cycle in glacial systems but there is relatively little research available for that environment.

1.5.1 Nitrogen Cycle

Although nitrogen is not as abundant in biomass as carbon, it is a major component of amino acids (and therefore proteins) and nucleic acids (Rudolf and Kroneck 2005). Little is known about biological cycling of nitrogen in glaciers and most studies have focused on NO_3^- and denitrification. In alpine and sub-polar glaciers, snow melt is the primary input of NO_3^- to the subglacial environment (Tranter et al. 1994). Interestingly, the rapid decline in the NO_3^- concentration of glacial runoff was one of the first indications that microbes may live beneath glaciers (Tranter et al. 1994). Since then, evidence for denitrification has been found in several glaciers at Svalbard (Hodson et al. 2005) and depletion of NO_3^- due to anaerobic nitrate reducing bacteria has been observed in incubated basal ice from John Evans Glacier, Nunavut (Skidmore et al. 2000), while both nitrification and nitrate reduction were observed in microcosms of subglacial sediment from Robertson Glacier, Alberta (Boyd et al. 2011). Amendment of anoxic basal ice sediment slurries from the Greenland ice sheet with $^{15}N\text{-}NO_3^-$ produced $^{15}N_2$, demonstrating the presence of an active population of denitrifiers (Yde et al. 2010). In June 1999 meteorological conditions led to increased air

pollution travelling to Svalbard, resulting in a record nitrogen deposition event when the catchment was frozen. Interestingly, microbial assimilation of NO_3^- and NH_4^+ still occurred, suggesting that subglacial microbial activity can occur even when terrestrial soil systems are unresponsive (Hodson et al. 2010). To date, nitrogen fixation has not been demonstrated in subglacial microbial communities, although *nifH* genes were detected in sediment from a temperate glacier (Boyd et al. 2011) and nitrogen fixing bacteria were isolated from subglacial sediments in New Zealand (Foght et al. 2004).

1.5.2 Sulfur Cycle

The sulfur cycle is known to occur in cold environments. Sulfate reducing bacteria (SRB) have been found in frozen Lake Fryxell, Antarctica (Karr et al. 2005), microbial sulfate reduction has been observed in anoxic glacial beds at Svalbard (Wadham et al. 2004), and SO_4^{2-} depletion due to biotic processes was observed in subglacial samples from John Evans Glacier, Nunavut (Skidmore et al. 2000). Sulfur springs have also been found on Ellesmere Island with sulfur-oxidizing bacteria oxidizing H_2S to S^o (Grasby et al. 2003).

Another important sulfur compound is dimethylsulfide (DMS), which is present in glacial melt water ponds in Antarctica (DeMora et al. 1996) and its distinctive odour has also been detected in some Antarctic glacial ice (Sharp 2007). DMS is produced through the catabolism of dimethylsulfoniopropionate (DMSP) (Todd et al. 2007), an osmoprotectant in many marine algae, and is the

primary volatile sulfur compound in the open ocean (Andreae 1990). In anaerobic marine sediments SRB and methanogens are responsible for the removal of DMS, with inhibition of methanogenesis resulting in a 20% decrease in DMS consumption and inhibition of sulfate reduction resulting in an 80% decrease in DMS consumption (Kiene 1988). However, in surface waters, abiotic photochemical decomposition is a major factor (Andreae 1990). The concentration of DMSP found in glacial melt ponds is low compared to the open ocean, possibly due to the relatively low productivity of algae, or accelerated bacterial DMSP degradation (DeMora et al. 1996).

Sulfur found in minerals such as pyrite (FeS_2) can be used as an electron donor by some chemolithotrophic microbes, a process important in weathering of rocks (Konhauser 2007). For many years it was thought that any subglacial weathering was due solely to inorganic reactions but, as we have seen in this review, the subglacial environment contains many active microbes. This has important ramifications for subglacial weathering. If microbes are oxidizing sulfides and carbon (discussed below) beneath the glaciers, this could be the dominant force in subglacial weathering (Sharp et al. 1999). Later research has suggested that the reduction of Fe(III) and oxidation of pyrite by microbes could have a profound effect on the rate of weathering in the subglacial environment, as opposed to weathering in most other earth surface environments, which is driven by atmospheric CO_2 (Tranter et al. 2002). Microbial oxidation of organic matter beneath Taylor Glacier, Antarctica, appears to be coupled to sulfur cycling with

Fe(III) as the terminal acceptor, where SO_4^{2-} is reduced to S^0 then re-oxidized to SO_4^{2-} with reduction of Fe(III) (Mikucki et al. 2009).

1.5.5 Carbon Cycle

As a component of all organic molecules and the source of energy for many organisms, carbon is the most essential element for life. Carbon is ubiquitous in the environment, but one of the largest stores of carbon is in the oceans in the form of dissolved organic carbon (DOC). DOC consists, in large part, of polysaccharides (~50% in surface water, ~25% in deep waters) and other carbohydrates (Benner et al. 1992), many of which are formed by microbes (Alldredge 2000). Polysaccharides can also be found in high concentrations in both sea ice (Krembs et al. 2002) and glaciers (Barker et al. 2006; Lafreniere and Sharp 2004). Much of the DOC in oceans and sea ice consists of EPS (Benner et al. 1992; Krembs et al. 2002), however although much of the DOC in glaciers is of microbial origin it is not known whether it is derived from EPS (Barker et al. 2006). This will be discussed in more detail in the following section.

Much of the research regarding microbially produced polysaccharides in ice has focused on sea ice. During the winter months there is very little microbial activity and primary production begins in the spring with sea ice algae at the interface between ice and water (Rysgaard and Nielsen 2006). Once the sea ice begins to break up, the pelagic organisms are exposed to sunlight and begin primary production (Rysgaard and Nielsen 2006). Over the course of the winter

large amounts of EPS also accumulate in the sea ice and are released when the ice thaws in the summer (Krembs et al. 2002). Interestingly, diatom EPS appears to be produced in the sea ice itself. Sea ice segments incubated at -5°C, -15°C, and -25°C for 3 – 14 months showed significant increases in EPS content.

There is such a wide variety of terrestrial systems that it is not feasible to cover carbon cycling for all of them in this review, so I will focus on cold ecosystems, particularly Antarctic Dry Valleys. Although the Dry Valleys are extremely cold and dry systems, they have functioning microbial ecosystems and carbon cycling does occur (Burkins et al. 2001). Due to the lack of higher plants and vegetation, carbon turnover and production of CO_2 in Taylor Valley, Antarctic soils are due to microbial processes and inorganic reactions (Burkins et al. 2001). The soil closest to the lakes in the Dry Valleys has the most carbon, the lowest turnover time and the highest rate of respiration, which is consistent with higher soil moisture because desiccation would be less of a problem for the microbes (Elberling et al. 2006). When amended with carbon, soil from the lake edge produced more than twice the amount of CO_2 per gram of soil than soil from other regions of a dry valley (Hopkins et al. 2006). CO_2 is not the only product of carbon mineralization in Dry Valley soils. In soils near water CH_4 emission has been observed at rates similar to those of temperate and high latitude wetlands in summer (Gregorich et al. 2006).

Very little is known about biological carbon cycling in glaciers. Glaciers contain large amounts of DOC, often derived from surrounding vegetation or, in

areas without vegetation, microbial activity (Lafreniere and Sharp 2004). Cryoconite holes are also an important localized source of primary production (Sawstrom et al. 2002). In addition to the organic carbon input from the glacier surface, the bed of the glacier may be a significant source of organic carbon derived from old plant materials (Hodson et al. 2008).

Chemical weathering beneath glaciers is also important in carbon cycling over glacial-interglacial time scales and subglacial microbial activity has the potential to influence these processes (Sharp et al. 1999). Many minerals, such as limestone, contain large amounts of carbonate, which is released through chemical and microbial weathering and is a significant carbon source for lakes and rivers (Konhauser 2007), and presumably any glaciers overlying bed rock with high carbonate content. It is estimated that microbially generated CO_2 and sulfide oxidation drive mineral dissolution and may be responsible for up to 80% of the solute flux in a high Arctic glacier (Wadham et al. 2010), although this likely depends on conditions that vary between glaciers.

Both CO_2 and CH_4 are important greenhouse gases and have a large influence on climate change. Permafrost is known to contain large amounts of organic carbon and has the potential to produce CO_2 and CH_4 as it begins to thaw (Rodionow et al. 2006). Although there has been less attention paid to glaciers, because they cover a large portion of the earth they have the potential to be a major source of CO_2, CH_4, and organic carbon that could be released as global temperatures rise. It is estimated that the subglacial reservoir of organic carbon

contains 63 Pg C that is available for conversion to CH_4 over a glacial cycle (Wadham et al. 2008). This demonstrates the importance of factoring the subglacial environment into considerations of global carbon cycling.

1.6 Extracellular Polymeric Substances (EPS)

Extracellular polymeric substances are secreted by many microbes, including bacteria, archaea and algae. Although EPS consists primarily of high molecular weight polysaccharides, it can also contain proteins, nucleic acids and lipids. However, the relative abundance of these components can be quite variable (Table 1). Most studies of EPS have concentrated on the exopolysaccharides, which can vary in both quantity and composition depending on the producing microbe's species and strain (e.g. Nichols et al. 2005a) or growth conditions (Corsaro et al. 2004; Nichols et al. 2005b). Unfortunately, because most studies have focused on the polysaccharide component there is little information available regarding the rest of the EPS, especially the protein.

EPS can be closely associated with a microbe (as capsule) or loosely attached and easily released into the environment (as slime) (Sutherland 1982; Whitfield 1988). As a result, much of the organic matter found in ocean surface waters is composed of EPS from both bacterial and eukaryotic phytoplankton (Benner et al. 1992; McCarthy et al. 1996). Particles consistent with EPS have been found in the air 27 m above sea level in the Arctic ocean (Leck and Bigg 2005) and are abundant in Arctic sea ice (Krembs et al. 2002). In addition to being present in

marine environments as DOC, EPS can also exist as particulate organic matter. EPS in this form tends to form aggregates with other matter, such as feces, phytoplankton, bacteria and other particles that may be present. Eventually these aggregates reach a size (>50μm) where they begin to sink to the bottom of the ocean through sedimentation (Bhaskar and Bhosle 2005). Sedimentation is a vital part of carbon cycling within marine environments as it transfers atmospheric carbon to the deep ocean where it becomes available to deep sea organisms (Alldredge 2000; Bhaskar and Bhosle 2005).

The ubiquity of EPS in the marine environment is not important solely in open water. Krembs et al. (2002) calculated the carbon content in ice from the Chukchi Sea, Alaska that was from EPS and found a range from 0.6 to 5.4 g C m^{-2}, varying with both time and depth in ice. At these concentrations melting sea ice may play an important role in the Arctic marine carbon budget (Krembs et al. 2002). Although the role of EPS in carbon cycling has been extensively studied in the open ocean and recently in sea ice, to date there have been no studies of the role of EPS in glacial carbon cycles. There have, however, been some studies of dissolved organic carbon (DOC) in glacial systems, and much of the DOC appears microbial in origin, comprising polysaccharides and proteins (Barker et al. 2006; Lafreniere and Sharp 2004). This suggests that the source of the DOC may be EPS, but to date there have been no studies to confirm this.

