Molecular characteristics of cold adaptation and subzero growth in polar microorganisms

By

Isabelle Raymond-Bouchard

Natural Resource Sciences
McGill University, Montreal

July, 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of PhD

"The road to wisdom? - Well, it's plain
and simple to express:
Err
and err
and err again,
but less
and less
and less."

— Piet Hein

"I almost wish I hadn't gone down that rabbit-hole — and yet — and yet — it's rather curious, you know, this sort of life!"

— Alice in Wonderland (Lewis Carroll)

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny…'"

— Isaac Asimov

Acknowledgments

I would like to thank, first and foremost, my supervisor Lyle Whyte. Thank you for taking me on as a doctoral student, for introducing me to the wondrous and exciting worlds of polar microbiology and astrobiology, and for your support and guidance throughout the years. Your strength of character is inspiring. I will always be eternally grateful for the opportunities and experiences that this lab brought me, both as a scientist and an individual.

Thank you to the members of my supervisory committee, Charles Greer and Sebastien Faucher, for your guidance, ideas, and critical feedback. A special acknowledgment to all my collaborators and co-authors. Your contributions to my PhD research were greatly appreciated and my work benefited from your expertise. I am grateful to the National Sciences and Engineering Research Council of Canada and the Canadian Astrobiology Training Program which provided the funding that supported my doctoral studies.

A special thank you to all the members of the Whyte lab, past and present, with whom I shared so much throughout my PhD. You made my time in the lab an experience I will never forget and I will be eternally grateful for your help, insight, laughter, and inspiration. A very special mention and thank you to Jesse Colangelo-Lillis, Ianina Altshuler, Rachel Backer, Jackie Goordial, Hana Trigui, Jen Ronholm, Haley Sapers, Shaun Turney and Krittika Mittal for your friendship, support, and all that you brought me during my PhD.

Cassandra, even though we were worlds apart during my doctoral studies, I feel like we walked this path side by side! I value your friendship and support more than you know. Thank you for always being there, for listening, and for all the great skype calls.

Un gros merci à tous mes amis et ma famille pour votre amour et soutien pendant toutes ces années. A big thank you to all my friends and family for your love and support during all these years. Thank you Karène and Christiane for your help in translating the abstract to French. David, quiet but always present. Thank you for your love and support. Caro, ma chère sœur, tu es une inspiration et je suis éternellement heureuse de t'avoir dans ma vie, de savoir que tu es toujours là pour m'écouter, m'aimer et m'encourager.

À Karène, merci grandement pour ton soutien, ton encouragement et ton affection ces derniers mois, durant la période la plus importante. Ta présence dans ma vie a rendu mon travail de rédaction de thèse plus plaisant et m'a énormément aidée.

Le plus gros des merci, profondément, à ma mère, Christiane, et mon père, Raymond, pour toutes ces années d'amour, d'écoute, d'encouragement, et de soutien. Il n'y a pas de mot pour d'écrire l'admiration, l'amour, et la gratitude que j'éprouve pour vous et pour tout ce que vous m'avez apporté et appris.

Je dédie cette thèse à la jeune fille, curieuse et passionnée, qui, après avoir vu une cellule pour la première fois, a voulu tout savoir. I dedicate this thesis to the young woman, curious and passionate, who, upon seeing a cell for the first time, wanted to know it all.

Table of Contents

Abstract

What is the cold limit of life? How do microbes survive and grow in cold subzero environments? Most of the earth's ecosystems exist in permanently cold environments where the mean temperature never rises above 15°C. Permafrost, soil that has been frozen for more than two consecutive years, accounts for 27% of all soil ecosystems on earth. These environments host diverse, living microbial communities. However, our understanding of microbial life in these habitats, their growth, activities, and adaptations, remains limited. Polar habitats serve as some of the best terrestrial analogues to Mars, Europa, and Enceladus and can provide important insights into the potential for subzero life and the cold limits of life both here on Earth and in our universe. There is also significant interest in the potential discovery of novel antimicrobial compounds from polar microbes and the biotechnological applications of cold-active enzymes. Given the overall lack of research focusing on microbes isolated from permafrost and permafrost-affected soils, the goal of the research in this thesis was to characterize cold growth, especially subzero growth, and cold adaptive properties of subzero growing microorganisms isolated from permafrost environments.

The bacterium *Planococcus halocryophilus* is capable of growth down to -15°C, making it ideal for studying adaptations to subzero growth. Proteomic analysis, comparing growth at -10°C, 23°C, and 23°C with salt, revealed a closely tied relationship between salt and cold stress adaptation. This is not unexpected given that *P. halocryophilus* likely inhabits small pockets of salty liquid water in permafrost. Cellular processes which displayed the largest changes in protein abundance at -10°C, and which are likely to play an important role in subzero growth, were peptidoglycan and fatty acid (FA) synthesis, translation processes, methylglyoxal metabolism, DNA repair and recombination, and protein and nucleotide turnover. We identified intriguing targets for further research at -10°C, including PlsX and KASII (FA metabolism), DD-transpeptidase and MurB (peptidoglycan synthesis), glyoxalase family proteins (reactive electrophile response), and ribosome modifying enzymes (translation turnover). PemK/MazF may have a crucial role in translational reprogramming under cold

conditions. At -10°C *P. halocryophilus* induces stress responses, uses resources efficiently, and carefully controls its growth and metabolism to maximize subzero survival.

We next examined features of cold adaptation in bacteria capable of subzero growth (cryophiles), isolated from permafrost, through comparative genomic analyses with closely related mesophiles. The cryophiles possess many genes typically associated with cold adaptation, including cold shock proteins, RNA helicases, oxidative stress proteins, and carotenoid synthesis enzymes, as well as cell wall and membrane modifications. Higher abundances of genes associated with compatible solute and sodium transport, traits important for osmoregulation, were observed and likely necessary for survival in the brine veins of permafrost. We investigated the presence of amino acid (AA) substitutions in the permafrost isolates that would indicate increased flexibility at cold temperatures, but found few significant differences between the cryophiles and mesophiles. However, there were exceptions, and we found significantly higher proportions of AA changes that would confer increased flexibility at cold temperature in several cryophiles, namely differences in proline, serine, glycine, and aromaticity. Comparing the average of all cryophiles to all mesophiles, we found that overall cryophiles had a much higher ratio of cold adapted proteins for serine (higher serine content), and to a lesser extent, proline and acidic residues (fewer prolines/acidic residues).

Lastly, we performed transcriptomic analyses on two cryophilic permafrost isolates with different growth profiles in order to characterize and compare their low temperature growth and cold-adaptive strategies. The two organisms, *Rhodococcus* sp. JG3 and *Polaromonas* sp. Eur3 1.2.1, shared several common responses during low temperature growth, including induction of translation and ribosomal processes, upregulation of nutrient transport, increased oxidative and osmotic stress responses, and stimulation of polysaccharide capsule synthesis. Recombination appeared to be an important adaptive strategy for both isolates at low temperatures, likely as a mechanism to increase genetic diversity and the potential for survival in cold systems. While *Rhodococcus sp. JG3* favored upregulating iron and amino acid transport, sustaining redox potential, and modulating fatty acid synthesis and composition, *Polaromonas* sp. Eur3 1.2.1 increased transcription involved in primary energy metabolism and the electron

transport chain, in addition to signal transduction and peptidoglycan synthesis. The increase in energy metabolism may explain why *Polaromonas* sp. Eur3 1.2.1 is able to sustain growth rates at 0°C comparable to higher temperature growth. For *Rhodococcus* sp. JG3, flexibility in use of carbon sources, iron acquisition, control of fatty acid composition in membrane, and modulating redox and co-factor potential may be ways in which this organism can grow at subzero temperatures in addition to sustaining growth over a wider range of temperatures (-5 to 30°C).

The results outlined in this thesis describe mechanisms and adaptations that are important for growth and survival of microorganisms in the subzero environment of permafrost. Overall, they point to the existence of multiple adaptive strategies that can support growth at subzero temperatures. While some appear to be shared, namely, sustaining translation processes, use of genomic redundancy and isozyme exchange, accumulation of compatible solutes, and induction of various stress responses, others are specific to each organism and reflect the potential for multiple approaches towards subzero adaptation and growth in cryophiles.

Résumé

Quelle est la limite de la vie dans le froid? Comment les microorganismes font-ils pour survivre et se reproduire dans les environnements sous zéro? La majorité des écosystèmes terrestres existent dans des environnements qui sont froids en permanence, où la température ne dépasse jamais 15°C. Le pergélisol, sol qui reste à une température inférieure ou égale à 0°C pendant au moins deux années consécutives, constitue 27% de tous les écosystèmes terrestres. Ces environnements contiennent diverses communautés microbiennes vivantes. Cependant, notre compréhension de la vie microbienne dans ces habitats, c'est-à-dire la croissance des microorganismes, leurs activités et leurs adaptations, reste limitée. Les environnements polaires sont considérés comme d'excellents analogues de Mars, Europe et Encelade et peuvent nous renseigner grandement sur la possibilité de la vie sous zéro et les limites de la vie dans le froid sur Terre et dans notre univers. Il existe également un intérêt important pour la découverte de nouveaux composés antimicrobiens qui proviennent de microbes polaires ainsi que pour les applications biotechnologiques d'enzymes actifs au froid. Compte tenu de l'absence générale de recherche axée sur les microbes isolés du pergélisol et des sols touchés par le pergélisol, l'objectif de la recherche menée dans le cadre de cette thèse était de caractériser la croissance dans le froid, en particulier sous zéro, et les adaptations au froid des microorganismes capables de croissance sous zéro isolés des environnements de pergélisol.

La bactérie *Planococcus halocryophilus*, qui est capable de croître jusqu'à -15°C, est idéale pour étudier les adaptations permettant la croissance sous zéro. Nous avons entrepris des analyses protéomiques nous permettant de comparer les croissances à -10°C, 23°C et 23°C avec du sel. Celles-ci ont révélé que les adaptations au froid et au sel sont étroitement liées. Ces résultats ne sont pas surprenants, étant donné que l'habitat naturel de *P. halocryophilus* est formé de petites poches d'eau liquide salée dans le pergélisol. Les processus cellulaires où l'on a détecté les plus grands changements dans l'abondance des protéines à -10°C, et qui jouent probablement un rôle important dans la croissance sous zéro, sont la synthèse des peptidoglycanes et des acides gras, les procédés de traduction, le métabolisme du méthylglyoxal, la réparation et la recombinaison de l'ADN et le renouvellement des protéines et

des nucléotides. Nous avons identifié des protéines qui seraient de bonnes candidates pour d'autres recherches dans le futur, y compris PlsX et KASII (métabolisme d'acides gras), DD-transpeptidase et MurB (synthèse de peptidoglycanes), des protéines de la famille des glyoxalases (les espèces réactives électrophiles) et des enzymes modifiant les ribosomes (contrôle de la traduction). PemK/MazF pourrait jouer un rôle crucial dans la reprogrammation translationnelle dans des conditions de froid. À -10°C, *P. halocryophilus* induit des réponses au stress, utilise efficacement les ressources et contrôle soigneusement sa croissance et son métabolisme pour maximiser la survie sous zéro.

Nous avons ensuite examiné les caractéristiques d'adaptation au froid de bactéries capables de croissance sous zéro (cryophiles), isolées du pergélisol, en faisant des analyses génomiques comparatives avec des mésophiles du même genre. Les cryophiles possèdent de nombreux gènes généralement associés à l'adaptation au froid, notamment les protéines de coup de froid, les hélicases à l'ARN, les protéines contre le stress oxydatif et les enzymes de synthèse des caroténoïdes, ainsi que les protéines associées aux modifications de la paroi et de la membrane cellulaire. Un plus grand nombre de gènes associés au transport de soluté compatible et de sodium, trait important pour l'osmorégulation, a été observé et est probablement nécessaire pour la survie dans les veines de saumure du pergélisol. Nous avons étudié la présence de substitutions d'acides aminés (AA) dans les isolats de pergélisol, qui indiquerait une flexibilité accrue dans les températures froides, mais nous avons trouvé peu de différences significatives entre les cryophiles et les mésophiles. Cependant, il y avait des exceptions, et nous avons trouvé des changements significatifs d'AA au niveau des prolines, sérines, glycines et résidus aromatiques, qui procuraient à plusieurs cryophiles une plus grande flexibilité dans des conditions froides. En comparant la moyenne des cryophiles à celle des mésophiles, nous avons constaté que les cryophiles avaient globalement un ratio beaucoup plus élevé de protéines adaptées au froid pour la sérine (plus de résidus de sérine) et, dans une moindre mesure, les résidus acides et la proline (moins de résidus acides/de proline).

Enfin, nous avons effectué des analyses transcriptomiques sur deux isolats de pergélisol cryophiles qui possèdent des profils de croissance différents afin de caractériser et de comparer

leur croissance à basse température et leurs stratégies d'adaptation au froid. Les deux organismes, *Rhodococcus* sp. JG3 et *Polaromonas* sp. Eur3 1.2.1, ont présenté plusieurs traits communs lors de la croissance à basse température, entre autres l'induction de la traduction et des processus ribosomiques, la régulation rapide du transport d'éléments nutritifs, l'augmentation des réponses au stress oxydatif et osmotique et la stimulation de la synthèse des capsules de polysaccharides. La recombinaison semble être une stratégie d'adaptation importante pour les deux isolats à basse température, qui fonctionne probablement comme mécanisme pouvant augmenter la diversité génétique et le potentiel de survie dans les systèmes froids. Alors que *Rhodococcus* sp. JG3 se concentre, pendant la croissance sous zéro, sur l'activation du transport de fer et d'acides aminés, le maintien du potentiel redox et la modulation de la synthèse et de la composition des acides gras, *Polaromonas* sp. Eur3 1.2.1 se concentre sur l'augmentation de la transcription impliquée dans le métabolisme de l'énergie primaire et la chaîne de transport d'électrons, en plus de la transduction des signaux et de la synthèse des peptidoglycanes. L'augmentation du métabolisme énergétique pourrait expliquer pourquoi *Polaromonas* sp. Eur3 1.2.1 est capable de maintenir des taux de croissance à 0°C comparables à la croissance à des températures plus élevées. La flexibilité dont fait preuve *Rhodococcus* sp. JG3 dans l'utilisation des sources de carbone, l'acquisition du fer, le contrôle de la composition des acides gras dans la membrane et la modulation du potentiel redox et de cofacteur pourrait quant à elle expliquer comment ce microorganisme arrive à croître à des températures allant de -5 à 30°C.