1.6.1 The Role of EPS in Microbial Survival and Growth

In addition to carbon cycling, it has been proposed that EPS plays a role in the survival and growth of microbes. One of the major functions of EPS is to aid in adhesion of cells to surfaces (Cammarota and Sant'Anna 1998; Tsuneda et al. 2003). Once the bacteria have attached to a surface they can begin to form a biofilm, a process for which EPS is essential (Cammarota and Sant'Anna 1998; de los Rios et al. 2004). Biofilms are difficult to penetrate or remove, offering protection for the microbial community. A major advantage of biofilms for communities is that they provide relatively close contact with both surfaces and other microbes, and stable environments that encourage synergistic relationships (Flemming and Wingender 2001). Microenvironments form within the biofilm (Hunter and Beveridge 2005) allowing organisms with different environmental requirements (aerobic, anaerobic, pH, etc.) to coexist. Complex microbial mats have been found in ice-covered Antarctic lakes (Van Trappen et al. 2002) and mixed communities of phototrophs and heterotrophs have been observed in biofilms in streams of glacial meltwater. The biofilms appear to prolong storage of substrates accumulated from the water and the byproducts of photosynthesis, which can later be used by the heterotrophs (Battin et al. 2001), however it is unlikely that biofilms of this complexity exist in subglacial streams due to lower temperatures and cell abundance in subglacial sediments (Battin et al. 2001; Foght et al. 2004; Lanoil et al. 2009).

Close contact with surfaces is also necessary for lithoautotrophs that obtain electrons from minerals such as iron or sulfur. These bacteria attach to a mineral surface and begin to dissolve the metal sulfides in a process called leaching (Sand

et al. 1995). The microenvironments within the biofilm allow the bacteria to increase acidity next to the mineral surface, resulting in dissolution of the minerals (Sand et al. 1995). Iron can also adsorb to the negatively charged EPS and serve as an electron shuttle, increasing the rate of mineral dissolution (Sand et al. 1995).

Microbes that are not attached to a surface can also have an indirect effect on mineral dissolution because the binding of dissolved metals to their EPS lowers the saturation state of the solution (Welch et al. 1999). Although it has not been investigated, it has been suggested that the polyanionic nature of EPS may allow it to play a role in metal scavenging in metal limited environments (Mancuso Nichols et al. 2004). The basal ice of glaciers is rich in sediment, and thus likely rich in metals, but the ice above it is relatively dilute and free of sediment. In such environments metal scavenging may be vital to survival. In addition to its potential for metal scavenging, the ability of EPS to bind metals also allows the bacteria to grow in areas where the concentration of heavy metals would normally be inhibitory (Aislabie and Loutit 1986).

These properties are not only important for the survival of the bacteria, but may also be important in metal cycling (e.g. Aislabie and Loutit 1986; Dean et al. 1981) and result in increased weathering of rocks (Welch et al. 1999), which is of great interest in glaciology. Glaciers have covered much of the earth for long periods of time and it has traditionally been thought that biological weathering processes stopped during this time in areas overlain by glaciers (Gibbs and Kump

1994). However, with the relatively recent realization that active microbes exist in subglacial waters it seems likely that microbes may have contributed to weathering during these periods. Understanding chemical weathering of modern rocks will thus lead to a better understanding of geological history.

EPS is not only important in attachment to surfaces, but also in forming aggregates of cells (Liu et al. 2004). Aggregation provides benefits similar to biofilms, such as protection from environmental changes, storage of substrates, and close proximity to other microbes, allowing the development of synergistic relationships. However, aggregation does not require a surface to which the bacteria can attach, which is advantageous in water or ice with low amounts of sediment. A 3-fold increase in EPS production was observed in *Pseudomonas* sp. 30-3, isolated from Antarctic soils, when incubated at 22 or 4°C in trypticase soy broth, relative to EPS production at 37°C (Panicker et al. 2006). A dramatic increase in aggregation was also observed at 4°C.

Unfortunately, very little is known about the role that EPS plays in survival at low temperatures (<15°C) beyond the fact that EPS production increases with decreasing temperatures (Nichols et al. 2005a; Panicker et al. 2006) and that increased EPS, or addition of EPS to a culture, appears to correspond to an increase in survival (Junge et al. 2004; Junge et al. 2006).

Other studies have also found "Ice-Active Substances" (IAS) that appear to consist of carbohydrates and proteins in similar ratios to EPS (Raymond and Fritsen 2001). IAS have been found associated with Antarctic cyanobacteria,

algae, and moss, and although they do not lower the freezing point of water, they can alter the shape of ice crystals and prevent crystals from growing (Raymond and Fritsen 2001; Raymond and Knight 2003). Much of the damage caused by freezing is due to the growth of crystals that penetrate their membranes and kill the cells. Therefore, the ability to prevent the growth of crystals is an important cryoprotective measure. It would be interesting to examine IAS further to determine if they are related to, or are a component of EPS.

Most of these studies have dealt with oceans, sea ice, or terrestrial environments. Unfortunately, to date there have not been any studies examining the role of EPS in microbial survival in the glacier environment. Due to the wide ranging roles that EPS may have in glacial systems, from nutrient cycling, to weathering, to survival of microbes, it is surprising that so little work has been conducted in this area. This has left a significant gap in our knowledge of the system that it is important to address.

1.7 Objectives and Overview of Thesis

The overarching objective of this project is to demonstrate the role(s) of EPS in microbial cryotolerance in the subglacial environment. To determine the potential roles of EPS in subglacial environments I will test two hypotheses. My first hypothesis is that production of EPS will be associated with increased cell surface ligand variety and density, presented in Chapter 2. My second hypothesis (Chapter 3) is that production of EPS will be associated with an increase in survival of a freeze-thaw cycle and that the presence of sediment will result in an

additional increase in microbial survival. Chapter 4 presents a synthesis of my research with suggestions for future research on EPS-associated changes to cell surface chemistry in a variety of psychrotolerant organisms and the potential role of EPS in microbial community resistance to freeze-thaw stress.

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2. THE ROLE OF EXTRACELLULAR POLYMERIC SUBSTANCES IN THE SURFACE CHEMICAL REACTIVITY OF *HYMENOBACTER AEROPHILUS*, A PSYCHROTOLERANT BACTERIUM[1]

2.1 Introduction

Acid-base titrations are frequently used to characterize microbial cell surface reactivity, in particular the ability of the cell to adsorb and desorb protons (e.g. Heinrich et al. 2007; Hetzer et al. 2006; Hetzer et al. 2006; Tourney et al. 2008). This ability is conferred by the presence of proton-reactive surface functional groups that are also responsible for the surface adsorption of other cations, including dissolved metals. Thus a microbe's ability to immobilize metals and influence metal transport is largely dependent on the nature of the reactive sites found at the cell-water interface, namely their concentrations and chemical affinities (in terms of equilibrium surface stability constants) for cations such as protons and metals.

Both Gram negative and Gram positive bacteria have been extensively characterized using acid-base titration to determine their reactivity with respect to geochemical processes (e.g. Haas 2004; Hong and Brown 2008; Leone et al. 2007). To date, most work has focused on mesophilic and strictly heterotrophic model organisms, however some work has also been done with cyanobacteria (Lalonde et al. 2008; Phoenix et al. 2002) and thermophiles (Heinrich et al. 2007;

[1] A version of this chapter has been published. Baker, M.G., Lalonde, S.V., Konhauser, K.O., and Foght, J.M. 2010. Appl. Environ. Microbiol. 76:102-109.

Tourney et al. 2008). While proton sorption assays provide information on surface site densities and acidity constants, a more direct assessment of a microbe's ability to interact with aqueous metals is the metal adsorption assay, where a cell's ability to adsorb metal ions from solution is measured over a range of pH. Metal adsorption assays have been used to characterize microbes from a wide variety of environments to determine their potential for bioremediation of heavy metal contamination (Hetzer et al. 2006; Komy et al. 2006; Rosales et al. 2012), their influence on geochemical cycling (Borrok et al. 2004; Gelabert et al. 2007; Moon and Peacock 2011), and their ability to serve as nucleation sites for mineral authigenesis (Benning et al. 2004; Phoenix et al. 2003). Although more than 80% of the Earth's biosphere is cold (Margesin et al. 2007), to my knowledge there have been no published studies of acid-base surface chemistry for psychrotolerant bacteria, although recently studies have been published examining metal adsorption (Zamil et al. 2008; Zhou et al. 2009).

The production of EPS can be important in mediation of environmental interactions, such as adhesion to surfaces and aggregation (Liu et al. 2004; Tsuneda et al. 2003); mineral weathering (Konhauser et al. 1994; Welch et al. 1999); microbial tolerance of toxic metals through sequestration of metal ions outside the cell (Aislabie and Loutit 1986; Clarke et al. 1997); and biomineralization (Konhauser et al. 1998). Indeed, the stability of metal-surface complexes is great enough to affect metal mobility in many aqueous systems (Fein et al. 1997), which can, in turn, affect the distribution of metals in the environment (Ledin et al. 1999).

The physical and chemical characteristics of EPS have usually been studied on cells with intact EPS or on purified EPS (Boyette et al. 2001; Braissant et al. 2007; Guibaud et al. 2005; Liu and Fang 2002; Paperi et al. 2006; Welch et al. 1999). Interestingly, few studies have compared cells with and without surface layers such as EPS (Cao et al. 2011; Phoenix et al. 2003; Tourney et al. 2008; Wei et al. 2011) despite the fact that EPS and other external layers alter the cell surface presented to the environment, potentially changing both the type and quantity of functional groups available for environmental interactions. Accordingly, the purpose of this study was to determine the changes in cell surface reactivity resulting from the production of EPS by *Hymenobacter aerophilus*, a psychrotolerant bacterium. Acid-base titrations and cadmium adsorption assays were used to compare the number and type of functional groups on the surface of bacterial cells presenting intact EPS and those from which EPS had been removed mechanically.

2.2 Materials and Methods

2.2.1 Growth conditions and preparation of cells. *Hymenobacter aerophilus* (DSMZ 13606 EU155008) is a psychrotolerant aerobic, rod-shaped, gram-negative bacterium belonging to the division *Bacteroidetes* (Buczolits et al. 2002). Cultures were incubated in R2 broth (per L: yeast extract 0.5 g; proteose peptone no. 3 0.5 g; casamino acids 0.5 g; dextrose 0.5 g; soluble starch 0.5 g; sodium pyruvate 0.3 g; K_2HPO_4 0.3 g; anhydrous $MgSO_4$ 0.05 g) for 4 d at 14°C with

shaking, transferred to fresh broth with 1/50 dilution and incubated for a further 4 d. Stock cultures were maintained at 4°C on R2A agar (Difco).

Cells with intact EPS for titration analysis (WC) were harvested and washed three times in 0.01 M $NaNO_3$ by centrifugation at 20,000 × *g* for 20 min at 4°C. Cells stripped of EPS (SC) were sonicated for 1 min at 20 W using a Branson Sonifier 450 probe sonicator (Branson Ultrasonics), then centrifuged at 30,000 × *g* for 20 min at 4°C. The supernatant was retained for extraction of EPS. After harvesting, cell pellets for use in titrations and cadmium adsorption assays were washed three times in 0.01 M $NaNO_3$ by centrifugation at 10,000 × *g* for 10 min at room temperature. India ink capsule stains were performed using phase contrast microscopy (1000x magnification) to ensure removal of EPS.

2.2.2 Extraction of EPS. Supernatant from high speed centrifugation was filtered using Millex-HA 0.45 µm cellulose ester syringe filters (Millipore). Three volumes of cold (4°C) 98% ethanol were added to the filtrate and briefly mixed by swirling. The mixture was incubated overnight at 4°C to precipitate the EPS. Sintered glass was used to filter the white precipitate, which was then dried at 37°C. The dried EPS was re-dissolved in 5 to 10 mL of distilled water, depending on the yield of EPS. Once completely dissolved, the EPS was dialyzed against distilled water for 24 h before lyophilization. The lyophilized EPS was stored at 4°C in the dark until it was used in titrations or analyzed using Fourier transform infrared spectroscopy (FT-IR).

2.2.3 Acid-base titrations. Site densities and pK_a values for proton-reactive functional groups were determined by acid-base titrations. All solutions were made with 18.2 MΩ water. After washing, 10 g of concentrated cell suspension (WC, 0.02 g dry wt.; SC, 0.06 g dry wt.) was transferred to an acid-washed 125 mL Erlenmeyer flask and brought to a final mass of 30 g with 0.01 M $NaNO_3$. The suspension was acidified to pH ~3 with 25 µL of 2 M HCl. For titration of EPS, 10 – 20 mg of lyophilized EPS was weighed and dissolved in 0.01 M $NaNO_3$. Three independent replicates of all titrations were performed on different days. Blanks without cells or EPS were run on each day that titrations were performed.

Titrations were performed from pH ~2 to pH 11 using a QC-Titrate autotitrator (Man-Tech Associates, Inc.) using 0.01 M NaOH that had been freshly purged with N_2 for 20 min to remove CO_2. NaOH (0.01 M) was variably delivered for ~0.1 pH unit increments with electrode stability criteria of <0.5 mV/s. The system was continuously purged with N_2 and stirred magnetically at room temperature. After titration, the biomass was measured by filtering the suspension through Whatman GF/C #42 filters (Whatman Inc.), which were then air dried at room temperature to constant weight. Glass pH electrodes were calibrated using commercial standards.

Linear program modeling (LPM) was used to determine ligand pK_a values and site densities, as described previously (Lalonde et al. 2008). Briefly, possible pK_a values were fixed at intervals of 0.2 units from 3 – 11 and site densities for

each pK_a value were optimized to best fit the titration data based on iterations comparing the charge excess measured versus the charge excess predicted for the potential set of ligands. Titration data were additionally modeled using FITEQL (Herbelin and Westall 1999), where a predetermined number of possible sites (from 1 to 4 in this study) are assumed, and ligand pKa values and site densities are both simultaneously optimized to best fit the data.

2.2.4 Cadmium adsorption assays. Cd adsorption assays were performed in a bulk manner using Cd-bacterial suspensions that were sampled multiple times over a sequence of pH adjustments. After washing, 0.2 – 0.3 g (wet weight) of cells were placed in an acid-washed beaker into which approximately 0.5 g of 1000 ppm cadmium solution in 2% HNO_3 (SPEX Certiprep) was added and the total mass brought to 100 g with 0.01 M $NaNO_3$. The sample was acidified to pH ~2 with one drop of concentrated trace metal grade HNO_3 (Fisher Scientific).

After equilibrating for 30 min with magnetic stirring, the pH was measured using glass electrodes and an 8 mL sample was removed. Samples were filtered through 0.22 µm nylon membrane filers (GE Water and Process Technologies) in syringe-driven 25 mm Swinnex polypropylene in-line filter holders (Millipore) into 20 mL polyethylene scintillation vials containing one drop of concentrated trace metal grade HNO_3. Samples were taken at ~1 pH unit intervals between pH 2 – 10, with 30 min to equilibrate after each adjustment of pH with 0.019 – 1.9 M NaOH. Samples were stored at 4°C until analyzed using atomic absorption spectrometry (Perkin Elmer Elan 6000 Quadrupole-ICP-MS or

Perkin Elmer 4000 AAS). Cadmium adsorption assays were performed in three independent replicates on different days.

Cd-ligand stability constants were modeled in FITEQL 4.0 (Herbelin and Westall 1999) for WC and SC cell suspensions using the best-fit FITEQL ligand model determined from titration data of each condition.

2.2.5 Fourier transform infrared spectroscopy. Lyophilized EPS was analyzed by FT-IR using a Nic-Plan FT-IR microscope attached to a Nicolet Magna 750 FT-IR spectrophotometer (Thermo Scientific). EPS was scanned over a wavenumber range of 4000 – 800 cm^{-1}.

2.3 Results

2.3.1 Acid-base titrations. *H. aerophilus* cells with intact EPS (WC) or stripped of EPS (SC) and purified EPS were titrated to determine changes in the abundance and variety of proton-reactive ligands on the cell surface due to the presence of EPS. Considerable variation in both the shape of the curve and the overall excess charge was observed between replicates for purified EPS and WC, compared to SC (Fig. 1). For SC, there was little deprotonation of surface functional groups until approximately pH 6.5 (Fig. 1c), in contrast to EPS and WC, where charge excess began to accumulate at pH 4 and 5, respectively. This is consistent with the presence of a variety of ligands with a broad range of pK_a values for WC and EPS and a lack of ligands with pK_a values below 6.5 for SC.

The steepest part of the curve for SC was between pH 6.5 and 8.5 (Fig. 1c), corresponding to the pair of ligands at pK_a 6.5 and 7.9 (Fig. 2c). It is clear from Fig. 1 that the reactivity of EPS is variable even between replicates, but the reactivity of the underlying cell surface is more consistent. Variation in the excess charge of WC and EPS may be due to slight changes in growth conditions between replicates, which were grown in separate batches and titrated on different days (Haas 2004; Lalonde et al. 2008). The excess negative charge of SC was also 10 to 20 fold lower than WC or EPS (Fig. 1).

Determination of discrete ligands through linear programming also showed similar patterns for WC and EPS titrations that differed from SC (Fig. 2). Table 1 summarizes the categories of ligands present in EPS and on the cell surface. Five distinct sites were observed for both WC (ligand classes A – E) and EPS (F – J). These sites are presumed to correspond to carboxyl (pK_a 2-6) (Cox et al. 1999), phosphoryl (pK_a 5.7 – 7.2) (Cox et al. 1999) and amine (pK_a 8-11) (Phoenix et al. 2002) groups. Removal of EPS from cells resulted in a decrease in the number of sites present on the SC surface (classes K – M). Carboxyl groups were not detected on the surface of SC, however there was a site at pK_a 9.9 that could correspond to either amine or hydroxyl groups (Cox et al. 1999). Phosphoryl groups were the most abundant ligands for all treatments. As indicated by charge excess (Fig. 1), SC showed a site density 10-fold lower than either WC or EPS (Fig. 2).

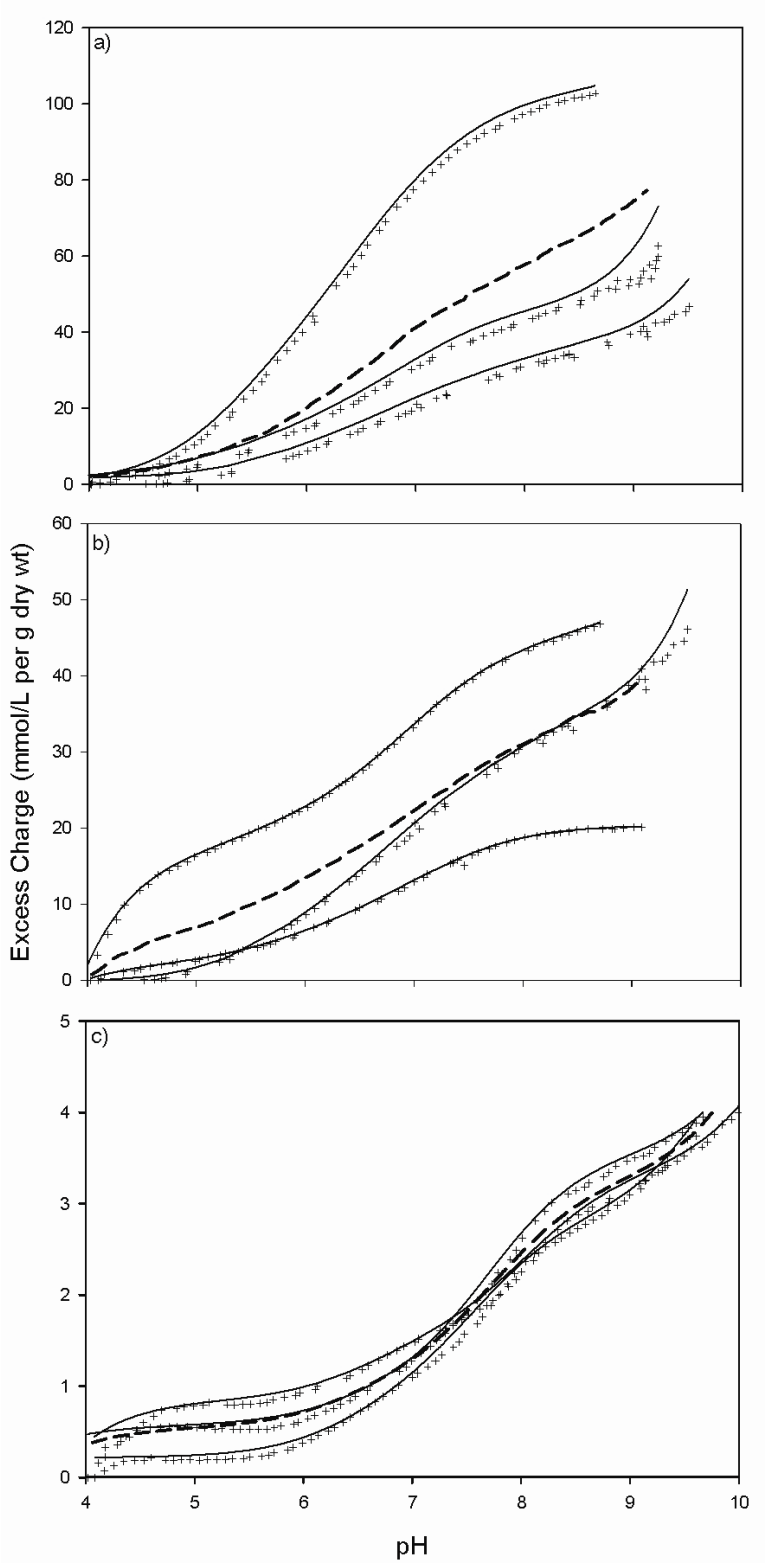

Figure 2-1: Titration excess charge (crosses), modeled excess charge (solid lines) and average excess charge (dashed lines) for replicate samples given in units of mmol/L per g dry weight of WC (a), EPS (b), SC (c).