Cette thèse expose les adaptations et les mécanismes qui importent pour la survie et la croissance des microorganismes dans l'environnement sous zéro du pergélisol. Globalement, elle décrit l'existence de multiples stratégies d'adaptation qui peuvent soutenir la croissance à des températures inférieures à zéro. Certaines adaptations, telles le maintien des processus de traduction, l'utilisation de gènes redondants et l'échange d'isozymes, l'accumulation de solutés compatibles et l'induction de diverses réactions au stress, apparaissent dans tous les cryophiles étudiés ; d'autres adaptations, par contre, sont propres à chaque organisme et démontrent le potentiel des approches multiformes qui favorisent et permettent la survie et la croissance sous zéro des cryophiles.

Contributions to knowledge

1. This thesis includes the first proteomic study performed at -10°C on a subzero growing permafrost isolate (*Planococcus halocryophilus*), thus providing a better understanding of the mechanisms and functions important during subzero growth.

2. The first large-scale study characterizing amino acid changes associated with cold adaptation in subzero growing permafrost isolates across several genera. It identifies potentially unique and conserved amino acid modifications involved in subzero growth. In addition, the study also describes numerous whole genome traits associated with cold adaptation present in these isolates.

3. This is the first transcriptomic comparison of a subzero growing eurypsychrophile and a stenopsychrophile isolated from permafrost, giving insight into the similarities and differences in cold growth strategies between psychrophilic isolates with different growth profiles.

List of Figures

List of Tables

List of Abbreviations

AA – amino acids

ABC – ATP-binding cassette

Arg/R – arginine

BLAST – Basic local alignment search tool

COG – clusters of orthologous groups

CSP – cold shock proteins

FA – fatty acid

GO – glyoxal

GRAVY – grand average of hydropathicity

HSP – heat shock proteins

KEGG – Kyoto encyclopedia of genes and genomes

Lys/K – lysine

MDV – McMurdo Dry Valleys

MGO – methylglyoxal

nSpc – normalized spectral counts

OM – organic matter

RES – reactive electrophile species

ROS – reactive oxygen species

TA – toxin/antitoxin

TCA – tricarboxylic acid

TG – transglycosylases

TP – transpeptidase

UV – ultraviolet

Chapter 1. Introduction and Literature Review

Portions of this literature review appear in:

Isabelle Raymond-Bouchard and Lyle G. Whyte. (2017). From Transcriptomes to Metatranscriptomes: Cold Adaptation and Active Metabolisms of Psychrophiles from Cold Environments. In Psychrophiles: from biodiversity to biotechnology, 2nd Ed., Springer International, pp 437-457

Contributions of authors: I.R.-B. developed the overall structure and format and wrote the review. L.G.W provided general content guidance and editing.

1.1 Introduction: Polar microbiology

What is the cold limit of life? How do microbes survive and grow in cold environments? How important are polar microbes in carbon cycling, and what role will they play in global warming? These questions lie at the heart of polar microbiology research today. Most of the earth's ecosystems exist in permanently cold environments where the mean temperature never rises above 15°C (Kirby et al. 2012). These include subzero cryoenvironments, where the temperature rarely rises above zero. It was once thought that these environments were almost sterile, devoid of microbial life. But we now know that this is not the case; in fact, cold-adapted microorganisms are widespread in these environments (Gunde-Cimerman et al. 2012). The discovery of living, growing communities of microbes in cryoenvironments led to the emergence of polar microbiology. Over the past 30 years there has been a rapidly increasing interest in studying and understanding the microbiology of polar systems: the activities and survival of microbes in cold environments, their ecological interactions, and the role that microbial communities may play in global warming. Polar habitats serve as some of the best terrestrial analogues to Mars, Europa, and Enceladus (Osinski et al. 2006; Pollard et al. 2009). As such, these unique polar sites can provide important insights into the potential for life, both here on Earth and in our universe. From an astrobiology perspective, gaining a better understanding of the microbes that inhabit these environments is critical to our understanding of subzero survival and the cold limits of life in the Universe. Lastly, there is also significant interest in the potential discovery of novel antimicrobial compounds from polar microbes and the biotechnological applications of cold-active enzymes, namely due to increased catalytic activity at low temperatures and thermolability at higher temperatures.

1.2 Polar cryoenvironments

Cryoenvironments occur predominantly in polar and alpine regions and include permafrost, subzero saline springs and lakes, ice sheets and glaciers, sea ice, and lithic communities (Kirby et al. 2012). Polar habitats can be found in the Arctic, which covers 14.5 million km^2 and consists of the Arctic Ocean and parts of Canada, the United States (Alaska), Russia, Denmark (Greenland), Norway, Sweden, Finland and Iceland, and on the continent of Antarctica, which is

almost the same size, covering 14 million km^2. Microbial communities living in polar cryoenvironments and in subzero conditions must overcome extremely harsh conditions, the primary of which is limited liquid water availability and desiccation. Water is vital to life, necessary for the most basic of cellular functions. In addition, the cold temperatures themselves severely limit life processes. Microbes must contend with ice crystals and freezing, sometimes highly oligotrophic conditions, increased oxidative stress, prolonged background radiation, and thermodynamic effects on cell function (Steven et al. 2006; Bakermans et al. 2012). Permafrost, one of the largest and most important source of microbial polar cryoenvironments, accounts for 27% of all terrestrial ecosystems on earth (Williams and Smith 1989). All of the bacteria selected for study in this thesis were isolated from Canadian high Arctic permafrost cryoenvironments, except for one, which was isolated from dry permafrost in the McMurdo Dry Valleys, Antarctica. As such, this introduction will focus more specifically on permafrost as a cryoenvironment.

1.3 Permafrost

Permafrost is defined as soil that is permanently frozen for more than two consecutive years. It covers large portions of both the Arctic and Antarctica. Permafrost is covered by an active layer, varying in depth between 0.6 and 4 m, that thaws seasonally during summer (Kirby et al. 2012). In Eureka, Nunavut, permafrost ambient *in situ* temperatures have been measured at ~-17°C (Steven et al. 2008). A number of unique features can be found within permafrost including ice wedges and polygon terrain, cryopegs, taliks, pingos, and ground ice. Ice wedges, found in wet permafrost environments, often form V-shapes in the ground (2-4 m width and 5-10 m depth), which are created over thousands of years by thermal contraction, repeated seepage, and freezing of water in the soil (Wilhelm et al. 2012). This gives rise to the unique depressions that are characteristic of polygon terrains (Figure 1.1). Microbial permafrost habitats are believed to consist of thin films of salty water surrounding soil particles (Gilichinsky et al. 2003; Steven et al. 2006). The higher solute concentration in these brine veins acts as a freezing point depressant, allowing these environments to remain unfrozen at ambient permafrost temperatures. Overall, Canadian Arctic permafrost salt concentrations have been estimated at ~14.6 g/kg (Steven et al.

2007a), while salt concentrations in Siberian cryopeg brine lenses have been measured at 170-300 g/L (17-30%) (Gilichinsky et al. 2003). In addition, microbial life in permafrost must be able to survive oligotrophic conditions, along with limited water activity (aw), which has been measured at about 0.85 (Gilichinsky et al. 1993; Vishnivetskaya 2009).

Some of the earliest studies on the microbial flora of Arctic permafrost and active layer date back to the 1950s-1970s (Boyd 1958; Boyd and Boyd 1962, 1964, 1971). These studies were entirely culture-dependent and primarily concerned with elucidating the viability of microbes in permafrost in northern Canada and Alaska. In a western Arctic permafrost sample, Boyd and Boyd (Boyd and Boyd 1964) found bacterial cell counts of about 130 cells/g at a depth of 1.8 m. A wide variety of species including bacteria (*Bacillus* spp., *Azotobacter* spp., *Streptomycetes* spp., sulfate reducers) fungi and protozoa were found. More recent studies on Siberian and Canadian permafrost have revealed microbial biomass ranging from 10^2-10^8 cells/g (Shi et al. 1997; Vorobyova et al. 1997; Steven et al. 2007a; Gilichinsky et al. 2008; Wilhelm et al. 2011, 2012). Permafrost samples from Eureka, Nunavut on Ellesmere Island (Canada) had about 10^3-10^4 viable cells (CFU g^{-1}), but total bacterial cell counts of over 10^7 g^{-1} (Steven et al. 2007a). Viable cell counts in the active layer are thought to be about 100-1000 greater than in the permafrost, where conditions are generally harsher (Gilichinsky et al. 2008). In comparison to Arctic permafrost and associated soils, much less is known about Antarctic permafrost environments. What is known has been obtained mostly from studies of the McMurdo Dry Valleys (Gilichinsky et al. 2007; Tamppari et al. 2012; Bakermans et al. 2014; Goordial et al. 2016a). While levels of viable cells in Antarctic active layer soils are similar to those from Arctic surface soils (10^6-10^8 cells/g), permafrost biomass is generally lower in Antarctic soils (10^3-10^4 cells/g) (Gilichinsky et al. 2007).

Advances in next generation sequencing revolutionized polar microbiology and allowed researchers to investigate microbial diversity and activity in ways never possible before (Goordial et al. 2012). Despite harsh environmental stressors, the diversity of microbial communities in permafrost is fairly high. RNA and protein-based studies focusing on active community profiles from permafrost include microarrays and real-time PCR from high Arctic

and Antarctic soils (Yergeau et al. 2007, 2009, 2010; Blanco et al. 2012), rRNA transcript analyses from Arctic permafrost, the Dry Valleys and from thawing permafrost (Steven et al. 2008; Bakermans et al. 2014; Crevecoeur et al. 2015), and metaproteomic and metatranscriptomics of permafrost and permafrost-affected soils (Coolen and Orsi 2015; Hultman et al. 2015; Lau et al. 2015; Tveit et al. 2015; Buelow et al. 2016). Omic based studies of permafrost cryoenvironments are described in more detail in further sections below.

Overall, the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria dominate most molecular investigations of permafrost in the Arctic and Antarctica (Gilichinsky et al. 2007; Steven et al. 2009; Yergeau et al. 2010). It is believed that these bacteria may be better suited to tolerate the permafrost environment; Firmicutes and Bacteroidetes are spore forming and as such may be better able to endure the subzero conditions, while Actinobacteria are well known to be able to metabolize at low temperatures (Goordial et al. 2012). Bacteria isolated from permafrost in Eureka, Ellesmere Island, cover three phyla (Firmicutes, Actinobacteria, and Proteobacteria) and include bacteria in the genera *Bacillus, Paenibacillus, Sporosarcina, Micrococcus, Kocuria, Rhodococcus* and *Pseudomonas* (Steven et al. 2007a). While the Firmicutes were the most abundant isolated bacteria, the culture independent 16s rRNA analysis revealed that Actinobacteria and Proteobacteria, as well as halophilic archaea, were the most dominant phylotypes. The bacterial community in a Canadian high Arctic permafrost ice wedge was also found to be dominated by Proteobacteria and Actinobacteria (Wilhelm et al. 2012). In northeast Siberia, similar findings have been reported, with isolated bacteria belonging to the Actinobacteria, Proteobacteria and Firmicutes (Shi et al. 1997; Hinsa-Leasure et al. 2010).

1.4 Survival in cryoenvironments

The presence of microbial communities in polar habitats raises many interesting questions with regards to how these organisms are able to survive and grow in these extreme environments. In addition to the environmental stressors mentioned above, microbes must tolerate a number of thermodynamic and biochemical challenges, such as decreased membrane fluidity, reduced enzyme activity, stable inhibitory secondary RNA/DNA structures, and protein denaturation and

misfolding (Doyle et al. 2012; De Maayer et al. 2014). Liquid water in subzero environments occurs primarily in salty brine veins, where increased solute and salt concentrations act as freezing-point depressants, and as such, the survival of microbes in these environments requires the presence of mechanisms to tolerate physical and osmotic stress (Mader et al. 2006; Chin et al. 2010). In depth studies, particularly omic-based studies, of microorganisms isolated from cryoenvironments has given us insight into the growth, molecular mechanisms, and metabolisms that fuel the adaptation and survival of microorganisms at cold temperatures.

1.5 Psychrophiles and cold adaptation

Organisms capable of growth at lower temperatures are labelled as either eurypsychrophiles (formerly psychrotrophs), for those organisms that possess a broader growth range (Tmax >20 °C), and who, while capable of growth at very low temperatures (-15°C), usually retain optimum growth rates at temperatures above 20°C, or stenopsychrophiles (formerly 'true psychrophiles'), which exhibit narrower growth ranges (Tmax <20°C), and who usually grow best at temperatures ranging between 5-15°C (Cavicchioli 2016). In this introduction, the term psychrophile will be used when referring to both eury- and steno-psychrophiles. The term cryophile is generally reserved for those psychrophiles capable of growth at subzero temperatures. Studies looking to elucidate the adaptation that allow these microbes to survive in extreme cold environments have included genomic, transcriptomic and proteomic investigations, as well as investigations into cellular physiological changes, and the specific structural and functional properties of cold-adapted proteins.

To date over 70 euro- and steno-psychrophilic genomes have been sequenced, primarily bacterial and archaeal, providing us with important clues regarding their unique capabilities (Bakermans et al. 2012; De Maayer et al. 2014). This represents about 0.2% of all prokaryotic genome sequences. However, of these, only a few are capable of subzero growth and even fewer have been investigated using post-genomic (transcriptomic, proteomic, enzymatic) techniques (Bakermans et al. 2012). In addition, most of these organisms were isolated from marine environments, and as a result, analyses of these genomes may not reflect the cold adaptations of terrestrial isolates, whose environments are distinctly different from those of

marine isolates (Bakermans et al. 2012). In order to truly understand the cold adaptations of cryophiles and perform comparative analyses, it is important that we acquire additional genome sequences, especially of cryophiles from terrestrial habitats. Comparative analyses of genomes will become increasingly important, as they will allow us to identify those adaptations that are common to all cryophiles, and those that are specific to cryophiles from a particular environment. In addition to genomic sequences, new transcriptome and proteome studies from microbes actively growing at subzero temperatures will allow us to investigate expression patterns and cell mechanisms that ultimately allow cold-adapted microbes to reproduce in cryohabitats.