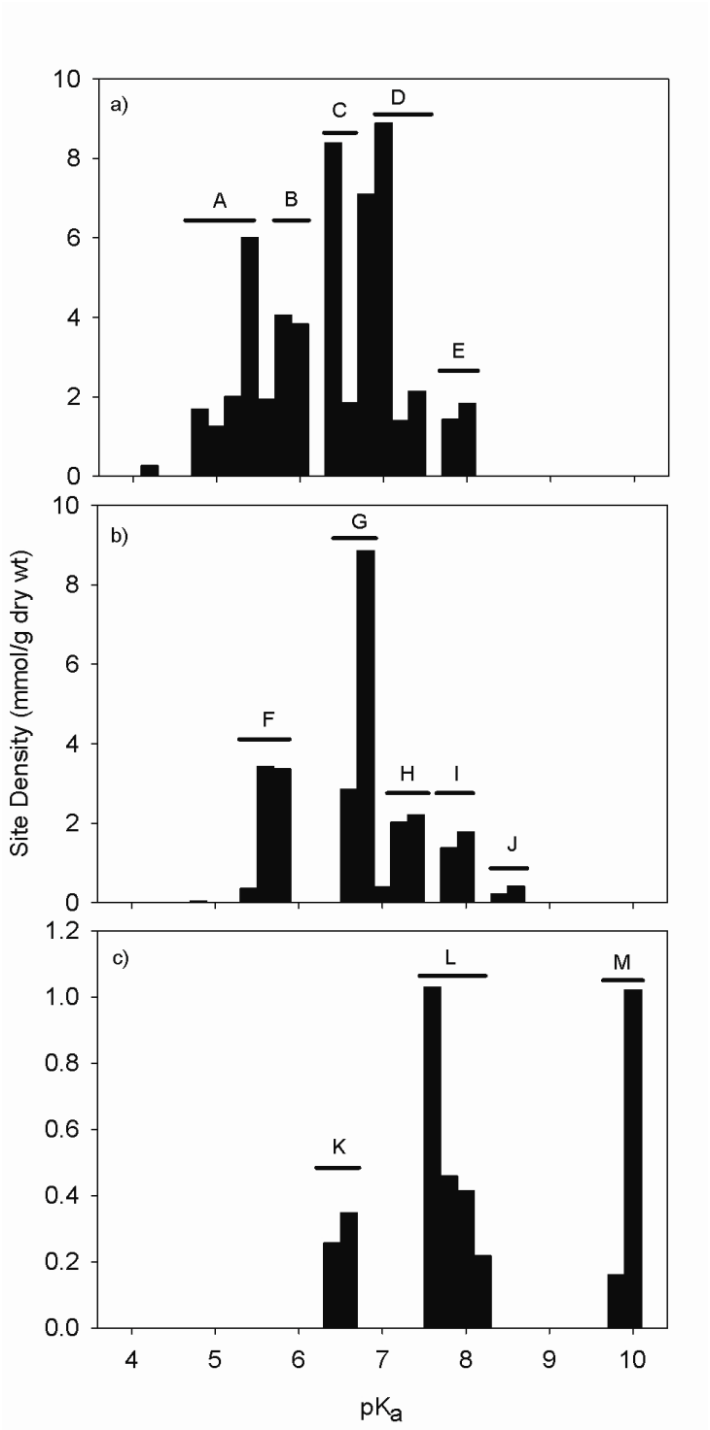

Figure 2-2: Site density (mmol/g dry weight) and pK_a values for ligands modeled in MATLAB based on titrations of WC (a), EPS (b) and SC (c). Letters A-M refer to ligand classes defined in Table 1.

Table 2-1. Summary of titration data (from Fig. 2) for cells with intact EPS (WC), purified EPS and cells stripped of EPS (SC).

Treatment	Ligand Class	Mean pK_a (SD)[a]	Mean site density, mmol/g[b] (SD).[a]	Proposed functional group represented
WC	A	5.10 (0.26)	2.74 (2.20)	carboxyl
	B	5.80 (0.20)	3.28 (1.16)	carboxyl or phosphoryl
	C	6.50 (0.14)	5.12 (4.63)	phosphoryl
	D	7.10 (0.26)	4.88 (3.68)	phosphoryl
	E	7.90 (0.14)	1.63 (0.29)	amine
EPS only	F	5.60 (0.20)	2.38 (1.75)	carboxyl
	G	6.70(0.14)	5.65 (3.96)	phosphoryl
	H	7.30 (0.14)	2.12 (0.14)	phosphoryl
	I	7.90 (0.14)	1.58 (0.28)	amine
	J	8.50 (0.14)	0.31 (0.15)	amine
SC	K	6.50 (0.14)	0.30 (0.064)	phosphoryl
	L	7.90 (0.26)	0.63 (0.34)	phosphoryl or amine
	M	9.90 (0.14)	0.59 (0.61)	amine or hydroxyl

[a] Standard deviation is indicated in parentheses.
[b] Site density is normalized to dry mass.

2.3.2 Cadmium adsorption assays. Figure 3 shows the adsorption of cadmium by WC and SC, modeled using pK_a values and concentration calculated in FITEQL (summarized in Table 2). WC replicates showed a greater range of maximum cadmium adsorption (0.53– 1.73 mmol/L per g dry wt) than SC (0.53–0.59 mmol/L per g dry wt) and adsorbed up to three times the amount of Cd than SC.

Table 2-2. pKa values, concentrations and cadmium affinity constants for ligands predicted by FITEQL.

Treatment	FITEQL predicted ligands	Mean pK_a [a]	Mean site density (mmol/g) [a, b]	Cd affinity constant [a]
WC	L_1	5.78 (0.42)	36.35 (28.93)	2.94 (0.19)
	L_2	7.21 (0.52)	24.50 (10.15)	N/A [c]
	L_3	9.58 (0.65)	53.13 (40.26)	6.20 (0.49)
SC	L_1	3.81 (0.91)	0.55 (0.34)	-0.05 (0.17)
	L_2	7.04 (0.96)	1.50 (0.49)	2.98 (0.18)
	L_3	8.51 (1.73)	1.81 (0.22)	4.19 (0.28)

[a] Standard deviation is indicated in parentheses.
[b] Site density is normalized to dry mass.
[c] Affinity constant not calculated in model

For WC, Cd adsorption began to increase at approximately pH 4.5, based on the FITEQL modeling (Fig. 3). The models and data were incongruent below pH 6, although above pH 6 the models accurately predicted adsorption (average fit over entire pH range ± SD: 26.38 ± 8.39). This is likely due to adsorption by ligands that have pKa values below 3 - 4, which are not detectable by titration. These include some carboxyl and the first of two phosphoryl deprotonations. In contrast, the modeled adsorption values and experimental data for SC were congruent along the entire length of the curve (average fit 3.65 ± 1.08), showing low levels of Cd adsorption up to pH 6, above which adsorption steadily increased.

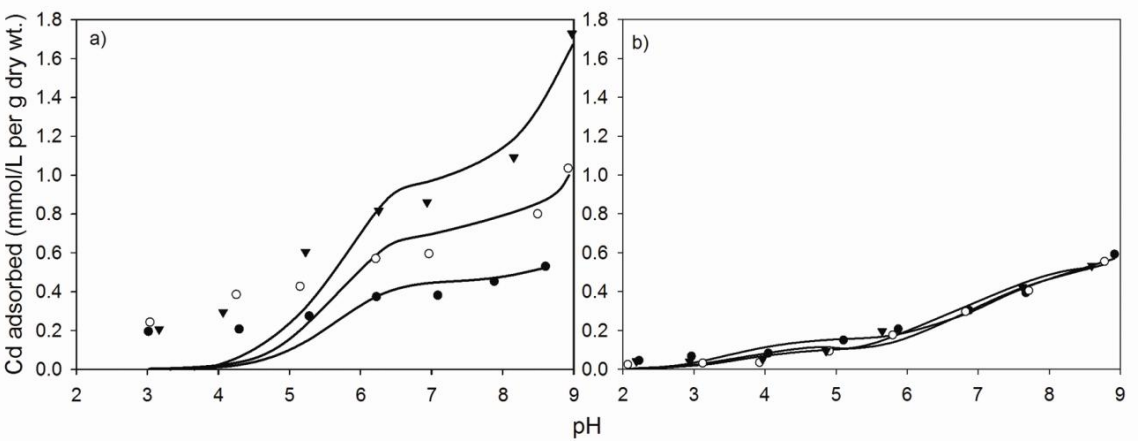

Figure 2-3: FITEQL models (solid lines) of cadmium adsorption for triplicate analyses of WC (a) and SC (b), as well as experimental data points for each replicate, indicated by different symbols.

Although the adsorption of Cd by WC was best modeled in FITEQL using a 2-site model that attempts to explain the experimental data by calculating cadmium affinity constants for two of three ligands predicted by FITEQL modeling of titration data, SC was best fit using a three site model. The cadmium affinity constant (-0.05) for the predicted SC ligand with a pK_a of 3.81 indicates that the ligand did not play any appreciable role in Cd adsorption (Table 2). Thus, for WC and SC, only two of three potential ligands were involved in Cd adsorption. In the presence of EPS, *H. aerophilus* cells adsorbed Cd using both acidic (pK_a 5.78) and basic (pK_a 9.58) ligands. After removal of EPS, only neutral (pK_a 7.04) and basic (pK_a 8.51) ligands were involved in adsorption of Cd by SC cells. For both WC and SC the most basic ligand had the highest affinity for Cd (6.20 and 4.19, respectively).

2.3.3 FT-IR analysis of EPS. Lyophilized EPS from *H. aerophilus* was analyzed by FT-IR to confirm the functional groups inferred from acid-base titrations of

EPS (Table 3). Four dominant peaks were observed (Fig. 4), with the broadest peak (3276cm^{-1}) corresponding to the O-H bond in water (Peng et al. 2003). The greatest absorbance was observed at 1045cm^{-1} and can be attributed to the C-O bond in polysaccharides (Yee et al. 2004). Peaks at 1654cm^{-1} and 1560cm^{-1} correspond to amide I and II bonds in proteins, respectively (Wei et al. 2004; Yee et al. 2004). These results are consistent with the inference of carboxyl, phosphoryl, and amine groups by acid-base titration of *H. aerophilus* biomass. The remaining peaks do not correspond to bonds characteristic of specific molecules.

Table 2-3. Summary of proposed bonds in EPS identified by FT-IR.

Wavenumber (cm^{-1})	Proposed bond	Reference
~3276	O-H in water	(Peng et al. 2003)
~2925	C-H stretching of CH_2 or CH_3 groups	(Yee et al. 2004)
~1654	C=O stretching in amide I, associated with proteins	(Wei et al. 2004), (Yee et al. 2004)
~1560	N-H bending in amide II	(Wei et al. 2004), (Yee et al. 2004)
~1420	C-H deformation of CH_2 or CH_3 groups	(Yee et al. 2004)
~1376	CH_3/CH_2 deformation; Stretching COO^-	(Ojeda et al. 2008)
~1256	C-O stretching in COOH	(Yee et al. 2004)
~1045	C-O of polysaccharide	(Yee et al. 2004)

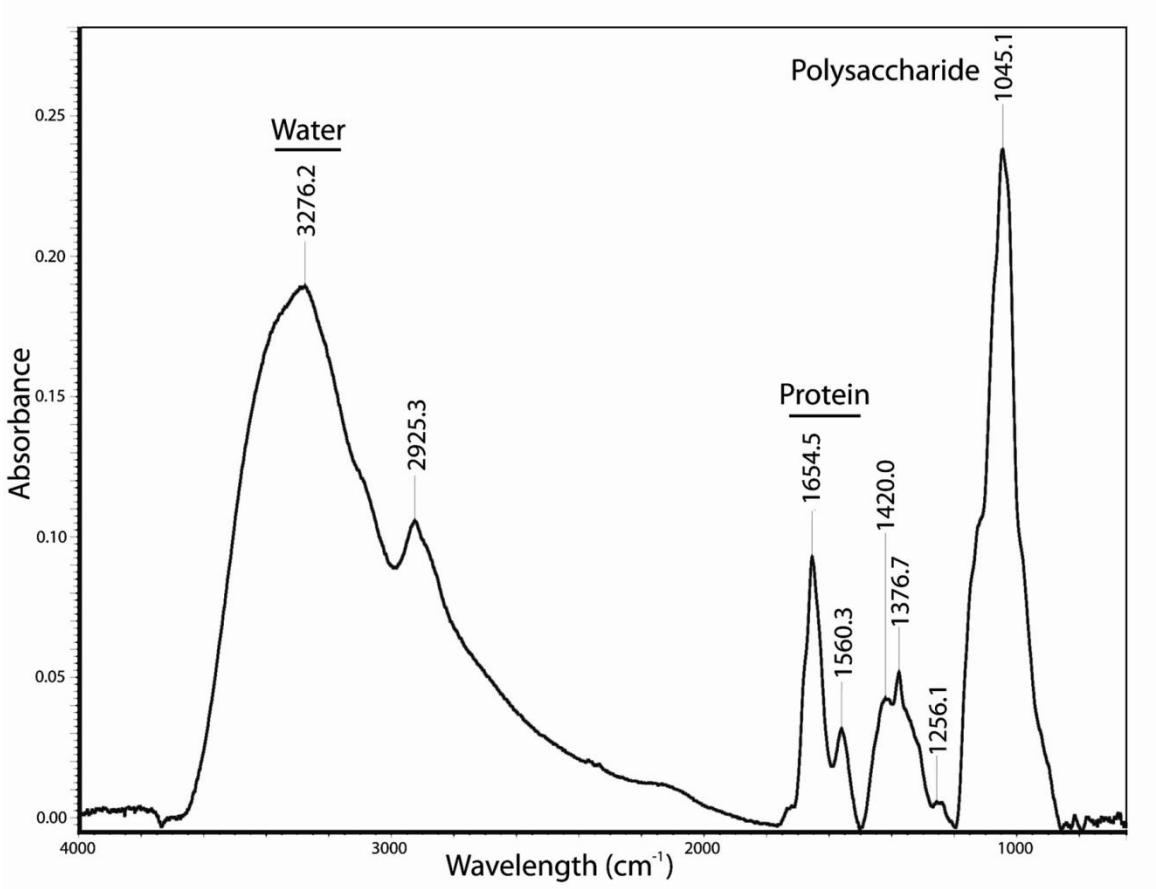

Figure 2-4: FT-IR spectrum for purified EPS.

2.4 Discussion

The purpose of this study was to characterize how the production of EPS alters the cell surface reactivity of *H. aerophilus* by evaluating the density of ligands present on the cell surface and their ability to adsorb cadmium from aqueous solution. The relative increase in excess charge of WC and EPS compared to SC, indicated an increased ability to adsorb or desorb protons. Although the greatest maximum excess charge measured for WC was two-fold greater than for EPS, it should be noted that the maximum excess charge of the other two WC replicates were similar to those of EPS. It is also possible that

some of the extra excess charge on WC can be attributed to ligands present on the cell surface beneath the EPS, which would not have been present in the purified EPS preparation. The excess charge for the highest replicate of WC is also high relative to published values for other organisms and SC is low (Lalonde et al. 2007a; Lalonde et al. 2007b; Lalonde et al. 2008; Liu and Fang 2002), however there is substantial variation among these values. Cell surface chemistry has been found to vary with growth conditions (Haas 2004; Hong and Brown 2008; Lalonde et al. 2008). Many bacterial species have been found to increase production of EPS with decreasing temperatures (Kiliç and Dönmez 2008; Mancuso Nichols et al. 2004), although Kiliç and Dönmez (Kiliç and Dönmez 2008) found that *Micrococcus* sp. increases production with temperature. Medium composition can also affect EPS production (Marx et al. 2009; Neal et al. 2007) and composition (Corsaro et al. 2004; Neal et al. 2007). Thus, slight variations in growth conditions or medium composition could account for some of the variation in WC and EPS.

The buffering capacity of a substrate at a specific pH corresponds to the slope of a charge excess curve at that point, thus the shape of the acid-base titration curve is related to buffering capacity. It is notable that not only the magnitude of the excess charge varies when EPS is present on the cell surface, but also the shape of the curves (Fig. 1). The variation in the shape of the titration curve indicates that EPS alters the buffering properties of the cell surface, contributing an increased number of ligands that are active at a lower pH.

Although there is considerable variation in the shapes of the curves, both among replicates and between WC and EPS, the average curves are very similar. This is reflected in their linear programming ligand models. The steepest section of the average curve, for both WC and EPS, corresponds to the section with the highest density of ligands, consisting primarily of carboxyl and phosphoryl groups. As with the titration data, there is a high degree of similarity in the ligand patterns of WC and EPS, which are both distinctly different from the pattern observed in SC. This indicates that the differences in ligands are due to the presence of EPS. The most obvious difference between SC and WC or EPS is the absence of carboxyl groups. Carboxylic sites on cell surfaces have been attributed to peptidoglycan (Cox et al. 1999), however *H. aerophilus* is Gram negative: it is unlikely that there will be exposed peptidoglycan in WC and purified EPS will certainly not contain peptidoglycan. Thus, it is most likely that the carboxyl groups are due to the polysaccharide component of EPS. Phosphoryl groups, which are the most prevalent groups, are likely to be present on sugars and nucleic acids, both of which are common components of EPS, although nucleic acids occur in very small quantities (Flemming and Wingender 2001). In the absence of EPS it is possible that phosphoryl groups on SC are due to phospholipids and lipopolysaccharides (LPS) in the outer membrane. Amino groups are most likely due to the presence of proteins in the EPS or outer membrane, although they may also be present in sugars. Previous studies of EPS and bacterial surfaces have found the most common functional groups to be carboxyl, phosphoryl and amine groups (Burnett et al. 2006; Hong and Brown

2008; Liu and Fang 2002; Phoenix et al. 2002; Tourney et al. 2008). This is consistent with the WC data, although SC does not show carboxyl groups as modeled using linear programming. Carboxyl groups on Gram negative bacteria are attributed to LPS and proteins (Hong and Brown 2008) so it is likely that carboxyl groups are present on the surface of *H. aerophilus* SC cells but are perhaps too low in number to detect. In contrast to linear programming, FITEQL predicts the presence of carboxyl groups, albeit at lower densities than phosphoryl or amine groups. FT-IR confirmed the presence of polysaccharides and protein in EPS (Fig. 4). Unfortunately, the presence of phosphoryl groups could not be confirmed by FT-IR, because phosphoryl groups characteristically absorb wavelengths between 935 – 1240 cm^{-1} (Wei et al. 2004) that would be masked by the large polysaccharide peak.

When interpreting titration data it is important to acknowledge the limitations of the procedure, in particular, that uncertainty increases as extremes in pH are approached (Smith et al. 1999). Therefore, although the titrations were carried out from approximately pH 2 – 11, only values between pH 4 – 10 were used in analysis. This is particularly relevant for the linear programming model of SC, where a ligand appears with a pK_a of 10. Because this ligand is on the edge of the acceptable range, it is necessary to be cautious when considering the site. Given the consistency of results for titrations of SC it was likely that the presence of a ligand with that pK_a would be observed in WC, however that was not the case. This could be due to assignment of a non-existent ligand as a result

of experimental uncertainty at high pH values or because the site density is small enough (1 mmol/g) relative to WC that the ligand is masked by the EPS.

FITEQL (least-squares optimization) and MATLAB (linear programming optimization) were both used to model pK_a and concentrations of ligands for WC and SC. Both methods were equally able to fit the experimental data (data not shown). Although the predicted ligand pK_a values are slightly different between models, the ligand classes represented are the same, with the exception of ligand L_1 predicted by FITEQL for SC, which corresponds to a carboxyl group (pK_a 3.81 ± 0.91). However, this value lies outside the reliable range of pH 4 – 10. In addition, both models show the site densities for SC as an order of magnitude lower than WC. Individual classes of functional groups have a range of characteristic pK_a values that would be reflected on the surface of the cell or in EPS, as opposed to a single pK_a for each class of functional groups, as observed with FITEQL. Thus the results from MATLAB were chosen for graphical representation of the differences in surface chemistry between WC, SC and EPS. However, the FITEQL models were used in Cd adsorption assays because this simplification is required for modeling.

As with the titration data, the Cd adsorption experiments reveal more variation between replicates of WC than of SC. It is possible that this is an artifact of the cell washing process as there is some loss of loosely attached EPS from the cell surface during each wash and inconsistencies between replicates could explain the differences observed. Alternately, it could be due to the

complex and varied nature of EPS, with slight differences in concentration and composition between separate cultures of the same organism. Excess charge curves of purified EPS (Fig. 1b) show variation in both shape and magnitude of curve, suggesting the reactivity of EPS is variable. This second possibility is further supported by the consistency in the shape of the WC curve between replicates and its distinct shape compared to SC. Although the washing process likely causes a small amount of variation between replicates, variability in the composition of EPS itself cannot be ruled out.

The production of EPS by *H. aerophilus* clearly results in an increase of both cell surface reactivity and extends the pH range over which the cells are able to adsorb cations. This partially contrasts with the results of Tourney et al. (2008), where EPS was found to increase the site concentrations on a mass-normalized basis but had no effect on the functional groups present on a thermophilic *Bacillus licheniformis* strain. In the current study, both titrations and Cd adsorption assays showed an increase in ligands available at lower pH values in the presence of EPS. *H. aerophilus* was grown at pH ~7.2 (starting pH of medium), at which point only the less abundant of SC's two major ligands would be deprotonated. The production of EPS, however, increases the number of ligands available, potentially aiding in accumulation of necessary cations such as Mg^{2+}. Since the original publication of this work, two new studies have been published concurring with increase in ligand availability and metal adsorption due to the presence of EPS (Cao et al. 2011; Wei et al. 2011). Phosphoryl groups have been shown to play a more important role in metal adsorption at the bacterial

surface than carboxyl groups (Boyanov et al. 2003; Kelly et al. 2002), which is consistent with the FITEQL models (Table 2).

The psychrotolerant lifestyle has the potential to affect cell surface chemistry. Growth at low temperatures can lead to changes in membrane composition (Beranová et al. 2008; Jagannadham et al. 2000) that could affect the type and density of functional groups present on the cell surface. In addition to changes in fatty acid composition and carotenoid content, Corsaro et al. (2004) observed an increase in phosphorylation of lipooligosaccharides and exopolysaccharides with an increase in growth temperature for *Pseudoalteromonas haloplanktis*, an Antarctic bacterium. It is clear that in the case of this psychrotolerant bacterium, the production of EPS confers additional and abundant surface sites that influence both the acid-base behaviour and metal binding capacity of the cells. It is important to consider adaptations resulting from psychrotolerance because microbes have been implicated in geochemical processes in many cold environments (Bargagli et al. 2007; Konhauser et al. 1994; Sharp et al. 1999; Tranter et al. 2002) and Arctic microbial biofilms have a direct role in sequestration of iron and phosphorus (Konhauser et al. 1994) that will be affected by surface reactivity.