1.6 Omic insights of cold adaptation in psychrophiles

Much of our current knowledge of cold adaptive strategies in psychrophiles has been obtained through transcriptomic and proteomic studies looking at active transcription of genes and presence of proteins in low (<10°C) temperature conditions. To date, about 20 transcriptomes and 15 proteomes have been described for eurypsychrophiles and stenopsychrophiles including bacteria, archaea, fungi, algae, ciliates and diatoms, isolated from polar and cold environments (Tables 1.1 and 1.2). These omic studies include microarray and sequencing-based transcriptomes, and proteomic studies making use of two-dimensional gel electrophoresis and/or mass spectrometry/mass spectrometry analyses. Psychrophiles have been isolated from diverse cold environments, including permafrost, active layer, marine systems, ice and lakes. In almost all cases, the aim of current omic studies has been to gain insight into the metabolisms and adaptations that are important for growth at colder (<10°C) temperatures. Currently, only five transcriptomes exist for growth at temperatures below zero, in the bacteria *Exiguobacterium sibiricum* (-2.5°C) (Rodrigues et al. 2008), *Psychrobacter arcticus* (-6°C) (Bergholz et al. 2009), *Psychrobacter* sp. PAMC 21119 (-5°C) (Koh et al. 2017), *Planococcus halocryophilus* (-15°C) (Mykytczuk et al. 2013), and in the psychrophilic diatom *Fragilariopsis cylindrus* (-1°C) (Mock et al. 2005); and only 3 proteomes, in the bacteria *Colwellia psychrerythraea* (-1°C and -10°C) (Nunn et al. 2015), *Psychrobacter cryohalolentis* K5 (-4°C) (Bakermans et al. 2007), and *Psychrobacter* sp. PAMC 21119 (-5°C) (Koh et al. 2017). *Colwellia*

psychrerythraea has not been shown to grow at -10°C but it does remain active in ice at that temperature. Interestingly, while these subzero growing organisms are capable of growth at some of the coldest temperatures recorded to date, all, except *Fragilariopsis cylindrus* and *Colwellia psychrerythraea ,* are considered eurypsychrophiles rather than 'true' psychrophiles and grow best at temperatures higher than 15°C (Bakermans et al. 2007; Rodrigues et al. 2008; Bergholz et al. 2009; Mykytczuk et al. 2013; Koh et al. 2017). The remaining psychrophilic transcriptomes and proteomes studies were conducted at above zero temperatures (4-5°C or higher) as their cold representative temperature.

1.6.1 Cold shock proteins and chaperones

Cold shock proteins (CSP), which bind to nucleic acids and function as regulators of various cellular functions including translation, transcription, protein folding and RNA degradation (De Maayer et al. 2014), and other predicted chaperones, such as heat shock proteins (HSP; protein chaperones) and DEAD-box and associated RNA helicases, are amongst the most studied features of cold shock and adaptation in microorganisms, owing in large part due to their significant importance in aiding transcription and translation processes. Almost all transcriptome and proteome studies conducted on psychrophiles to date have found increased transcript levels of at least one of the CSPs, HSPs, DEAD-box and associated helicases and other chaperones at colder temperatures (La Terza et al. 2001; Mock et al. 2005; Gao et al. 2006; Bergholz et al. 2009; Campanaro et al. 2011; Chen et al. 2012; Mazzon et al. 2012; Kim et al. 2013; Dall'Agnol et al. 2014; Liu and Huang 2015; Koh et al. 2016). The psychrophilic alga, *Chlamydomonas* sp. ICE-L possesses 39 DEAD-box RNA helicase genes, over 10 of which are strongly upregulated at lower temperatures (Liu and Huang 2015). Four of the six CSPs found in the genome of *Exiguobacterium antarcticum* B7 are expressed at 0°C (Dall'Agnol et al. 2014). In the bacteria *Psychrobacter arcticus* (Bergholz et al. 2009) and *Shewanella oneidensis* (Gao et al. 2006), two isozymes of a DEAD-box RNA helicase and a CSP, respectively, are differentially expressed at colder and warmer temperatures. *Psychrobacter* sp. PAMC 21119 also makes use of differentially expressed CSP isozymes at -5°C and 20°C, with the -5°C isozyme showing signs of possible cold adaptation with increased protein flexibility (Koh et al. 2017). CSPs and

chaperones are not always upregulated at low temperatures. They may be constitutively expressed across all temperatures in some organisms, suggesting perhaps a readiness of these organisms to react quickly to changing temperatures. A CSP (cspA) from *P. arcticus* was found constitutively expressed at high levels across all temperatures (22°C, 17°C, 0°C and -6°C) (Bergholz et al. 2009). *Euplotes focardii*, an Antarctic stenopsychrophilic ciliate from benthic coastal sediments, grows optimally at 4-5°C and, while it possesses many copies of hsp70, expression of these proteins changes only minimally over a large change in temperature, contradictory to its relative, the eurypsychrotroph *Euplotes nobilii*; this indicates that *E. focardii* may have lost thermal adaptation mechanisms as a result of long term influence of the thermally (~ -2°C) stable waters from which it was isolated (La Terza et al. 2001).

1.6.2 Translation and ribosomal processes

Large changes in translation processes at lower temperatures are commonly observed in psychrophiles, perhaps unsurprisingly, as organisms must undergo a number of cellular modifications to the changing environmental conditions (De Maayer et al. 2014). It has also been postulated that increases in abundance of ribosomal and translation proteins may help compensate for lower protein synthesis rates, a common obstacle of growth at low temperatures as a result of reduced diffusion rates (Piette et al. 2011b). Translational and ribosomal processes dominate low temperature responses in several organisms, including *Psychrobacter* sp. PAMC 21119 at -5°C (Koh et al. 2017), *Colwellia psychrerythraea* at -10°C (Nunn et al. 2015), *Pseudoalteromonas haloplanktis* TAC125 at 4°C (Piette et al. 2010) and *Fragilariopsis cylindrus* Grunow at -1.8°C (Mock et al. 2005). In addition to cold shock proteins and RNA/DNA helicases discussed in the previous paragraph, increases have been described for translation initiation and elongation factors (Gao et al. 2006; Bakermans et al. 2007; Kawamoto et al. 2007; Dall'Agnol et al. 2014), tRNA synthethase (Zheng et al. 2007), and numerous ribosomal proteins (Seo et al. 2004; Bakermans et al. 2007; Williams et al. 2010).

1.6.3 Cell wall and cell membrane

Modifications to fatty acid biosynthesis and cell wall structure allow organisms to overcome decreased membrane fluidity at lower temperatures, and perhaps increase protection from environmental challenges such as ice crystals. As a result, elevated transcription of genes and increases in abundance of proteins involved in cell wall membrane and fatty acid biosynthesis is observed (Gao et al. 2006; Rodrigues et al. 2008; Bergholz et al. 2009; An et al. 2013; Kim et al. 2013; Mykytczuk et al. 2013). For example, the bacteria *Exiguobacterium sibiricum*, isolated from Siberian permafrost, increases expression of fatty acid desaturase and peptidoglycan biosynthesis genes, leading to a decrease in saturation and length of fatty acids in its membrane and a thickening of the cell wall, respectively, at subzero temperatures (Rodrigues et al. 2008). The extreme cryophile *Planococcus halocryophilus*, capable of growth down to -15°C, undergoes extensive modifications in its cell wall and membrane at subzero temperatures leading to the formation of a unique cellular envelope, initially observed through microscopy analyses, and confirmed by transcriptomic analysis showing significant increase in cell wall biosynthesis transcripts at -15°C (Mykytczuk et al. 2013, 2015).

1.6.4 Universal and oxidative stress responses

Universal stress response genes and genes involved in the oxidative stress response have also been established as important for cold adaptation (Chen et al. 2012; De Maayer et al. 2014). Universal stress proteins are small cytoplasmic proteins often transcribed in response to multiple cell stressors including nutrient starvation, heat shock, cell growth inhibition, presence of reactive oxygen species (ROS) (Kvint et al. 2003), and to cold conditions (Gao et al. 2006; Aliyu et al. 2016). An association between cold stress and oxidative stress has been observed, and ROS concentrations are known to increase at lower temperatures, likely the result of increased gas solubility and higher rates of enzyme activity to compensate for reduced reaction rates (Chattopadhyay et al. 2011; De Maayer et al. 2014). As a result, the oxidative stress response, and expression of genes encoding antioxidative enzymes, is often induced at lower temperatures by many psychrophiles (Bergholz et al. 2009; Chen et al. 2012; Mykytczuk et al. 2013; Tripathy et al. 2014; Aliyu et al. 2016). In contrast, in *Pseudomonas extremaustralis,*

genes involved in the oxidative stress response are repressed at colder temperatures, although iron-related proteins, including uptake and iron-binding proteins, were upregulated, which is theorized to play a redundant role in the reduction of oxidative stress (Tribelli et al. 2015).

1.6.5 Compatible solutes and antifreeze proteins

Compatible solutes, small water soluble organic compounds such as glycine betaine, ectoine and trehalose, are used by bacteria to maintain turgor pressure during growth in saline habitats. In cold-adapted bacteria, compatible solutes play an important role in allowing psychrophiles to resist osmotic pressure and prevent water loss caused by extracellular ice formation and increases in salinity often associated with cold environments (Doyle et al. 2012). Compatible solutes also play a role as cryoprotectants and increase the stability of macromolecules, membranes, and proteins, as well as enhancing folding and ligand binding in the latter (Thomas et al. 2001; Yancey 2005). Genes for the biosynthesis or transport of compatible solutes are widespread in psychrophilic microbes and increase in expression at cold temperatures in several instances (Rodrigues et al. 2008; Bergholz et al. 2009; Campanaro et al. 2011). Ice nucleating proteins or antifreeze proteins also function as cryoprotectants; they bind directly to the ice surface and prevent the growth of ice crystals (Davies et al. 2002). Higher expression of these proteins at cold temperatures is described in a number of psychrophilic organisms, including the Antarctic green algae *Chlorella vulgaris* NJ-7 and Arctic *Chlamydomonas* sp. ArF0006 (Li et al. 2009; Liu et al. 2011; Kim et al. 2013).

1.6.6 Metabolisms and energy/nutrient acquisition

Temperature can have a significant impact on expression of genes involved in metabolism and energy and nutrient acquisition. While psychrophiles are able to grow at low temperatures, their optimum growth temperatures usually remain above 10°C, and in the case of eurypsychrophiles, maximum productivity is usually in the 20-30°C range (Lauro et al. 2011; Siddiqui et al. 2013). Given that eurypsychrophiles are generally psychrotolerant rather than truly psychrophilic and tend to have growth rates that are highest above 20°C, it is not surprising that at lower temperatures transcripts for metabolic pathways and energy

production will often be downregulated. Energy production and most biosynthetic pathways are downregulated in *Psychrobacter articus* 273-4 (Bergholz et al. 2009), while in *Pseudomonas extremaustralis*, primary metabolism, the TCA cycle and amino acid metabolisms are repressed (Tribelli et al. 2015). Energy metabolism is also repressed in *Planococcus halocryophilus* at -15°C (Mykytczuk et al. 2013). Transcripts for methanogenesis and energy production in the methanogens *Methanococcoides burtonii* and *Methanolobus psychrophilus* R15 are more abundant at higher temperatures, as was core carbon and nitrogen metabolism in *M. burtonii* (Campanaro et al. 2011) and biosynthesis pathways in *M. psycrhophilus* R15 (Chen et al. 2012). This is not the case for most stenopsychrophiles, however, historically recognized as true psychrophiles, which may grow optimally at relatively low (5-15°C) temperatures. In these organisms, the opposite trend can be observed. The stenopsychrophilic algae *Chaetoceros neogracile* is very well adapted to its natural Antarctic environment, growing better at 4°C than at higher temperatures (10°C). This is in part due to an increase in photosynthesis and photosynthesis efficiency at lower temperatures (Hwang et al. 2008). More than 15% of all down-regulated genes at 10°C belonged to part of the photosynthesis pathway. Photosynthesis systems were >3-fold higher at 4°C in the Antarctic algae *Chlorella* sp. UMACC 234 (Chong et al. 2011). Similar results were seen for *Fragilariopsis cylindrus*, a stenopsychrophilic diatom that increases photosynthesis and abundance of some photosynthesis transcripts at -1°C compared to 7°C (Mock and Hoch 2005; Mock et al. 2005).

1.7 Omics of psychrophiles in cryoenvironments

Omic-based studies of polar environments, namely metagenomics, metatranscriptomics, and metaproteomics have emerged in recent years as powerful tools to not only characterize community members, but to determine the functional and metabolic potential of these communities, to identify and genome sequence novel microbes, and in the case of metatranscriptomics and metaproteomics, to characterize the active members, functions, and metabolisms of psychrophilic communities. Since the research described in this thesis centers on active mechanisms of cold growth in psychrophiles, this section will focus primarily on available metatranscriptomic and proteomic studies of cryoenvironments. Most of these

studies have emerged in the last 4-5 years and have given us significant in situ knowledge of the cold active mechanisms and metabolisms of psychrophiles (Table 1.3). At present, these omic studies include those focused on permafrost and permafrost-affected soils (Coolen and Orsi 2015; Hultman et al. 2015; Lau et al. 2015; Tveit et al. 2015; Buelow et al. 2016) and those looking at polar sea water, sea ice, and saline lakes (Ng et al. 2010; Williams et al. 2012; Toseland et al. 2013; Bertrand et al. 2015; Pearson et al. 2015).