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3. COMPARING *FLAVOBACTERIUM* SP. SURVIVAL OF A FREEZE-THAW CYCLE, WITH AND WITHOUT EPS, IN THE PRESENCE OF PARTICULATE SUBSTRATA

3.1 Introduction

Over the past several decades microbial cryotolerance has been researched extensively, including changes in membrane structure (Morgan-Kiss et al. 2006), gene expression (Qiu et al. 2006) and protein structure (Georlette et al. 2004), and production of proteins that interact with ice crystals (Raymond and Knight 2003). Although some bacteria have been shown to be metabolically active at temperatures below 0°C (e.g. Bakermans et al. 2003; Bakermans and Skidmore 2011; Breezee et al. 2004; Junge et al. 2006), much of the work on cryotolerance has focused on temperatures above 0°C. Of the work that has been done on microbes at subzero temperatures, surprisingly little focus has been placed on tolerance and survival of environmental freeze-thaw cycles. Interestingly, much of the literature involving free-thaw cycles comes from food microbiology and concerns pathogens surviving frozen storage (Azizoglu et al. 2009; Azizoglu and Kathariou 2010; Durack et al. 2011), however these organisms are of little environmental relevance when considering subglacial sediments.

Frequently, environmental studies have been focused on the changes to microbial community structure caused by freeze-thaw cycles, rather than the effect on the cells themselves. In general, repeated freeze-thaw cycles have been

found to reduce both total cell numbers and diversity of communities that are not exposed to frequent freeze-thaw cycles (Stres et al. 2010; Walker et al. 2006). As one would expect, the diversity and activity of Arctic and Antarctic soil communities are resistant to freeze-thaw cycles, although these studies have not assessed changes in cell numbers (Mannisto et al. 2009; Yergeau and Kowalchuk 2008). Comparison of freeze-thaw responses of microbial communities from high-alpine and temperate regions shows that although abundance and diversity initially decrease in temperate soils, the community adapts over time, reaching a state physiologically, if not phylogenetically, similar to the high-alpine soils (Stres et al. 2010).

In addition to the cold adaptations outlined above, EPS is thought to play a role in cryotolerance, and thus freeze-thaw tolerance. Addition of cell free EPS to cultures has been linked to increased activity at subzero temperatures (Junge et al. 2006) and to improved growth after freezing under long term storage conditions (-80°C for 48 h) (Marx et al. 2009). In each of these cases the additional EPS was derived from the strain tested. To determine if exogenous EPS could aid in stress tolerance Knowles and Castenholz (2008) added EPS derived from *Nostoc* sp. to cultures of either *Chlorella* sp. or *Chroococcidiopsis* sp. Although the addition of exogenous EPS was found to be beneficial for both species for desiccation resistance, results were mixed for freezing tolerance, with *Chlorella* sp. showing decreased survival after addition of EPS in 2 of 3 trials (Knowles and Castenholz 2008).

The presence of particles, such as sediment, has also been linked to improved cryotolerance. In a study of microbial activity in sea ice Junge et al. (2004) found that the percent of active cells associated with particles increased with decreasing temperature, with nearly all active cells being associated with particles at -20°C. It is unknown whether the benefit of particle association is due purely to surface attachment or to increased access to nutrients that may be adsorbed to the particle surface.

Understanding the mechanisms of freeze-thaw tolerance in the subglacial environment is important, particularly in temperate polythermal glaciers where microbes are likely to be exposed to freeze-thaw cycles due to changes in pressure at the interface between the ground and ice. The experiment presented here attempts to determine both the effect of EPS on freeze-thaw tolerance, and the importance of particle association in the subglacial environment. *Flavobacterium* A97, an isolate from Antarctic glacial ice, is subjected to a freeze-thaw cycle in the presence of clean quartz sand or glacial sediment and the absence of any particles, before and after physical removal of EPS. To my knowledge this is the first experiment to examine survival of freezing after removal of EPS rather than addition of extra EPS. It is expected that the presence of EPS will result in increased survival, regardless of the presence or absence of particles, but that cells frozen in the presence of glacial sediment will benefit more than those without particles or with only sand.

3.2 Materials and Methods

3.2.1 Growth conditions and preparation of cells. *Flavobacterium* A97 was isolated by Jonathan Klassen from ice collected by Joel Barker at Victoria Upper Glacier, Antarctica. *Flavobacterium* A97 cultures were incubated in R2 broth (per L: yeast extract 0.5 g; proteose peptone no. 3 0.5 g; casamino acids 0.5 g; dextrose 0.5 g; soluble starch 0.5 g; sodium pyruvate 0.3 g; K_2HPO_4 0.3 g; anhydrous $MgSO_4$ 0.05 g) for 3 d at 10°C without shaking, transferred to fresh broth with 1/100 dilution and incubated for a further 4 d. Stock cultures were maintained at 4°C on R2A agar (Difco).

3.2.2 Survival of freeze-thaw conditions. Figure 1 is a flow diagram illustrating the freeze-thaw procedure. A 1/100 dilution of a three day old *Flavobacterium* sp. A97 culture was grown to late log phase (OD_{600} ~0.1–0.2). Cell concentrations were calculated using a Petroff-Hauser chamber and it was determined that at OD_{600} = 0.1, 30 µL of culture was required to inoculate each sample with 10^6 cells. To strip cells of EPS (SC), 20 mL of culture was sonicated at 20 W for 1 min, then centrifuged at 30,000 x *g* for 20 min. The supernatant was discarded and the cells were resuspended in 20 mL of R2 broth. Thirty microliters (or less, depending on OD_{600}) of the suspension was resuspended in sterile R2 broth, up to 1.0 mL. For samples with EPS intact (WC) and killed controls (K), 30 µL (or less, as above) of culture were resuspended in sterile R2 broth. The tubes were centrifuged (Denver Instrument Force 7 Microcentrifuge) at maximum speed for 20 min. The supernatant was discarded and the pellet

resuspended in 5 mL of phosphate buffered saline (PBS). The killed control was autoclaved at 121°C for 10 min.

Textural analysis of glacial sediment (GS), from the surface of John Evans Glacier, Ellesmere Island, was performed by the Natural Resources Analytical Laboratory at the University of Alberta (Sheldric and Wang 1993). Analysis showed GS to consist of 6% clay (<2 µm), 59% silt (2 – 50 µm), and 35% sand (>50 µm). One gram of quartz sand (QS) (Mesh 50-70; Sigma) or glacial sediment was added to 2-mL screw-cap microcentrifuge tubes and autoclaved at 121°C in a 25-min wrapped cycle. Five hundred microliters of WC, SC, or K cell suspension containing 10^6 cells total were added to each of 27 tubes containing QS, SG, or no sediment (NS). All tubes were placed in a circulating glycol bath (Cole Parmer Polystat 1268-10) at -5°C and incubated for 5 d before being moved to a 4°C incubator for a further 4 d. Three tubes were removed for analysis before freezing, during transfer to 4°C and after incubation at 4°C.

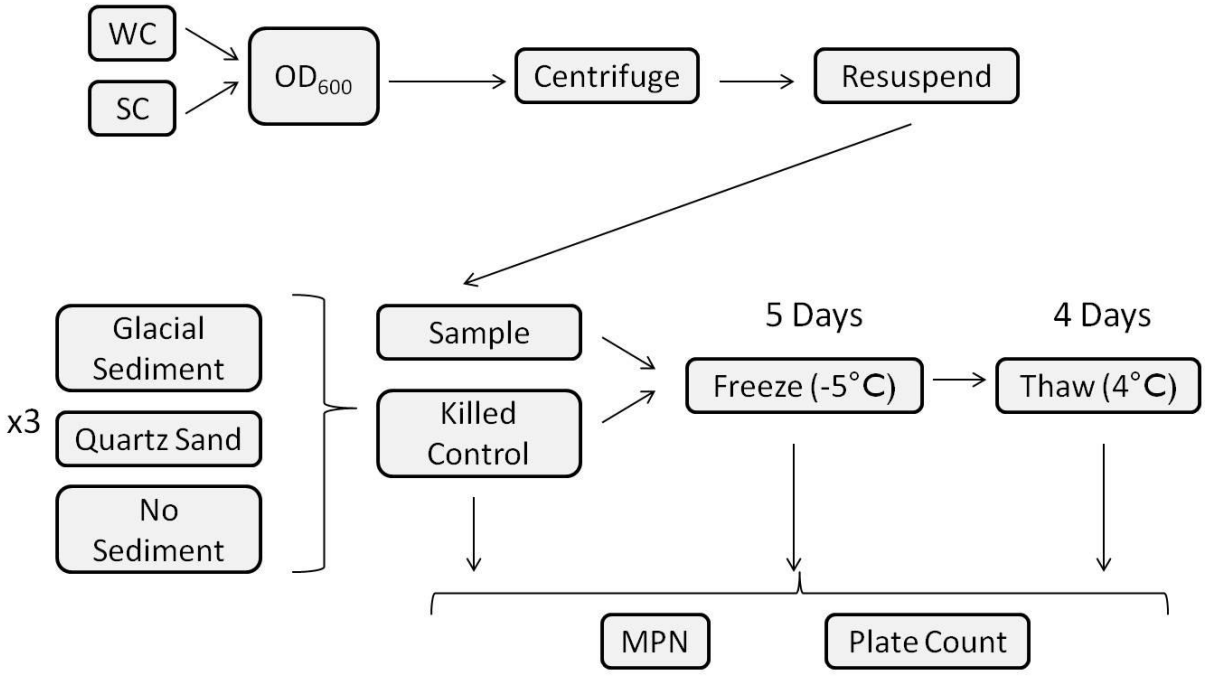

Figure 3-1: Flow diagram outlining the general procedure for testing freeze-thaw survival of *Flavobacterium* A97.

Cell numbers were determined by 5-tube Most Probable Number (MPN) and plate counts. Sodium pyrophosphate was added to each tube at a final concentration of 0.01 M to assist in removal of cells from particles (Velji and Albright 1986). After vortexing for 15 sec, the tubes rested at room temperature for 15 – 30 min before being vortexed for another 15 sec. A 1/100 dilution was made in 9 mL R2 broth. After vortexing, 1 mL was dispensed into each of five tubes containing 9 mL R2 broth. Serial 1/10 dilutions were made in cold R2 broth to a maximum dilution of 10^{-9} for samples before and after freezing, and 10^{-10} for samples taken after incubation at 4°C. Only 10^{-3} dilutions were made for killed

controls. Dilutions were plated onto R2A with 0.1 mL spread in duplicate. For samples taken before freezing or after four days incubation at 4°C MPN dilutions of 10^{-3} and 10^{-4} were plated. For spread plates of samples taken after incubation at -5°C, MPN dilutions of 10^{-2} and 10^{-3} were used. For all killed controls 0.1 mL was plated directly from the sample (final plate dilution of 10^{-1}). Plate counts and MPNs were incubated at 10°C for a minimum of two weeks before reading.

3.2.4 Statistical analysis. Comparisons between pairs of MPNs were performed using Cochran's method (Cochran 1950). The statistical equation is:

$$Z = \frac{|\log MPN_1 - \log MPN_2|}{c\sqrt{[(\log a_1 / n_1) + (\log a_2 / n_2)]}}$$

where Z is the test statistic, c is a constant (0.55), a_i is the dilution ratio, and n_i is the number of samples per dilution. The critical value for Z at P=0.05 is 1.96 and Z=2.58 at P=0.01. In cases where the MPN value is zero, 1 is added to each MPN value.

3.3 Results

3.3.1 Survival of free-thaw conditions. Known quantities of *Flavobacterium* A97 cells, with and without EPS, were subjected to a single freeze-thaw cycle and their survival was assessed by MPN and plate counts. MPNs show similar initial numbers of viable cells for WC and SC added to QS and GS (Fig. 2a,b; Table 1; Table 2), indicating similar efficiency in recovery of cells from both substrata.

However, there is significant variation between WC and SC in NS samples (Z=4.89, P=0.01) (Table 2; Fig. 2c). For the range of dilutions tested (10^{-3} – 10^{-10}) very few cells remained viable after freezing or the four day recovery period at 4°C (Table 1; Fig. 2). The same trend was observed with plate counts, although no plates contained colony numbers in the generally accepted statistically valid range of 30-300 (data not shown).

It is interesting to note that there is significant variation in the number of cells for each WC and SC test condition with the different substrata (Table 3). For GS and NS, this is likely due to loss of cells while washing during cell preparation. However, the number of cells recovered from QS is 10– to 100–fold higher than either GS or NS (Fig. 2). This is most likely an artifact resulting from scoring tubes after only two weeks when growth was weak. I later learned, when looking at results from another data set, that R2 broth sometimes has precipitates at the bottom of a tube, mimicking weak growth. Because the initial QS cell numbers were similar to the quantity of cells initially added in the freeze-thaw tests, I did not realize there was a problem and terminated the experiment prematurely. All other MPNs were scored after a minimum of three weeks to account for precipitates.

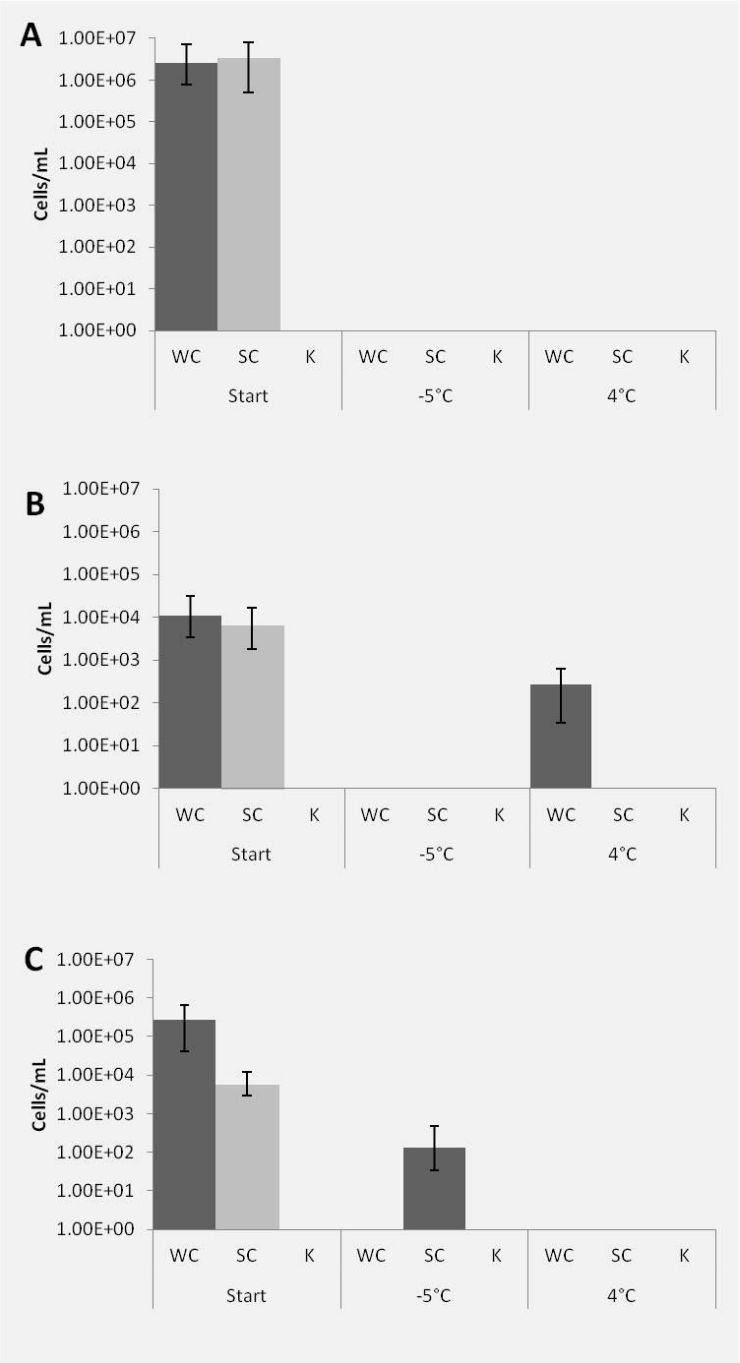

Figure 3-2: Enumerated MPN cells/mL of *Flavobacterium* A97 with EPS (WC), stripped of EPS (SC) and killed controls (K) recovered from a) quartz sand, b) glacial sediment, and c) no sediment at the start of the experiment, after five days incubation at -5°C, and after four days incubation at 4°C. Values are averages of triplicate samples. Bars indicate 95% confidence limits.

Table 3-4: Averaged enumerated MPN cells/mL of *Flavobacterium* A97 with EPS (WC), stripped of EPS (SC) and killed controls (K) recovered from a) quartz sand, b) glacial sediment, and c) no sediment at the start of the experiment, after five days incubation at -5°C, and after four days incubation at 4°C.

Substrata	**Time**	**Cell Treat.**	**MPN**	**St. Dev.**	**Upper L.**	**Lower L.**
QS	Start	WC	$2.57x10^{6}$	$1.27x10^{6}$	$7.03x10^{6}$	$8.00x10^{5}$
		SC	$3.37x10^{6}$	$4.03\ x10^{6}$	$8.03x10^{6}$	$5.00x10^{5}$
		K	0	0	0	0
	-5°C	WC	0	0	0	0
		SC	0	0	0	0
		K	0	0	0	0
	4°C	WC	0	0	0	0
		SC	0	0	0	0
		K	0	0	0	0
GS	Start	WC	$1.07x10^{4}$	$1.02x10^{4}$	$3.10x10^{4}$	$3.40x10^{3}$
		SC	$6.60x10^{3}$	$2.25x10^{3}$	$1.63x10^{4}$	$1.83x10^{3}$
		K	0	0	0	0
	-5°C	WC	0	0	0	0
		SC	0	0	0	0
		K	0	0	0	0
	4°C	WC	$2.67x10^{2}$	$4.62x10^{2}$	$6.33x10^{2}$	$3.33x10^{1}$
		SC	0	0	0	0
		K	0	0	0	0
NS	Start	WC	$2.74x10^{5}$	$3.73x10^{5}$	$6.58x10^{5}$	$4.07x10^{4}$
		SC	$5.47x10^{3}$	$7.41x10^{3}$	$1.21x10^{4}$	$2.90x10^{3}$
		K	0	0	0	0
	-5°C	WC	0	0	0	0
		SC	$1.33x10^{2}$	$1.15x10^{2}$	$4.67x10^{2}$	$3.33x10^{1}$
		K	0	0	0	0
	4°C	WC	0	0	0	0
		SC	0	0	0	0
		K	0	0	0	0

Table 3-5: Z statistical values for pairwise comparisons between averaged MPNs within a substrata set based on the method of Cochrane (1950).

Substrata	**Pair**	**Z[a]**
Quartz Sand	WC Start + WC -5°C	18.4
	WC Start + WC 4°C	18.4
	SC Start + SC -5°C	18.8
	SC Start + SC 4°C	18.8
	WC Start + SC Start	0.34*
Glacial Sediment	WC Start + WC -5°C	11.6
	WC Start + WC 4°C	11.6
	SC Start + SC -5°C	11.0
	SC Start + SC 4°C	11.0
	WC Start + SC Start	0.61*
No Sediment	WC Start + WC -5°C	15.6
	WC Start + WC 4°C	15.6
	SC Start + SC -5°C	10.8
	SC Start + SC 4°C	10.8
	WC Start + SC Start	4.89

[a]The critical value for Z at P=0.05 is 1.96 and Z=2.58 at P=0.01.
* indicates a statistically significant difference

Table 3-6: Z statistical values for pairwise comparisons between averaged MPNs within a cell treatment.

Cell Treatment	Pair	Z[a]
WC	QS + GS	6.84
	QS + NS	2.79
	GS + NS	4.04
SC	QS + GS	7.78
	QS + NS	8.02
	GS + NS	0.24*

[a]The critical value for Z at P=0.05 is 1.96 and Z=2.58 at P=0.01.
* indicates a statistically significant difference

3.4 Discussion

The results of the freeze-thaw experiment do not support my hypothesis that cells with EPS are better able to survive freezing. In most cases, the MPN

tests and plate counts showed no detectable levels of viable cells after five days at -5°C. The two cases in which growth was observed after freezing are deceptive (Fig. 2). In each case growth was not observed in all replicates and was only at the lowest dilution represented, which is confirmed by large standard deviations (Table 1). It is important to note, however, that the MPN dilutions used in this experiment ranged only from 10^{-3} to 10^{-10}. It is therefore possible that viable cells did remain, but at concentrations low enough that they were below the detection limit. This is supported by the presence of growth in a small number of MPN tubes at 10^{-3} dilution, suggesting that dilutions between 10^{0} and 10^{-2} may have yielded useful results. Although it is not possible to confidently test my hypothesis using these data, the consistent lack of growth after freezing shows that, regardless of the presence of EPS, at least 99.9% of *Flavobacterium* A97 cells cannot be recovered after freezing for 5 d in PBS in the presence or absence of a mineral substratum. This is in stark contrast to previous observations of psychrotolerant and psychrophilic bacteria where there is approximately only a 10-fold decrease in viability for untreated cells (Knowles and Castenholz 2008; Walker et al. 2006). However there are some substantial differences between the freeze-thaw experiment tested here and the literature procedures, the greatest being the use of a growth medium when freezing cells (Walker et al. 2006). Previous studies have also used microscopic assessment of viability using SYTOX Green (Knowles and Castenholz 2008). While developing my method I found that R2 broth would not reproducibly freeze at -5°C and I had to use PBS to ensure consistent freezing, so it is possible that the increased level of solutes in

the freezing medium used by Walker et al. (2006) would have resulted in less overall freezing (i.e. larger liquid water veins between ice crystals) at higher sub-zero temperatures. The use of PBS, as opposed to a dilute growth medium, also prevents the addition of excess nutrients that may result in survival or resuscitation that is not representative of environmental conditions. It is also possible that bacteria too damaged for growth, but which still had intact membranes, would have been detected and classified as viable by microscopy but not by MPN. In fact, I chose to use MPNs as my primary quantification method, despite its high statistical variability, in order to specifically quantify the bacteria that are viable as opposed to bacteria that just have intact cell membranes. Although plate count analysis also offers this advantage, it is generally acknowledged that liquid cultures are less stressful and better for resuscitation of cells. In the future it may be better to combine MPN and plate counts with microscopic techniques, such as BacLight live/dead staining or CTC staining that measures active respiration (Junge et al. 2004), to get a more complete picture of microbial survival. Additionally, the use of positive and negative control organisms with characterized responses to freeze-thaw cycles would assist in comparisons of future research to the published literature.