1.7.1 Permafrost and associated soils

In Alaskan permafrost, the cold transcriptional response of psychrophiles is dominated primarily by amino acid transport and metabolism, energy production, and stress specific mechanisms, focusing on survival and long term cellular maintenance (biofilm formation, pilus assembly, virulence, horizontal gene transfer, DNA repair and the general SOS response) (Coolen and Orsi 2015). By contrast, thawed Alaskan soils have increased microbial activity as indicated by higher levels of transcripts involved in translation, ribosomal structure, biogenesis, extracellular protein degradation, and carbohydrate transport and degradation. Microorganisms from the Proteobacteria, Acidobacteria, and Actinobacteria are the dominant active members in frozen Alaskan permafrost, compared to the spore forming phyla Bacteriodetes and Firmicutes, which increased in abundance in thawed samples (Coolen and Orsi 2015). Increases in metabolic activities, as determined by the ratio of functional gene transcripts to genes, was also seen in thawed soils sequenced as part of a comparison study of three types of soils, permafrost, active layer, and thermokarst, from Fairbanks, Alaska (Hultman et al. 2015). Indeed, the active layer community exhibited more functional diversity than the permafrost and thermokarst bog samples. Many transcripts involved in specific biogeochemical cycles including methane oxidation, nitrate reduction, denitrification, and iron reduction were detected in the active layer samples. Fewer functional processes could be assigned to the permafrost, although dissimilatory ferric iron reduction was noted as a potentially important metabolic process. Cold shock proteins were detected in both permafrost and active layer. Proteins involved in chemotaxis and motility were also observed in permafrost, as were transporters, but these were much more abundant in active layer, suggesting greater potential

for transport of nutrients in thawed soils (Hultman et al. 2015). Overall, there were fewer proteins linked to cold tolerance and stress responses in the bog soils.

High Arctic peat soils from Svalbard, Knudsenheia, show active hydrogenotrophic and acetotrophic methanogenesis from Methanobacteriales and Methanosarcinaceae, respectively (Tveit et al. 2015). When artificially warmed (1-30°C gradient), active hydrogenotrophic and acetotophic members shifted to Methanomicrobiales and Methanosaetaceae, respectively, and CH_4 production increased rapidly. Methanosarcinaceae showed a high degree of flexibility in these soils over the tempreature range, and changed their methanogenic metabolism from acetotrophic to methylotrophic, likely due to increased substrate availability, as temperatures increased. Thermokarst bog soil from Alaska, which experiences higher temperatures than neighboring permafrost soil (~6 to -2°C for bog vs ~ -0.05 to -2°C for permafrost) has a high relative abundance of transcripts involved in methanogenesis, with a correspondingly very high measured rate of methane production, compared to permafrost. This was attributed partly to *Methanosarcina* spp., which comprised 6.8-10.5% of all 16s rRNA gene sequences (Hultman et al. 2015). Similarly, transcripts for heterotrophic methanogenesis, using acetate, methanol, and methylamine were predominant in thawed Alaskan permafrost compared to the frozen sample, while acetogenesis was exclusive to the thawed samples (Coolen and Orsi 2015). It has been suggested that organisms within these complex communities have adapted the capacity to shift their metabolisms, and consequently community metabolic contributions, across a temperature gradient, allowing them to remain competitive for available substrates (Hall et al. 2008; Tveit et al. 2015).

In the oligotrophic soils of the lower elevation McMurdo Dry Valleys (MDV), surface soils have active heterotrophic organisms, as well as active photoautotrophic cyanobacteria and algae (Buelow et al. 2016). Transcripts indicate the use of simple carbon sources including maltose, maltodextrin, and methylotrophy (serine-glyoxylate cycle), as would be expected in these low nutrient soils. These soils contain high concentrations of inorganic nitrate and this was reflected in the transcripts of the nitrogen metabolism subsystem, 31% of which could be assigned to nitrate and nitrite ammonification and 48% to ammonia assimilation. Interestingly, when water

and nutrients (organic matter, OM) was added to MDV surface soils, losses occurred in both taxonomic and functional diversity. There was a large decline in abundance of eukaryotes and significant losses of transcripts could be assigned to specialized pathways in bacteria and cellular function in eukaryotes. Pathways for glycolysis, solely assigned to the Actinobacteria, and maltose/maltodextrin utilization, mapping mostly to Actinobacteria (67%) and Gammaproteobacteria (30%), were more often under-expressed in the amended soils. Positive transcriptional responses were only seen in the OM amended soils, specifically the pentose phosphate pathway (mostly Actinobacteria and Firmicutes), sulfur metabolism (Actinobacteria) and transport systems (Firmicutes and Proteobacteria), perhaps indicative of the increased nutrient availability in the amended soils.

1.7.2 Sea water, sea ice, and lakes

Phytoplankton are the dominant primary producers in marine ecosystems and are important contributors to global biogeochemical cycles, especially carbon cycling systems (Field et al. 1998). Differential transcript expression by similar microbial populations across different temperature regimes in the environment, highlighting the importance of temperature on metabolic activity, can be illustrated by eukaryotic phytoplankton from polar, temperate, and tropical temperature zones of the ocean (Toseland et al. 2013). Translation of proteins is significantly affected by temperature, with ribosomes and transcripts of key enzymes involved in purine and pyrimidine metabolism highest in polar waters, though the rate of protein synthesis decreases at lower temperatures, indicating that translation may be the rate-limiting step for phytoplankton protein synthesis at lower temperatures. Evidence for cold adaption is seen in colder waters with heat shock proteins, DEAD-box RNA helicases, and fatty acid desaturases abundantly transcribed.

Diatom rich communities in sea ice from the Wilkins Ice Shelf in the Southern Ocean are clearly in the more stressful environment, when compared to neighboring waters from the Bransfield Straight and the western Weddell Sea, with transcriptomic data showing lower levels of carbohydrate and energy metabolism compared to the pelagic communities (Pearson et al. 2015). Organisms in sea ice show increased levels of ice-binding (antifreeze) proteins and cold

shock transcription factors. In the Western Antarctic Peninsula of the Southern Ocean, the most abundant proteins were those involved in transport, likely important for nutrient scavenging, ribosomal subunits, for translational processes, and various chaperones (Williams et al. 2012). Similarly to the work described in the above paragraph, temperature was found to have a strong impact on transcript expression in Southern Ocean water communities, especially transcription, translation and ribosome processes, which decreased with increasing temperature, highlighting the potential pressure that cold sea environments place on efficient protein synthesis (Pearson et al. 2015).

The cold hypersaline lakes, Ace Lake and Deep Lake, in the Vestfold Hills of Antarctica are characterized by distinct microbial communities dominated by green sulfur bacteria (*Chlorobiaceae*) and haloarchaea of the family Halobacteriaceaea, respectfully. Green sulfur bacteria in Ace Lake show presence of cold adaption proteins involved in DNA/RNA binding and protein folding, such as chaperones and helicases, as well as proteins involved in DNA modification, polysaccharide biosynthesis, and certain ABC transporters (Ng et al. 2010). There is also evidence of synthesis of monounsaturated fatty acids to modulate membrane fluidity. The green sulfur bacteria possess specific bacteriochlorophylls with extremely efficient chlorosomes, and exhibit a syntrophic relationship with sulfate-reducing bacteria. Together, these traits underscore the ability of green sulfur bacteria to dominate their environment. Deep Lake is the coldest aquatic environment known to support life (temperatures down to -20°C) (Tschitschko et al. 2016). Three members of the family Halobacteriaceae dominate Deep Lake, *Halohasta litchfieldiae*, *Halorubrum lacusprofundi,* and strain DL31 from an unknown genus closely related to *Halolamina*. Metaproteomic data from Deep Lake reveals presence of proteins involved in motility, likely for nutrient acquisition, and antioxidative enzymes for protection from UV light. Presence of specific transport and metabolic proteins reveals a preference of *Hht litchfieldiae* for carbohydrates, while DL31 and *Hrr lacusprofundi* appear more reliant on free amino acids and peptides. Overall, numerous proteins were detected that could support a range of metabolic functions.

1.8 Cold adapted proteins

At lower temperatures, psychrophiles must contend with decreased flexibility of proteins, which can have significant impacts on function. Therefore, the ability of cold active proteins to maintain flexibility is one of the most crucial of cold adaptations. Some of the general features commonly observed in cold adapted proteins that have been theorized to impart increased flexibility include fewer salt bridges, a lower content of hydrophobic and proline residues, less hydrogen bonds, a reduced Arg/(Arg+Lys) ratio, and increases in serine and glycine content and amino acids with small side chains (Table 1.4) (Russell et al. 1998; Aghajari et al. 1998; Georlette et al. 2000; Huston et al. 2004; Collins et al. 2005; Ræder et al. 2008; Metpally and Reddy 2009). For example, the elongation factor 2 protein of the archaea *Methanococcoides burtonii* exhibits greater structural flexibility as a result of fewer salt bridges, less densely packed hydrophobic cores and a reduction in proline residues in surface loops (Thomas and Cavicchioli 2000). A lipase from the Antarctic bacterium *Psychrobacter immobilis* B10 also contains a low number of salt bridges and proline residues, along with a reduced proportion of arginine to lysine residues, a small hydrophobic core and a very small number of aromatic-aromatic interactions (Arpigny et al. 1997). As these examples highlight, the adaptive changes vary somewhat from enzyme to enzyme and not all of the features listed above are used by a single enzyme. In general, many of the described changes act to reduce the number of potential hydrogen bonds and ionic interactions within the protein and this is thought to help counteract the increased rigidity at low temperatures. Prolines, covalently bonded to the nitrogen atom of the peptide group, create rigid kinks in peptide chains because of rotational constraints about the N-C_α bond of the backbone (Aghajari et al. 1998). Enzymes in several cold-adapted microbes, including the bacteria *Pseudomonas haloplanktis*, and *M. burtonii,* have been shown to have modified k_{cat} (rate of catalysis) or K_m (the substrate concentration at which the reaction proceeds at half its maximum rate) values when compared to their meso- or thermophilic homologs (Feller et al. 1992; Georlette et al. 2000; Thomas et al. 2001), properties which would increase the overall enzyme catalytic efficiency (k_{cat}/K_m) of the proteins at lower temperatures. This becomes important at lower temperatures where overall reaction rates would be lower due to reduced diffusion rates.

While certain amino acid compositional changes have been consistently noted for a number of psychrophilic proteins, such as reduced proline and arginine residues, these changes do not appear in all psychrophilic proteins. The isocitrate dehydrogenase from the psychrophile *Desulfotalea psychrophila* doesn't appear to possess the changes commonly associated with these proteins at the amino acid level, although a different set of potential adaptations, including clusters of methionine and acidic residues near the catalytic site were reported (Fedøy et al. 2007). There is also some contradiction in findings between different psychrophilic proteins. While some studies show decreases in hydrophobicity (Arpigny et al. 1997; Huston et al. 2004), others observe increases in hydrophobic residues in certain regions of the protein, notably surface exposed regions (Russell et al. 1998; Aghajari et al. 1998) and yet a third set report replacement of large hydrophobic residues with smaller hydrophobic amino acids (Thomas and Cavicchioli 1998; Watanabe et al. 2005).

Moving beyond individual proteins, a few studies have attempted to determine amino acid features of cold adaptation on a genome-wide scale through comparative predicted-protein analyses between psychrophile and mesophile and/or thermophile genomes (Table 1.4). These studies include those focused on psychrophilic protein comparisons against large nonredundant protein databases and comparisons against select specific mesophiles (Saunders et al. 2003; Rabus et al. 2004; Methé et al. 2005; Metpally and Reddy 2009; Zhao et al. 2010; Ayala-del-Río et al. 2010; Mykytczuk et al. 2013; Goordial et al. 2016b). Methods for comparison have included those that compare total amino acid counts across an entire genome between mesophiles and psychrophiles, to those that compare amino acid counts only between previously matched homologous proteins. Another potential adaption in psychrophilic proteomes, a higher content of non-charged polar residues, especially threonine and serine, has been reported in several of these studies (Saunders et al. 2003; Methé et al. 2005; Metpally and Reddy 2009). Overall, however, results from these studies have been somewhat inconsistent. While some identified a number of changes in amino acid composition or proportions in psychrophilic/psychrotrophic genomes, consistent with those identified in psychrophilic proteins (Saunders et al. 2003; Zhao et al. 2010; Ayala-del-Río et al. 2010), others have found limited changes (Methé et al. 2005; Mykytczuk et al. 2013; Goordial et al. 2016b),

and in some cases, none (Rabus et al. 2004; Yang et al. 2015). Contradictory findings have also been described, including variation in the reported content of charged residues (Russell et al. 1998; Saunders et al. 2003; Methé et al. 2005; Fedøy et al. 2007) and alanine (Metpally and Reddy 2009; Zhao et al. 2010), showing both increased and decreased numbers in different psychrophiles.

Nevertheless, some general changes stand out has having been detected in many of these psychrophilic protein analyses, whether in individual proteins or across genomes, such as the ratio of Arg/Lys, acidic residues, and proline and serine content. It remains somewhat unclear however, partly due to the lack of available genomic data on psychrophiles, whether these amino acid adaptations are conserved in most psychrophiles and generally representative of cold adaptation in proteins. It also remains to be determined if these amino acids changes exist and can be conclusively identified on such a large scale across whole genomes. Furthermore, in those studies that perform comparisons using distantly related organisms, it is not always apparent if the differences observed are the result of cold adaptation or simply phylogenic divergence. In this vein, comparisons using organisms in the same genus may provide a more stringent analysis, as we expect these members to share close common ancestry, and therefore observed amino acid differences between psychrophiles and their close mesophilic relatives are more likely to be the result cold adaptation.

1.9 Conclusion

The last 20 years have brought us significant knowledge and understanding of microbial life and activity in cold environments. Yet, there remains comparatively little known about the specific adaptations of sub-zero growing microbes, especially at -5 and below, and those that are isolated from permafrost. The work described in this thesis will aim to bridge that gap. In the future, it will be important to look more closely at the relationship and interplay between the growth ranges of psychrophiles, their metabolisms, their differing environments, and the range of temperatures and conditions they are likely to encounter in these habitats, when we look to make larger statements about cold adaptation and ecosystem function. Each of these will, individually, impact activity and function of psychrophiles and may explain some of the

differences and contradictions observed in omic studies of psychrophiles and their environment.

1.10 Objectives of this thesis

The goal of my research is to characterize cold growth and cold adaptive properties of subzero growing (cryophilic) microorganisms isolated from polar permafrost environments. The main questions that my research aims to address are: 1) *What are the molecular mechanisms utilized by cryophilic organisms for cold growth? What are the cold adaptive features present in cryophilic permafrost microorganisms that allow them to survive at subzero temperatures? Are there conserved features of cold adaptation and cold growth strategies common to cryophiles? Are there features of cold growth and survival that are linked to salt tolerance?*

The specific objectives of this thesis were:

1) To identify the proteins and pathways active and important for growth in subzero and high salt growth conditions in the halophilic cryophile *Planococcus halocryophilus*. Our goal was to use comparative whole cell proteomics and bioinformatic analyses to determine presence and abundance of proteins and functional mechanisms active during cold and high salt growth.