Generation of a *Flavobacterium* mutant incapable of producing EPS would be ideal for these experiments because it would enable the testing of multiple freeze-thaw cycles, as well as eliminating the concern that cells may produce extra EPS between being stripped of EPS and frozen. Transposon mutagenesis experiments related to this research were performed by Anh Dao, a

project student working under my supervision, in addition to my own mutagenesis trials. All attempts to generate *Flavobacterium* mutants incapable of producing EPS were unsuccessful. Several aspects of the procedure, including incubation time and temperatures, centrifugation, ratio of *E. coli*:*Flavobacterium*, and $CaCl_2$ concentration, were varied in an attempt to maximize conjugation efficiency. However, we were unable to generate any antibiotic resistant mutants, let alone mutants incapable of producing EPS. At this point I determined that the best course of action was to adapt the freeze-thaw experiments to be performed with cells stripped of EPS.

EPS plays an important role in resistance to a variety of environmental stresses, such as heavy metals (Aislabie and Loutit 1986; Gonzalez et al. 2010) and desiccation (Knowles and Castenholz 2008), although these effects are often studied in the context of biofilms rather than just as an effect of EPS alone (Harrison et al. 2007; Williams et al. 2009). One of the best known roles of EPS is facilitation of attachment to surfaces (Tsuneda et al. 2003). Combined with the increased resistance to environmental stress observed in attached cells versus planktonic cells, it was necessary to examine the possibility that cryotolerance associated with EPS may be due to surface attachment. Particulate substrata were chosen that would provide information not just about benefits due to presence or absence of particles, but also any effects that may result from adsorption of nutrients to the substratum surface. Quartz sand, purchased from Sigma-Aldrich, was chosen to represent the effect of the substratum alone, whereas glacial sediment was chosen to represent a more environmentally relevant situation in

which nutrients have adsorbed to the particles. This distinction made the use of PBS particularly important to avoid loading the quartz sand with extra adsorbed nutrients. However, there were no samples of subglacial sediment in storage in sufficient quantity for the experiment, so it was necessary to use mud taken from the surface of an Arctic glacier. Unfortunately, the data obtained were not sufficient to differentiate between the two substrata.

The unexpectedly high percentage of unrecoverable *Flavobacterium* A97 suggests that a number of procedural improvements could be made to improve detection. The most obvious modification would be to simply increase the number of cells added to each sample. However, the initial choice to use 10^6 cells was made to maintain environmental relevance because subglacial sediments have a cell density of 10^5 – 10^6 cells/g (Foght et al. 2004; Kastovska et al. 2007). Based on MPN results it is possible that a substantial portion of these initial cells were lost during preparation, likely at the washing stage. The consistently low cell numbers in samples without sediment confirm that the loss of cells is not due to entrapment within the sediment matrix. There are two options to remedy the loss of cells: 1) increase the initial number of cells added, or 2) eliminate the cell washing step. Simply increasing the initial number of cells would introduce greater variation into the experiment, relative to the second option, because the quantity of cells lost to washing would be inconsistent between samples. Thus, I believe that removing the washing step is the best course of action. Although preventing nutrient carryover from the medium to PBS is important, the volume of medium used is small enough (15-30 µL) that dilution in 5 mL of PBS should

decrease any effects of the nutrients. This would ensure that a consistent number of cells is added to all samples.

Another solution may be to use larger sample volumes for the freeze-thaw experiment. Because of limited space in the glycol bath, samples were aliquoted into 2-mL centrifuge tubes where approximately 200 µL of liquid was available for MPNs and spread plates. As a result, the lowest dilution available for MPN and plate counts was 10^{-3}, which I deemed reasonable at the time but which, in retrospect, proved too dilute. The use of larger sample tubes could have allowed for aliquoting from a 1/10 dilution and an MPN range starting at 10^{-2}. This could be further improved using 900 µL instead of 9 mL blanks for dilutions, although the small size may make it even more difficult to detect positive tubes. The increase in sample size would also require a larger glycol bath, which was not available to me. However, it would be possible to avoid scaling up the sample size by scaling down the MPN volume even further using 96-well plates with 90 µL of broth. These plates could then be scored by OD_{600}.

It was expected that incubation at 4°C after thawing would allow time for damaged cells to repair themselves, resulting in higher counts for the final time point, relative to enumeration immediately after thawing, however this was not observed (Fig. 2). There are three possible explanations: 1) the hypothesis is incorrect and no difference in numbers of viable cells would be observed, 2) all of the cells died, or 3) the recovery period is not long enough to observe a difference. Unfortunately with the data I generated it is not possible to determine which

possibility is correct, although the second seems unlikely, given the psychrophilic nature of *Flavobacterium* A97 and its isolation from glacial ice. I believe the third option is the most likely and would also have to be accounted for in order to conclude that the first assumption was correct. In my original experimental plan, MPNs were to be scored after one week, based on the 4-d incubations used to grow cultures. This was changed to two weeks and eventually three, so it seems reasonable to assume that the stress of freezing and thawing substantially slowed growth of the organism. I therefore recommend that future versions of this experiment allow a resuscitation period of at least two weeks, in order to be confident that any negative results are valid.

Although this experiment was not successful overall it did yield some interesting and important information, namely that the survival rate of the freeze-thaw process of the glacial isolate *Flavobacterium* A97 is substantially lower than has been observed in other psychrotolerant organisms. It is unfortunate that the data were not sufficient to elucidate a role for EPS in tolerance to freeze-thaw cycles, but I believe that the experimental principles are sound and that the procedural adjustments recommended above can serve as a valuable stepping stone to future investigators.

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4. SUMMARY AND SUGGESTIONS FOR FUTURE RESEARCH

4.1 Summary

Potential roles of EPS in psychrotolerant and psychrophilic organisms were investigated, both in terms of surface chemistry (Chapter 2) and protection from freeze-thaw cycles (Chapter 3). Potentiometric titration of EPS, cells stripped of EPS (SC) and cells with EPS intact (WC), in combination with cadmium adsorption assays for SC and WC, supported the hypothesis that production of EPS alters the cell surface chemistry of *Hymenobacter aerophilus*, a model psychrotolerant bacterium. A study comparing survival of a freeze-thaw cycle by WC and SC *Flavobacterium* A97 was not able to support or refute the hypothesis that production of EPS increases a cell's ability to survive the freezing process.

Very few studies have been published comparing the surface chemistry of microbial cells with and without EPS, and with the exception of this work, none have been published using a psychrotolerant microbe. An increase in the variety and abundance of surface ligands has the potential to dramatically impact how a microbe interacts with its environment, especially with regards to metal binding capabilities. This may be of particular importance in the subglacial environment where have been implicated in geochemical weathering processes (Sharp et al. 1999; Tranter et al. 2002).

The presence of EPS on *H. aerophilus* cells resulted in increased excess charge and a greater variety of proton-reactive ligands on the cell surface, with

similar patterns seen for both WC and purified EPS (Chapter 2). Linear programming models, estimating ligand site density, reflect this similarity; WC and EPS ligand patterns show a high degree of similarity and are both distinct from SC, indicating that EPS is responsible for the difference. According to the linear programming models carboxyl groups are present on WC and EPS cells but not SC cells, which contrasts with the results of Tourney et al. (2008) who found that although the presence of EPS results in increased site density it has no effect on the variety of ligands present. However, least squares modelling of *H. aerophilus* data did suggest the presence of carboxyl groups on the SC surface, although they occurred in an unreliable pH range (below pH 4) and are thus not conclusive (Chapter 2).

Least squares optimization of ligand site densities was used to model cadmium adsorption to *H. aerophilus* in the presence and absence of EPS. As expected based on ligand modelling, the presence of EPS corresponded to an increase in the amount of cadmium adsorbed and an increased pH range over which it is adsorbed (Chapter 2). Clearly, under these growth conditions, the production of EPS can influence the ability of *H. aerophilus* to interact with its environment through additional and abundant surface ligand sites.

Comparison of *Flavobacterium* A97 survival of a freeze-thaw cycle in the presence and absence of EPS did not yield conclusive results. Although previous studies found psychrotolerant organisms to have a free-thaw cycle survival rate of ~90% (Knowles and Castenholz 2008; Walker et al. 2006), my results showed a

loss of at least 99.9% of the cells (Chapter 3). This discrepancy may be due to high sensitivity of *Flavobacterium* A97 to freeze-thaw or to procedural differences such as assessment of viability through SYTOX Green staining (Knowles and Castenholz 2008) or use of broth as a freezing medium (Walker et al. 2006). Although the data were not able to support or refute either part of the hypothesis that EPS production would lead to an increase in survival and that the presence of particles would also be associated with survival, the dramatic loss of cells is interesting.

EPS is generally accepted to be involved in microbial attachment to surfaces so having an understanding of the changes to cell surface chemistry (i.e. how the cell interacts with environmental surfaces) should provide insight into microbial survival strategies if the presence of particles is associated with increased survival. Unfortunately, the data presented in Chapter 3 were unable to demonstrate a relationship between particle presence or type and survival of *Flavobacterium* A97.

4.2 Future Research

Future research into the influence of EPS on psychrotolerant cell surface chemistry should focus on examining a range of environmentally relevant isolates. Although *H. aerophilus* was a useful model psychrotolerant organism due to its relative ease of growing and copious production of EPS, it was not isolated from the subglacial environment. As a result, the conclusions arising from this work

regarding subglacial microbiology are limited. It is also well established that production and composition of EPS varies among microbial species and genera (Kiliç and Dönmez 2008). Repetition of the experiments in Chapter 2, using Gram negative and Gram positive examples of both psychrotolerant and psychrophilic subglacial isolates would provide a much deeper understanding of the potential roles that EPS plays in microbe-environment interactions. Additionally, the titrations and cadmium adsorption experiments should be repeated with varied growth conditions, especially temperature. Growth conditions such as pH, temperature and medium composition, have been shown to affect EPS production and composition (Haas 2004; Lalonde et al. 2008; Mancuso Nichols et al. 2004; Neal et al. 2007).

To tie these experiments more firmly to the subglacial environment, it would also be interesting to examine any changes to both cell surface chemistry and the EPS itself when cells are grown in the presence of glacial sediment. The type of minerals present in the glacier bed are a potential influence on microbial communities (Skidmore et al. 2005) so inclusion of a variety relevant sediments (e.g. evaporates, sulfides, carbonates) could be useful in determining types of interactions between particles and EPS. Such experiments, especially if combined with that described in Chapter 3, would be of great benefit in understanding microbial survival and environmental interactions in the subglacial environment.

Improvements to the freeze-thaw survival assay were suggested in Chapter 3 and the following suggestions for future research assume successful generation

of results from that experiment. Although I was unsuccessful at generating an EPS^- mutant, this is still a worthwhile avenue of research as it would create many opportunities for new experiments. Most importantly, it would allow for testing of survival of repeated freeze-thaw cycles, addressing the possibility that EPS is more involved in long-term survival, rather than a single freeze-thaw cycle.

If a link is found between EPS production and microbial survival of a freeze-thaw cycle it will be important to demonstrate what effect this may have on microbial communities as a whole. There is evidence that suggests bacteria living in a community have increased resistance to environmental stresses, such as toxic metals or antimicrobials, when compared to bacteria in a single species population (Turner 2012). Addition of exogenous EPS to cultures subjected to desiccation or freezing was found to be beneficial in some cases, depending on the species tested (Knowles and Castenholz 2008). It would therefore be interesting to repeat the freeze-thaw assays presented in Chapter 3 using a co-culture of a bacterial species that produces copious amounts of EPS with one or several species of bacteria that produce little to no EPS.

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