2) To characterize the genomic and amino acid traits of cold adaptation present in cryophilic permafrost isolates. With new cryophilic whole genome sequences, our goal was to use comparative bioinformatic analyses to identify features of cold adaptation, including potentially conserved or important amino acid changes, on a genome-wide scale. We also aim to investigate the presence of known cold adaptation genes and genomic redundancy in each cryophile.

3) To perform transcriptomic analyses during cold growth of two cryophilic permafrost strains, a eurypsychrophile and a stenopsychrophile, each with different growth profiles, in order to identify the actively transcribed genes present at low temperatures in these strains, and to determine, as well as compare and contrast, their cold growth strategies.

Figure 1.1. Characteristic polygon terrain of permafrost near the McGill Arctic Research Station (79°26’N, 90°46’W) on Axel Heiberg Island.

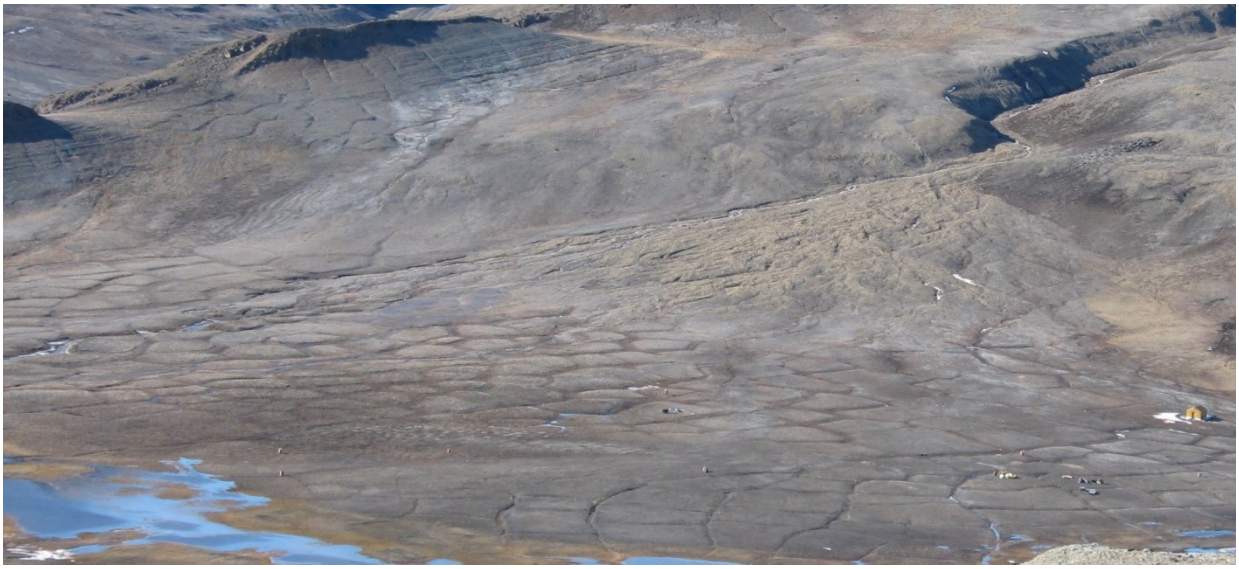


Table 1.1. Summary of transcriptomic studies from psychrophiles.

Organism	Location	Taxa	Temp. (°C)	Key findings	Reference
Nesterenkonia sp. AN1	Antarctic dry permafrost	Bacteria	5 and 21	Upregulation of oxidative response, universal stress proteins, chaperone functions, protein turnover, and cell membrane biogenesis, and induction of glyoxylate cycle at 5°C	Aliyu et al. (2016)
Psychrobacter arcticus 273-4	Siberian permafrost	Bacteria	-6, 0, 17 and 22	Transcription, translation, energy production, most biosynthesis pathways decreased at lower temps, but AA synthesis, RNases and peptidases increased. Evidence for isozyme exchange and shift to resource efficiency at lower temperatures	Bergholz et al. (2009)
Exiguobacterium antarcticum B7	Antarctic lake	Bacteria	0 and 37	Cold shock proteins most highly expressed transcripts at 0°C. Transcripts involved in translation, transcription, RNA helicases higher at 0°C	Dall'Agnol et al. (2014)
Shewanella oneidensis MR-1	Freshwater	Bacteria	8, 15, and 30	Lipid fluidity and catabolic processes for NADH and NADPH enhanced at lower temps. Differential regulation of cold shock proteins observed. Increase in translation, chemotaxis, chaperones and DNA metabolism at lower temperatures	Gao et al. (2006)
Psychrobacter sp. PAMC 21119	Antarctic permafrost	Bacteria	-5 and 20	Translation, ribosome structure and biogenesis upregulated at -5°C. Decrease in lipid transport, energy production, and metabolism.	Koh et al. (2016)
Planococcus halocryophilus Or1	Arctic active layer	Bacteria	-15 and 25	Cell envelope formation, membrane remodeling, and increased protein flexibility at -15°C. Isozyme exchange of genes involved in cell division, fatty acid synthesis, solute binding, oxidative stress response and transcriptional regulation	Mykytczuk et al. (2013)
Exiguobacterium sibiricum 255-15	Siberian permafrost	Bacteria	-2.5, 10 and 39	Constitutive adaptation to cold. Decrease in saturation and length of fatty acids in membrane, thickening of peptidoglycan at -2.5°C. Shift in carbon source utilization and isozyme exchange at different temperatures	Rodrigues et al. (2008)
Pseudomonas extremaustralis	Antarctic pond	Bacteria	8 and 30	Transcription regulation, signal transduction overexpressed at 8°C, but primary and AA metabolism repressed. Ethanol oxidation pathway induced and important for growth at low temperatures	Tribelli et al. (2015)
Methanolobus psychrophilus R15	Tibetan wetland	Archaea	4 and 18	Methanogenesis, biosynthesis and protein synthesis downregulated at 4°C, but translation, protein turnover and chaperones, and signal transduction increased	Chen et al. (2012)
Methanococcoides burtonii	Antarctic Ace Lake	Archaea	4 and 23	Transcriptional regulation, not translation, largely responsible for controlling gene expression. Increase of cell surface and secretory proteins, protein turnover, and maintenance of translation and initiation at 4°C. Energy generation and methanogenesis higher at 23°C	Campanaro et al. (2011)

Chlamydomonas sp. ICE-L	Antarctic algal mat	Algae	-20, -10, 0, 5, 10 and 15	Upregulation of 11 DEAD-box RNA helicase genes after 36 h cold stress. Fatty acid desaturases increased at low temps with higher proportion of polyunsaturated fatty acids	An et al. (2013); Liu and Huang (2015)
Chlorella UMACC 234	Antarctic snow	Algae	4, 20 and 30	Higher expression of photosystem II reaction center at 4°C	Chong et al. (2011)
Chaetoceros neogracile KOPRI AnM0002	Antarctic ocean	Algae	4 and 10	Metabolism and photosynthesis highest at 4°C. Photosynthesis genes made up 15% of downregulated genes at 10°C	Hwang et al. (2008)
Euplotes focardii and *Euplotes nobilii*	Antarctic pore waters	Ciliate	4 and 20	Heat shock protein not induced in response to heat shock; low constitutive expression at 4°C	La Terza et al. (2001)
Chlamydomonas sp. ArF0006	Norway pond	Algae	4	Cold response and photosynthesis prominent at 4°C, including heat shock proteins, transport functions, lipid biosynthesis, fatty acid desaturase, ribosomal proteins, antifreeze protein	Kim et al. (2013)
Chlorella vulgaris NJ-7	Antarctica	Algae	4 and 20	Abundant expression of antifreeze proteins, highest at 4°C, and higher than *C. vulgaris* UTEX259. Two novel cryoprotectants identified and highly expressed in NJ-7	Li et al. (2009); Liu et al. (2011)
Fragilariopsis cylindrus Grunow	Antarctic sea ice	Diatom	-1, -1.8, 5 and 7	Translation, ribosomal structure, biogenesis, post-translation modifications dominated transcripts. Expression of six different DNA/RNA helicases. Increase in numerous photosynthesis genes at -1°C	Mock et al. (2005); Mock and Hoch (2005)

Table 1.2. Summary of proteomic studies from psychrophiles.

Organism	Location	Taxa	Temp. (°C)	Key findings	Reference
Psychrobacter cryohalolentis K5	Siberian permafrost	Bacteria	-4, 4, and 16	31% of proteins identified varied significantly in abundance with temperature. Relief of translational stress via specialized ribosomal proteins and elongation factors, increased transport capacity, and energy production via glyoxylate cycle all important for subzero growth. Evidence for iron limitation during subzero growth.	(Bakermans et al. 2007)
Shewanella frigidimarina	Antarctica waters	Bacteria	4, 20, 28	Cell envelope, energy metabolism, redox homeostasis and cold acclimation proteins induced at 4°C. Evidence for isozyme exchange at the different temperatures. Evidence for low availability of nutrients and iron as main stress at low temperatures.	(García-Descalzo et al. 2014)
Shewanella livingstonensis	Antarctic seawater	Bacteria	4 and 18	Increased abundance of proteins at 4°C involved in RNA synthesis, protein synthesis and folding, membrane transport, and motility; necessary for efficient and accurate transcription, stable RNA folding, proper protein folding, and increase nutrient uptake efficiency.	(Kawamoto et al. 2007)
Psychrobacter sp. PAMC 21119	Antarctic permafrost	Bacteria	-5 and 20	Protein folding, metabolite transport, and membrane fluidity increased in abundance at -5°C. Isozyme exchange of cold shock proteins between temperatures. Limited overlap between transcriptome and proteome.	(Koh et al. 2017)
Acidithiobacillus ferroxidans D6	Elliot Lake, Canada	Bacteria	5 and 15	Chaperone functions, transport proteins and capsular polysaccharide export protein induced at cold temperatures, as well as maintaining iron oxidation through increased abundance rusticyanin.	(Mykytczuk et al. 2011)
Colwellia psychrerythraea	Arctic marine environments	Bacteria	-10, and -1	Presence of proteins involved in osmoregulation and polymer secretion at all temps. Reallocation of resources from DNA binding to DNA repair, and from motility to chemotaxis at subzero temperatures, as well as changes to protein synthesis and folding, iron and nitrogen metabolism, and the cell membrane.	(Nunn et al. 2015)
Pedobacter cryoconitis	Alpine cryoconite	Bacteria	1 and 20	Reduced abundance of proteins involved in stress response (chaperonin) and carbohydrate metabolism with concurrent increases in antioxidants at 1°C. 20°C nearing maximum growth temp and more stressful condition. Parallel up- and down-regulation of translation factors.	(Pereira-Medrano et al. 2012)
Pseudoalteromonas haloplanktis TAC125	Antarctic ice shelf	Bacteria	4 and 18	Translation, protein folding, antioxidants and membrane fluidity stimulated at low temps. Trigger factor, the first chaperone to interact with newly synthesized polypeptides, is most abundant protein at 4°C. Alternative chaperones are strongly repressed at cold temps.	(Piette et al. 2010, 2011a)

Exiguobacterium sibiricum 255-15	Siberian permafrost	Bacteria	4 and 25	Numerous cold acclimation proteins preferentially expressed at 4°C, including trigger factor, pyruvate dehydrogenase, phage shock protein A, and heat shock protein 70. Csps were constitutive at both 25 and 4°C.	(Qiu et al. 2006)
Bacillus psychrosaccharolyticus	Lowland marshes	Bacteria	0, 15, 30	Energy metabolism, translation, chaperones, and stress responses are globally upregulated at cold temps.	(Seo et al. 2004)
Sphingopyxis alaskensis	Cold marine waters	Bacteria	10 and 30	Dedicated protein-folding system identified at 10°C. Increased synthesis of unsaturated fatty acids in membrane, amino acid synthesis, transport, and iron homeostasis at low temps.	(Ting et al. 2010)
Psychrobacter articus 273-4	Siberian permafrost	Bacteria	4 and 22	5% NaCl and 4°C show combined effect on protein expression. Control of translation and ribosomal proteins important for the cold and salt response. Increased expression of cold shock proteins and chaperones at low temps.	(Zheng et al. 2007)
Methanococcoides burtonii	Ace Lake Antarctica	Archaea	4 and 23	Remodeling of cell envelope at low temperatures and focus on preserving translational capacity. Increase in chaperones and oxidative stress proteins at higher temperatures.	(Burg et al. 2010; Williams et al. 2010)
Antarctic microalgae ANF0048	Antarctic lake	Algae	4 and 15	Increased antioxidant activities and reduction in heat shock proteins at higher temperatures.	(Choi and Lee 2012)
Chaetoceros neogracile	Antarctic ocean	Algae	0 and 4	Exchange of primary chaperone protein utilized at the two temperatures. Changes in enzyme activity and isozyme profiles of antioxidant proteins.	(Park et al. 2008)

Table 1.3. Summary of metaproteomic and metatranscriptomic studies from permafrost, permafrost-affected, and polar marine environments.

Location	Sample type	Key active microbial members	Key microbial metabolic and functional activity	Reference
McMurdo Dry Valleys, Antarctica	Top soil (~12 cm)	Bacteria (largely dominant): Actinobacteria, Firmicutes, and Proteobacteria; Fungi: Ascomycetes and Basidiomycetes	Primarily chemoorganoheterotrophic bacteria, but also photoautotrophic cyanobacteria/algae; transcripts abundant for carbohydrate/nitrogen metabolism: serine-glyoxylate cycle, maltose/maltodextrin metabolism, and nitrate/nitrite ammonification	(Buelow et al. 2016)
	Amended top soil		Loss of functional diversity. Transcript loss assigned to specialized pathways in bacteria and cellular function in eukaryotes. Under-expressed: glycolysis and maltose/maltodextrin utilization. Over-expressed: pentose phosphate pathway, sulfur metabolism, and transport systems	
Kuparuk river, LTER field station, Alaska	Permafrost, acidic tundra	Bacteria: Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria; Archaea: Euryarchaeota; Fungi: Ascomycetes	Higher stress specific response: biofilm formation, DNA repair, SOS response, pilus assembly	(Coolen and Orsi 2015)
	Thawed permafrost	Bacteria: Firmicutes and Bacteroidetes; Archaea: Euryarchaeota; Fungi: Ascomycetes	Higher activity: abundance of transcripts for translation, ribosomes, biogenesis, carbohydrate degradation and transport, peptidases (Euryarchaeota, Bacteriodetes), hydrolases (fungi, Firmicutes), and methanogenesis (Euryarchaeota)	
Fairbanks, Alaska	Permafrost	Bacteria: Proteobacteria, Acidobacteria, and Firmicutes	Metabolic processes lower than active layer, but abundance of certain transcripts, i.e. methane oxidation, similar. Dissimilatory ferric iron reduction potentially important process	(Hultman et al. 2015)
	Active layer	Bacteria: Proteobacteria, Acidobacteria, and Actinobacteria; Archaea: Crenarchaeota	Increase in functional activity and diversity comparted to permafrost. Transcripts involved in a variety of metabolic processes, including methane oxidation, nitrate reduction, denitrification, and iron reduction	
	Thermokarst	Bacteria: Proteobacteria and	Higher abundance of transcripts for methanogenesis (*Methanosarcina*)	

	bog	Firmicutes; Archaea: Euryarchaeota		
Svalbard, Knudsenheia, Norway	Active layer peat soil	Bacteria: Firmicutes (Clostridiales), Archaea: Euryarchaeota (Methanobacteriales, Methanosarcinaceae)	Syntrophic relationship between propionate oxidation (Peptococcaceae) and methanogenesis (Methanobacteriales). Transcripts for hydrogenotrophic (Methanobacteriales) and acetotrophic (Methanosarcinaceae) methanogenesis	(Tveit et al. 2015)
	Warming peat soil	Bacteria: Bacteriodetes; Archaea: Euryarchaeota (Methanomicrobiales, Methanosaetaceae)	Decrease in Peptococcaceae (Clostridiales) for Bacteriodetes. Increase in CH_4 production and shift in active methanogen community to Methanomicrobiales (hydrogenotrophic) and Methanosaetaceae (acetotrophic). Increase in abundance of transcripts for methanogenesis from methylamines	
Axel Heiberg Island, Nunavut, Canadian high Arctic	Ice-wedge polygon	Bacteria: atmospheric methane-oxidizing bacteria	Active atmospheric methane sink; detection of transcripts for atmospheric CH_4-oxidizers (atmMOB). 100-fold higher atmMOB transcript reads in polygon trough compared to polygon interior. Rate of CH_4 uptake increases with temperature; theorized to increase by factor of 5-30 as Arctic warms	(Lau et al. 2015)
Southern Ocean (Wilkins ice shelf, Bransfield Straight, western Weddell Sea)	Ice	Heterokont algea: Dictyochophyceae	Carbohydrate and energy metabolism lowest in ice, concurrent active stress response: transcripts for antifreeze proteins and cold shock transcription factors increased. Nitrogen acquisition contrasted: transport of ammonia and urea high in Bransfield community, but absent in post-bloom Weddell sample. Cold temperature pressure on protein synthesis: Decreasing temperatures linked to increase in transcripts for translation, transcription, and ribosome processes	(Pearson et al. 2015)
	Seawater	Diatoms: Bacillariophyta		
Southern Ocean, West Antarctic Peninsula	Seawater	Phytoplankton; Bacterioplankton: Flavobacteria, Alphaproteobacteria, Gammaproteobacteria; Archaea: Crenarchaeota	Proteins involved in transport are abundant and have an important role in nutrient scavenging. Other prevalent proteins include ribosomal subunits and various chaperones. Recycling of glycine via metabolism of glycine betaine. Oxidation of ammonia to nitrite in winter conditions by active Crenarchaeota and Betaproteobacteria.	(Williams et al. 2012)
McMurdo Sound, Antarctica	Coastal seawater	Diatoms: *Fragilariopsis* and *Pseudonitzchia*; Bacteria: Gammaproteobacteria (*Oceanospirillaceae* and *Methylophaga*) and Bacteriodetes	Iron and cobalamin levels impact phytoplankton growth and are meditated by intricate bacterial-phytoplankton interactions. Transcript levels indicate diatoms dependent on cobalamin synthesis by bacteria (*Oceanospirillaceae*) for primary production, while bacteria dependent on diatoms for organic compounds	(Bertrand et al. 2015)

Southern and Arctic Oceans	Surface seawater	Diatoms: Bacillariophyta	Significant impact of temperature on phytoplankton metabolism. Transcripts show translation processes most impacted by temperature. Translation may be rate limiting step for protein synthesis at lower temperatures	(Toseland et al. 2013)
Barbados accretionary prism	Deep cold seep water	Bacteria: Epsilon- (*Sulfurovum*), Delta- (*Desulfo*. families) and Gamma-proteobacteria (*Vibrionales*), and methanotrophs (*Methylococcales*); Archaea: ANME-II Methanosarcinales	Primary productivity dependent on sulfide production primarily from AOM (ANME-II and Deltaproteobacteria). Supports sulfur oxidation and nitrate reduction by Ca. *Thiopilula* population and *Sulfuvorum* and *Sulfurimonas* spp.; Organoheterotrophy very active, especially *Photobacterium* spp, with carbon metabolism and transport functions	(Jones et al. 2015)
Ace Lake, Antarctica	Cold saline lake	Bacteria: Green sulfur bacteria	Evidence for cold adaptation from proteins involved in DNA/RNA binding and protein folding, DNA modification, polysaccharide biosynthesis, ABC transporters. Modulation of membrane fluidity via synthesis of monounsaturated fatty acids. Efficient photosynthesis via low light sensitive chlorosomes	(Ng et al. 2010)
Deep Lake, Antarctica	Hypersaline lake	Archaea: Halobacteriaceae (*Halohasta litchfieldiae*, *Halorubrum lacusprofundi*); Diatoms: *Dunaliella* spp.	Expression of proteins involved in motility for nutrient acquisition, and antioxidative enzymes as protective response to UV light. Light also used for photoheterotrophic growth. Transport and metabolic proteins reveal a preference of *Hht litchfieldiae* for carbohydrates, *Hrr lacusprofundi* for free amino acids and peptides. Proteins detected support a range of metabolic functions	(Tschitschko et al. 2016)

Table 1.4. Summary of cold adaptive amino acid changes of psychrophilic proteins.

Organism	Target	Key adaptations	Reference
Methanococcoides burtonii	Elongation factor 2	Fewer salt bridges, smaller residues in hydrophobic cores, and lower proline content in loops.	(Thomas and Cavicchioli 1998)
Antarctic bacterium	Citrate synthase	Compared to hyperthermophile, increased loop length with more charged residues and fewer prolines, increase in intramolecular ion pairs and solvent exposed hydrophobic residues.	(Russell et al. 1998)
Vibrio salmonicida	Uracil-DNA *N*-glycosylase	A few less arginines and prolines, more serine residues, lower Arg/(Arg+Lys) ratio and more charged residues.	(Ræder et al. 2008)
Colwellia psychrerythraea	Aminopeptidase	Fewer proline residues, ion pairs, and lower hydrophobicity	(Huston et al. 2004)
Colwellia maris	Isocitrate dehydrogenase	Overall amino acid sequence similar to mesophile. Replacement of larger hydrophobic residues with smaller hydrophobic residues in domain II, smaller number and content of proline residues in the third domain.	(Watanabe et al. 2005)
Alteromonas haloplanctis	α-amylase	Almost identical amino acid sequence between mesophilic and psychrophilic proteins in active site. Less proline residues in loops, decrease in arginine residues resulting in weaker interdomain contacts, and increased surface exposed hydrophobic residues	(Aghajari et al. 1998)
Desulfotalea psychrophila	Isocitrate dehydrogenase	Destabilizing clusters of methionine and acidic amino acids near catalytic sites, negatively charged surface residues to increase solvation and flexibility of catalytic site.	(Fedøy et al. 2007)
Psychrobacter immobilis B10	Lipase	Low arg/lys ratio, small hydrophobic core, and reduced proline content, salt bridges, and aromatic-aromatic interactions.	(Arpigny et al. 1997)
Methanogenium frigidum and *Methanococcoides burtonii*	Genome-wide protein content	Lower content of hydrophobic amino acids, especially leucine, and higher content of non-charged polar amino acid, especially glutamine and threonine. Tendency for fewer charged residues and more Gln and Thr in solvent-accessible areas.	(Saunders et al. 2003)
Shewanella spp.	Genome-wide protein content	Decrease in alanine, proline and arginine content of proteins.	(Zhao et al. 2010)
Desulfotalea psychrophila	Genome-wide protein content	No detectable signs of protein cold adaptive traits in genome proteins	(Rabus et al. 2004)

Multiple psychrophiles	Genome-wide protein content	Coil regions show significantly higher proportions of small or neutral residues, including serine, aspartic acid, threonine, and alanine. Underrepresentation of leucine and glutamic acid in helical regions, generally amino acids with aliphatic, basic, aromatic, and hydrophilic side chains.	(Metpally and Reddy 2009)
Planococcus halocryophilus	Genome-wide protein content	Less proline and fewer acidic and aliphatic residues compared to mesophilic genomes	(Mykytczuk et al. 2013; Ronholm et al. 2015)
Psychrobacter arcticus	Genome-wide protein content	Decreased content of proline, arginine, acidic residues, and hydrophobicity in a significant portion of genome. Most prominent in proteins involved in cell growth and reproduction.	(Ayala-del-Río et al. 2010)
Colwellia psychrerythraea	Genome-wide protein content	Trend for increase in polar residues, especially serine, the substitution of aspartate for glutamine, and decrease in charged residues on protein surfaces.	(Methé et al. 2005)
Rhodococcus sp. JG3	Genome-wide protein content	Most of the genome neutral with little sign of cold adapted protein, but still a significant proportion of cold to hot adapted proteins with less proline, acidic and aliphatic residues and a lower arginine/lysine ratio. Highest cold adaptation in cell division, energy and lipid metabolism, and transcription	(Goordial et al. 2016b)
Multiple psychrophiles	Genome-wide protein content	Meta-analysis comparing thermopiles, psychrophiles and mesophiles found no significant change in amino acid frequency for low temperature adaptation. Some evidence for substitution of polar uncharged amino acids for phenylalanine. For high temperature adaptation, significantly less serine and aspartic acid residues, and increases in arginine and proline residues. Low temperature adaptation is not the opposite of high temperature adaptation in terms of amino acid changes.	(Yang et al. 2015)

Connecting Text:

Given the lack of data on subzero growth of permafrost microorganisms and in order to increase our limited knowledge of molecular mechanisms that are important for subzero growth, especially below -5°C, we undertook a comprehensive proteomic analysis of the cryophilic halotolerant *Planococcus halocryophilus*, isolated from permafrost-associated soil in the Canadian high Arctic, comparing growth at -10 and 23°C and with high salt. A separate published study, aiming to characterize specifically the surface exposed proteins in *Planococcus halocryophilus* at low temperatures, was also conducted and is outlined briefly in Appendix 1.

Contributions of authors: The authors that contributed to this work are Isabelle Raymond-Bouchard[1], Karuna Chourey[2], Ianina Altshuler[1], Ramsunder Iyer [2,3], Robert L. Hettich[2], and Lyle G. Whyte[1]**.** I.R.-B. wrote the manuscript, carried out microbial culturing and protein extractions as well as all downstream analyses following protein identification. KC contributed to experimental design, performed liquid chromatography-mass spectrometry/mass spectrometry analysis of peptides and protein identification, and provided critical editing of manuscript. IA provided constructive experimental feedback and helped with creation of the cell figure. RI helped with creation of the heat maps and provided critical feedback. RH provided critical editing of manuscript. L.G.W contributed to overall experimental design and planning and provided critical editing.

[1]McGill University, Macdonald Campus, 21,111 Lakeshore Rd, Ste.-Anne-de-Bellevue, QC, Canada, H9X 3V9
[2]Chemical Sciences Division, Oak Ridge National Laboratory, 1 Bethel Valley Rd, Oak Ridge, TN, USA, 37831
[3]Graduate School of Genome Science and Technology, University of Tennessee, Knoxville, TN, USA, 37996

This manuscript has been accepted for publication with the journal Environmental Microbiology and is available in Early View online: DOI: 10.1111/1462-2920.13893

Chapter 2. Mechanisms of subzero growth in the cryophile *Planococcus halocryophilus* determined through proteomic analysis

2.1 Abstract

The eurypsychrophilic bacterium *Planococcus halocryophilus* is capable of growth down to -15°C, making it ideal for studying adaptations to subzero growth. To increase our understanding of the mechanisms and pathways important for subzero growth, we performed proteomics on *P. halocryophilus* grown at 23°C, 23°C with 12% w/v NaCl and -10°C with 12% w/v NaCl. Many proteins with increased abundances at -10 vs 23°C also increased at 23C-salt vs 23°C, indicating a closely tied relationship between salt and cold stress adaptation. Processes which displayed the largest changes in protein abundance were peptidoglycan and fatty acid (FA) synthesis, translation processes, methylglyoxal metabolism, DNA repair and recombination, and protein and nucleotide turnover. We identified intriguing targets for further research at -10°C, including PlsX and KASII (FA metabolism), DD-transpeptidase and MurB (peptidoglycan synthesis), glyoxalase family proteins (reactive electrophile response), and ribosome modifying enzymes (translation turnover). PemK/MazF may have a crucial role in translational reprogramming under cold conditions. At -10°C *P. halocryophilus* induces stress responses, uses resources efficiently, and carefully controls its growth and metabolism to maximize subzero survival. The present study identifies several mechanisms involved in subzero growth and enhances our understanding of cold adaptation.

2.2 Introduction

Most of the earth's ecosystems exist in permanently cold environments, including subzero environments, where the mean temperature never rises above 15°C (Kirby et al. 2012). The discovery of living, growing communities of microbes in polar habitats has raised numerous questions with regards to how microbes grow and survive at cold temperatures. Indeed, microorganisms living at subzero temperatures must overcome several kinetic, biochemical and physiological challenges, including decreased membrane fluidity, reduced enzyme activity, stable inhibitory secondary RNA and DNA structures , such as hairpins, and protein denaturation and misfolding (Doyle et al. 2012; De Maayer et al. 2014). In addition, since liquid water in subzero environments occurs primarily in salty brine veins, where increased solute and salt concentrations act as freezing-point depressants, the survival of microbes in these environments requires the presence of adaptive mechanisms that allow them to grow under cold and high salt conditions (Mader et al. 2006; Chin et al. 2010).

Studies looking into the cold-adaptations of microbes have included genomic, transcriptomic and proteomic investigations, as well as investigations into cellular physiological changes, and the specific structural and functional properties of cold-adapted proteins. Comparative studies looking at growth of cold-adapted microbes at both low (<10°C) and high (>10°C) temperatures, have found cold growth to be associated with significant changes in translation and transcription processes, with often elevated expression of ribosome and associated proteins, RNA helicases, and cold shock proteins. These studies also describe, at low temperatures, the presence and use of differentially regulated isozymes, upregulation of fatty acid, membrane transport and cell wall biosynthesis, accumulation of chaotropic metabolites, reduced expression of flagellar motility and carbon-metabolizing enzymes**,** production of antifreeze proteins, and increases in carotenoids and osmotic and oxidative stress responses (Chin et al. 2010; Miller and Whyte 2012; De Maayer et al. 2014).

To date about 75 eury- and steno-psychrophile genomes have been completely sequenced or have permanent sequence drafts (De Maayer et al. 2014). Of these, however, few are capable

of subzero growth and even fewer have been investigated using transcriptomic and proteomic techniques. In order to truly understand the cold adaptation and growth of psychrophiles it is important that we increase available transcriptome and proteomic studies, especially those that focus on microbes actively growing at subzero temperatures. A global picture of cold adaptation must also take into account differences between strains, and the environmental conditions from which the organism was isolated, which no doubt, will have a significant impact on the resulting methods of cold adaptation. In this context, it should be noted that many of the psychrophilic strains which have been sequenced to date were isolated from marine environments, and as a result, omic analysis of these genomes may not reflect the cold adaptations of terrestrial isolates, whose environments are distinctly different from those of marine isolates (Bakermans et al. 2012).

Planococcus halocryophilus, a cryophilic (capable of subzero growth) eurypsychrophile, halotolerant bacteria was recently isolated from the active layer of a permafrost core from Eureka (Nunavut) in the Canadian high Arctic (Mykytczuk et al. 2012). *P. halocryophilus* is capable of growth down to -15°C and respiration at least down to -25°C, the lowest temperature recorded to date for a bacteria (Mykytczuk et al. 2013), making it an ideal candidate to study cold adaptation in microbes. While capable of growth at subzero temperatures, *P. halocryophilus* grows optimally at 25°C, with a maximum temperature of 37 °C. The ability to grow over such a wide range of temperatures is intriguing and may reflect the harsh environment from which the organism was isolated, which experiences seasonal freeze and thaw cycles. *P. halocryophilus* is halotolerant, and so while salt is not required for growth, the bacterium is able to tolerate NaCl (w/v) concentrations of up to 19%, with optimal growth at 1.5% NaCl. While it is clear that *P. halocryophilus* would have had to adapt to withstand periods of subzero temperatures and high salt conditions, and has developed mechanisms that allow growth to continue even in these conditions, they are not optimum for growth, and constitute stressful conditions that the organism must contend with (Mykytczuk et al. 2013). Transcriptomic analyses comparing growth at -15°C and at 25°C revealed selective upregulation of genes involved in cell division, fatty acid synthesis, solute binding, oxidative stress response and transcriptional regulation in the subzero condition (Mykytczuk et al. 2013). In addition,

Mykytczuk et al. discovered unusual cell envelope modifications during subzero growth, leading to production of a crust like envelope composed of peptidoglycan (+ protein), choline and $CaCO_3$ (Mykytczuk et al. 2015). A study looking at the exposed surface proteins in *Planococcus halocryophilus* at -5°C and -10°C found differences in the composition of membrane, peptidoglycan, and transport proteins present at the surface (Ronholm *et al.*, 2015; Appendix 1). Interestingly, the composition of fatty acids in *P. halocryophilus* at lower temperatures was found to favour a higher ratio of saturated to branched fatty acids (Mykytczuk et al. 2013), contrary to what is observed in many cold adapted organisms. *P. halocryophilus* also shows evidence of genomic redundancy, including multiple copies of genes involved in osmolyte uptake. Considering its wide growth range (-15°C to 37°C), it is possible that these may be important and differentially expressed at different temperatures.

In order to gain a better understanding of the proteins and pathways active and important for growth in subzero and high salt conditions in this organism, we performed whole cell proteomic analyses of *P. halocryophilus*. This work is only the second proteomic study performed at such a low temperature (-10°C) (Nunn et al. 2015) and the first at -10°C for an organism isolated from permafrost. Given its wide growth range and ability to grow at subzero temperatures, gaining a better understanding of the mechanisms used by this bacterium for subzero growth is particularly interesting.

2.3 Results and Discussion

We used NanoLC-MS/MS to analyze whole cell lysates of *Planoccocus halocryophilus* grown in triplicate to late exponential phase in each of three conditions: 23°C, 23°C with 12% w/v NaCl (23C-salt), and -10°C with 12 w/v NaCl (-10 °C). Initial and final OD_{600} were 0.1 and 0.4 for -10°C, 0.1 and 0.4 for 23C-salt, and 0.1 and 1.0 for 23°C (subsequently diluted). Normalized spectral counts (nSpc) were obtained as outlined by Paoletti *et al.* (2006) and identification of two unique peptides per protein sequence was set as a prerequisite for protein identification. Only proteins that passed the Benjamini-Hochberg test ($q < 0.05$), had a fold change of at least 1.5,

and were identified in at least two of the three biological replicates in each growth condition were considered for further analysis.

Over 600 proteins were identified in each of the three conditions. Of these, 511 were present in all three conditions, while 34 and 156 were found to be unique to the -10°C and 23C-salt conditions, respectively, and 68 proteins were present in both conditions (Figure 2.1a). While most of the proteins identified were present in all three conditions, significant changes in abundance levels were observed in many of these proteins between the three conditions (Figure 2.1b and S2.1). A 1.5 or greater fold change in abundance (q value < 0.05) was observed for over 250 proteins at -10°C compared to 23°C and 23C-salt. Many of the proteins with high abundance at -10°C vs 23°C also showed enhanced protein abundance at 23C-salt compared to 23 °C, indicating an overall closely tied relationship between salt and cold stress adaptation in *P. halocryophilus*. Additionally, and correlating with a unique and important role in cold growth, almost all of those proteins highly abundant at -10°C compared to 23C-salt were also found increased compared to 23°C. While some overlap was observed for those proteins with lowered abundance at -10°C vs 23°C and -10°C vs 23C-salt, most of these differed between the two conditions (-10°C vs 23°C and -10 °C vs 23C-salt).

2.3.1 Pathways and mechanisms

Predicted COG categories and KEGG pathways for differently abundant proteins at -10°C vs 23°C or 23C-salt are shown in Figures 2.2 to 2.4. Proteins unique to a growth condition with no detectable spectral counts in the other conditions are not included in the KEGG analysis. KEGG pathways and modules assigned to proteins detected only at -10°C vs 23°C or 23C-salt are provided in Table 2.1. Since KEGG pathways which possessed the highest number of matched proteins were not always those with the highest change in abundance, Figures 2.3 & 2.4 show KEGG annotations in terms of both total number of proteins that map to each pathway and most significant abundance changes observed for each of the pathways. As is the case with other psychrophiles (Gao et al. 2006; Rodrigues et al. 2008) a significant proportion (~20%) of differentially abundant proteins at -10°C are either hypothetical or have general function

predictions only and do not readily map to known COG categories and KEGG pathways (Figure S2.1 Group R).

Of the proteins for which COG and KEGG assignments were possible, pathways related lipid and fatty acid metabolism, peptidoglycan synthesis, amino acid and nucleotide turnover and metabolism, ribosomal and translation processes, DNA recombination/repair, and energy acquisition were found to have the highest abundance in the -10°C condition, either in terms of greatest increases in abundance or total proteins mapped (Figures 2.2 to 2.4). However, decreases in protein abundance was also noted for other proteins that map to many of these processes, indicating that overall growth at -10°C generally involves significant changes in abundance of proteins in these cellular processes, rather than a simple increase or decrease of a specific process. Exceptions to this include peptidoglycan biosynthesis, nucleotide turnover, DNA repair, mRNA biogenesis, nicotinamide metabolism and fructose/mannose metabolism, for which only very minor to no decreases in abundance were seen for proteins in these categories, but for which there was a significant increase in several proteins (Figure 2.3 & 2.4). Overall fewer proteins were found to be differentially abundant in the -10°C vs 23C-salt condition (Figure 2.4), as compared to the -10°C vs 23°C condition (Figure 2.3). This is not surprising since, given the cold and salty permafrost affected environment from which *P. halocryophilus* was isolated, we would expect some overlap in the adaptions to both cold and salt stress. Some significant changes were seen however between -10°C and 23C-salt, with the most notable increases in -10°C grown cells observed for proteins involved in peptidoglycan biosynthesis, DNA repair, and sulfur, B6 and amino acid metabolisms (Figure 2.4). Those proteins with significant differential abundances at -10°C and which appear to have important roles for subzero growth (Tables 2.1-2.2, S2.1 and Figure S2.1) are discussed in more detail below with regards to their respective pathways and potential cellular functions.

2.3.2 Cold shock proteins, chaperones, and helicases

Cold shock proteins (CSP), which bind to nucleic acids and function as regulators of various cellular processes including translation, transcription, protein folding and RNA degradation, along with RNA helicases, which act as chaperones to destabilize potentially inhibitory

secondary RNA structures, and proteins chaperones, are amongst the most studied features of cold growth and adaptation in cold-adapted microorganisms, owing in large part to their significant importance in stabilizing cold translation and transcription processes. Increases in abundance of one of more of these proteins has been seen in many of the transcriptomic/proteomic studies performed on eury- and steno-psychrophiles to date (reviewed in: De Maayer et al. 2014 and Miller & Whyte 2012). The present study showed a 2-fold increased abundance of an RNA helicase at -10°C compared to the 23°C and 23C-salt condition, while a DNA helicase with homology to RuvA, involved in recombination, was increased >1.5 and >3.5 fold compared to 23°C and 23C-salt respectively (Table 2.2). The protein chaperone, ThiJ, with putative roles as a protein deglycase and in the oxidative stress response (discussed in more detail below) was ~3.25-fold increased. 2 cold shock proteins were detected in the organism (Table 2.2), but these were found to be abundant in all three conditions, perhaps signaling a preparedness of the organism to react quickly to changing temperatures, as would be expected in its natural active layer environment, which experiences large yearly freeze-thaw temperature fluctuations. This is not uncommon in psychrophiles; constitutive expression of these proteins across broad temperature ranges are reported in several psychrophiles (De Maayer et al. 2014), including the permafrost bacteria *Psychrobacter arcticus* 273-4 (Bergholz et al. 2009) and the soil isolate *Arthrobacter globiformis* (Berger et al. 1996).

2.3.3 Cell wall biosynthesis and remodeling

A D-alanyl-D-alanine carboxypeptidase (DD-transpeptidase) was differentially abundant by more than 9-fold at -10°C (no spectral counts at 23°C) (Table 2.1), while a multimodular transpeptidase-transglycosylase and a UDP-N-acetylenolpyruvoylglucosamine reductase (MurB) were strongly (> 2-fold) increased at -10°C compared to both 23°C and 23C-salt (Table 2.2). A second transpeptidase-transglycosylase was increased > 1.5-fold compared to 23°C. D-alanyl-D-alanine carboxypeptidases are transpeptidases (TP) responsible for cross-linking of peptide chains during the last steps of cell wall synthesis (Lee et al. 2003), while MurB is the enzyme responsible for the second committed step of peptidoglycan synthesis. The previous

transcriptome study of *P. halocryophilus* (Mykytczuk et al. 2013) found the first enzyme involved in synthesis, MurA, to also be increased, although this was not seen in the present proteomic study (i.e. it had no normalized spectral counts (nSpc) in this study). Multimodular transpeptidases/ transglycosylases (TG) are proteins which, in addition to crosslinking of peptide chains by TP, are also responsible for polymerization of the disaccharide units by the activity of TG (Ramachandran et al. 2006). Together, these results indicate that peptidoglycan synthesis at subzero temperatures in *Planococcus halocryophilus* is a potentially crucial adaptation mechanism and is consistent with previous studies detailing the complex encrustation observed around *P. halocryophilus* cells during subzero growth (Mykytczuk et al. 2013), which was shown to be composed of peptidoglycan (+ protein), choline and $CaCO_3$ (Mykytczuk et al. 2015). The increased peptidoglycan is believed to provide an abundant negatively charged matrix that allows for $CaCO_3$ mineralization at the surface. The role that this encrustation plays, if any, in subzero growth is unknown but it has been suggested that it may offer additional protection from the harsh environment (Mykytczuk et al. 2015). The psychrotroph *Exiguobacterium sibiricum*, isolated from Siberian permafrost, also increases expression of peptidoglycan biosynthesis genes at subzero temperatures leading to a thickening of the cell wall (Rodrigues et al. 2008). Two N-acetylmuramoyl-L-alanine amidases were also found to be more abundant at both -10°C and 23C-salt vs 23°C (Tables 2.1 & 2.2), but one of these (gi|495774369) was much greater (> 9-fold) at -10 °C. This is intriguing since amidases are responsible for the breakdown of peptidoglycans, hydrolyzing the link between N-acetylmuramoyl and L-amino acid residues. However, it is possible that in this case the amidases may function in enabling the significant remodeling of the peptidoglycan as shown previously by Mykutczuk et al. (Mykytczuk et al. 2013, 2015) leading, in combination with increased synthesis, to formation of the crust. These findings are somewhat contradictory to those from the surfaceomics study (proteomic analysis of the cell surface) of *P. halocryophilus*, which did not find a transpeptidase/transglycosylase or N-acetylmuramoyl-L-alanine amidases at subzero temperatures, but this may be due to the more limited number of proteins identified at -10°C in that published study (Ronholm et al. 2015).

2.3.4 Fatty acid and lipid synthesis and modifications

Membrane remodeling is one of the most wide-spread adaptations observed in cold growing organisms and upregulation of related enzymes has been seen in numerous psychrophiles (Gao et al. 2006; Rodrigues et al. 2008; Bergholz et al. 2009; Kim et al. 2013; Mykytczuk et al. 2013). Modifications to fatty acid and membrane biosynthesis allow organisms to overcome decreased membrane fluidity at lower temperatures. In *P. halocryophilus*, PlsX, a phosphate:acyl-ACP acyltransferase was very strongly increased (> 9 fold) at -10°C, with no observed abundance at 23°C or 23C-salt (Table 2.1). PlsX links the fatty acid (FA) synthase II pathway to the phospholipid synthesis pathway in gram-positive bacteria by converting acyl-ACP *de novo* end products to acyl-PO_4 derivatives (Parsons et al. 2014). A deletion mutant of PlsX in *Staphylococcus aureus* was a FA auxotroph (Parsons et al. 2014), while in *Streptococcus pneumoniae* and *Streptococcus mutans,* PlsX deletions led to increases in saturated fatty acids (SFA) and changes in fatty acid chain length (Parsons et al. 2015; Garcia et al. 2016). In *Bacillus subtilis*, a close relative of *P. halocryophilus*, depletion of PlsX led to cessation of both FA and phospholipid synthesis (Paoletti et al. 2007). These studies suggest a crucial role of PlsX in modulating FA and phospholipid synthesis, chain length, and for accumulation of unsaturated fatty acids in the membrane. In addition to PlsX, an acyl-coA hydrolase and a homologue to the KASII protein from *Escherichia coli* were also increased (> 2-fold) (Table 2.2). Acyl-CoA hydrolases are responsible for hydrolysis of fatty acyl-CoA esters to free coenzyme A and a carboxylate. *E. coli* KASII, a 3-oxoacyl-[acyl-carrier-protein] synthase, is important for temperature-dependent regulation of membrane fatty acid composition (de Mendoza and Cronan Jr 1983). KASII can perform all elongation steps in SFA synthesis and is uniquely required for the last step in the unsaturated pathway, the elongation of palmitoleate (C16 monounsaturated) to *cis*-vaccenate (C18 monounsaturated) (Campbell and Cronan Jr 2001). Deletion mutants were found to be deficient in synthesis of longer unsaturated fatty acids (UFA). A significant increase in abundance of PlsX, a hydrolase, and KASII in the present study likely points to major fatty acid membrane modifications at subzero temperatures. Indeed, previously published FA profiles of *P. halocryophilus* reveal significant shifts in FA and lipid composition at lower temperatures (Mykytczuk et al. 2013). However, curiously, *P.*

halocryophilus decreases unsaturated and branched chain FAs at lower temperatures in favor of saturated FAs, contrary to what is observed in many psychrophiles, who tend to favor unsaturated and branched chain fatty acids, as well as shorter chain FA, which allow for increased fluidity of the membrane. In addition, longer chain SFAs were observed at subzero temperatures in *P. halocryophilus*, while longer chain UFAs were not (Mykytczuk et al. 2013).

Carotenoid pigments have been proposed to play a role in modulating membrane fluidity and in conserving homeoviscosity (Rodrigues & Tiedje 2008; Chattopadhyay 2006). In this capacity, carotenoids act to increase membrane rigidity to balance the higher membrane fluidity as a result of changes in the composition of FA to favor branched chain and unsaturated fatty acids. The two enzymes involved in the biosynthesis of the carotenoid lycopene from phytoene, a prolycopene-producing pro-zeta carotene desaturase and a phytoene desaturase, were more abundant in the two stress conditions (Table 2.2). Since *P. halocryophilus* increases saturated fatty acids instead of branched or unsaturated, a mechanism as described above would not be consistent. However, this does not rule out the possibility that carotenoids associated with the membrane are playing an as yet unknown but important role in membrane activity. The proteins identified in this study no doubt play an important role in affecting fatty acid composition at these temperatures. However, the unexpected findings described above raise interesting questions with regards to the mechanisms used by *P. halocryophilus* to modulate membrane fluidity, thereby counteracting potential risks from increased membrane rigidity at low temperatures, and warrants further investigation.

2.3.5 Transporters

A hydrophobic/amphiphilic exporter belonging to the HAE1 family, and a multidrug efflux pump, were unique to -10°C vs the 23°C condition, while the exporter was also unique compared to 23C-salt (Table 2.1). This is in addition to three ABC transporter binding proteins, involved in cysteine, ferric iron, and manganese transport, which were all increased in abundance as compared to 23°C, and also increased vs 23C-salt in the case of manganese transport binding (Table 2.2). The same cysteine and ferric iron ABC transporter proteins (gi|495772257| & gi|495773646|) were previously detected at the surface of *P. halocryophilus*

in both cold and salt stress conditions (Ronholm et al. 2015). While the earlier transcriptome study of *P. halocryophilus* did not detect the increased transcription of these specific transporter proteins, similar increases were seen in efflux and multidrug permeases as well as ABC transporter binding proteins for sugar and xylose (Mykytczuk et al. 2013). In order to overcome lower rates of transport and diffusion across the membrane in colder environments (Nedwell 1999), transporters are commonly increased in psychrophiles. Permeases, exporters and ABC transporters involved in metabolite and amino acid transport have been detected in previous cold transcriptomic and proteomic studies (Mock et al. 2005; Campanaro et al. 2011; Koh et al. 2017), including a hydrophobe/amphilphile efflux protein and two ferric iron transporter-related proteins in *Psychrobacter cryohalolentis* K5 (Bakermans et al. 2007).

2.3.6 Translation and ribosomal processes

Translation and ribosomal processes saw the most significant changes in protein abundance, with an overall large increase in proteins involved in ribosome biogenesis, aminoacyl-tRNA biosynthesis, RNA degradation, and translation factors (Figures 2.2-2.4). A peptide chain release factor 2 was only detected in the -10°C condition (Table 2.1). This enzyme binds to the ribosome and is involved in ending mRNA translation at the stop codon and subsequent release of the completed peptide chain. Increases in abundance of this protein were also detected in the marine bacterium *Sphingopyxi alaskensis* (Ting et al. 2010). tRNA A37 threonylcarbamoyladenosine dehydratase (CsdL) was present only at -10°C and 23C-salt (Table 2.1); CsdL is responsible for the formation of cyclic threonylcarbomoyladenosine (ct_6A37) in tRNAs that read adenine and shown to be important for the recognition of noncognate codons and the preservation for decoding efficiency (Miyauchi et al. 2013). In addition, a > 5-fold increase in a protein involved in translation initiation and an RNA chaperone (Hamma and Ferré-D'Amaré 2006), the ribosomal large subunit pseudouridine synthase B, was observed at -10°C compared to 23°C (Table 2.2). RsmB and RsmE, rRNA small subunit methyltransferases, were increased almost 4 and 2-fold in abundance, respectfully (Table 2.2). Methyltransfereases are important regulators of translation and play important roles in stabilizing rRNA. Expression of certain methyltransferases, including RsmE, has been shown to change during temperature

stress (Baldridge and Contreras 2013), as is seen in this study. Noteworthy increases at -10°C were seen in 3 GTP-binding proteins, with probable roles in ribosome biogenesis and there was a change in abundance of ribosomal subunits with certain ribosomal proteins being reduced in favor of others, which included the 30S ribosomal protein S17, with a role in translation accuracy (Figures 2.3 & 2.4 and Tables 2.2 & S2.1). Variation in the content and number of ribosomal proteins has been shown to contribute significantly to ribosomal performance, as have rRNA modifications such as methylations (Sharma et al. 2003; Baldridge and Contreras 2013).Together, these results highlight an overall focus of *P. halocryophilus* at -10°C towards increasing translation rates and RNA turnover, all the while keeping translation processes tightly regulated and preserving stability and decoding efficiency. Large changes in translation processes at lower temperatures are commonly observed in psychrophiles, perhaps unsurprisingly, as organisms must undergo a number of cellular modifications to the changing environmental conditions (De Maayer et al. 2014). In addition, the overall abundance increase seen in numerous ribosomal and translation proteins would support an attempt by *P. halocryophilus* to compensate for lower protein synthesis rates, a common obstacle of growth at low temperatures as a result of reduced diffusion rates (Piette et al. 2011b). The proteomic response of the psychrophile *Colwellia psychrerythraea* at -10°C was also dominated by an abundance increase in translation processes and protein synthesis (Nunn et al. 2015), and we suggest that this maybe be an especially important adaptation to sustain continued growth at subzero temperatures. In certain psychrophilic communities, translation has been postulated to be the rate-limiting step for protein synthesis at low temperatures (Toseland et al. 2013).

2.3.7 Nucleotide and amino acid synthesis and turnover

Amino acid and nucleotide metabolism also saw strong changes in abundance (Figures 2.2-2.4). The largest change in nucleotide metabolism seen at -10°C was in the abundance of a uridine kinase, a key enzyme in the nucleotide salvage pathway, which was not detected at 23°C or 23C-salt (Table 2.1) and a 5-nucleotidase (Table 2.2), which converts 5'-nucleotides to their corresponding nucleosides and plays an important role in facilitating uptake of nucleotides (Bengis-Garber and Kushner 1982). Several proteins involved in amino acid biosynthesis were

shifted in abundance. A homocysteine S-methyltransferase, which catalyses production of methionine from homocysteine, was detected at -10°C but not in the 23°C condition and, while present in the 23C-salt condition, its abundance remained higher at -10°C (Table 2.1). Additional proteins involved in amino acid synthesis that were increased at -10°C and 23C-salt include chorismate synthase and D-3-phosphoglycerate dehydrogenase (Table 2.2). These results suggest the potential for increasing biosynthesis of certain amino acids at -10°C; however, it should be noted that abundance decreases in enzymes involved in the biosynthesis of other amino acids was also detected (Table S2.1 and Figure S2.1 Group E). There is evidence for the breakdown of extraneous amino acids, including the arginine utilization protein, RocB, implicated in the arginine degradation pathway (Calogero et al. 1994), and the alpha subunit of L-serine dehydratase, necessary for the breakdown of serine to pyruvate via the gluconeogenesis pathway (Table 2.2). Transcription of rocB was also increased in the transcriptomic study of *P. halocryophilus* (Mykytczuk et al. 2013). Interestingly, we also noted a concurrent decrease in arginase (Table S2.1), responsible for breaking down arginine to ornithine, suggesting that arginine utilization and degradation is tightly controlled via specific pathways. Protein turnover at -10°C was apparent with abundance increases in two aminopeptidases (Table 2.2). Increases in both amino acid biosynthesis and protein turnover rates may be important to maximize *de novo* protein synthesis rates and would agree with the increases seen in the nucleotide salvage pathway. The Antarctic archaeon *Methanococcoides burtonii* also induces protein turnover at low temperatures (Campanaro et al. 2011). Amino acid turnover would also allow extraneous amino acids to be made available as nitrogen sources or conserved as metabolic precursors during periods of slowed growth (Mykytczuk et al. 2013), which would be the normal growth status of *P. halocryophilus* in its permafrost habitat.

2.3.8 Replication, repair and transcription

Several components with roles in DNA replication, repair, and transcription also increased in abundance at -10°C in *P. halocryophilus* (Figures 2.2-2.4). A DnaD-like protein was only detected in the -10°C condition (Table 2.1). DnaD is a primosomal protein (part of the complex responsible for primer loading during replication) involved in loading of the replicative ring

helicase. DNA polymerase III subunits gamma/tau, ATPase components of the holoenzyme necessary for clamp loading and to coordinate activities and movement at the replication fork (Bloom 2009), increased more than 3 and 2 fold vs 23°C and 23C-salt, respectively. In psychrophiles, lower replication rates and higher concentrations of reactive oxygen species (ROS) can lead to accumulation of potentially damaging errors (Chattopadhyay et al. 2011; De Maayer et al. 2014) which are compensated for by inducing the antioxidative stress response and DNA repair (Bergholz et al. 2009; Chen et al. 2012; Mykytczuk et al. 2013; Tripathy et al. 2014; Aliyu et al. 2016). Endonuclease IV, the enzyme responsible for recognition and priming of the damaged site for repair in numerous bacteria (Hosfield et al. 1999), is increased almost 3-fold in the two stressed conditions compared to 23°C (Table 2.2). Several proteins involved in DNA repair were also detected at -10°C in *Colwellia psychrerythraea* (Nunn et al. 2015). In addition to potential increases in DNA damage, low temperatures and stress conditions can induce changes to DNA supercoiling, and since DNA topology has a substantial impact on gene expression (Pruss and Drlica 1989), mediating these changes would be expected. DNA gyrase, the subunit B of which is almost 4 fold increased at -10°C, can mediate recovery of normal supercoiling (López-García and Forterre 2000). Members of the transcriptional machinery were also affected, including, the RNA polymerase (RNAP) subunit epsilon, which was not detected at 23°C (Table 2.1), but highly abundant at -10°C, while the RNAP subunit alpha was approximately 1.5-fold higher at -10°C than 23°C. The expression of RNAP epsilon, a novel subunit of RNA polymerase in gram-positive bacteria (Keller et al. 2014) with an as yet unknown role, is intriguing, and further research would be worthwhile to elucidate the function of this potentially important protein at subzero temperatures. Overall, there was not a very significant observable abundance increase in many transcription factors, with the exception of the attenuator MtrB, required for transcription attenuation control of the Trp operon, suggesting that control of gene expression may be more important at the post transcriptional level at -10°C in *Planococcus halocryophilus*.

2.3.9 Oxidative and carbonyl stress

Cold environments have been shown to lead to an increase in the production of reactive oxygen species (ROS) due to the increased volatility of gases and increases in enzymatic activity to counteract lower reaction rates, and, as such, a strong oxidative stress response has been observed in a number of psychrophiles (De Maayer et al. 2014). This response can include stimulating DNA repair pathways, as well as antioxidative agents. In addition to a putative role in regulating membrane fluidity (discussed above), heightened synthesis of the carotenoid lycopene in *P. halocryophilus* under cold conditions would also be important in the oxidative response, as it is known to neutralize free oxygen radicals (Sharma and Goswami 2011). As a carotenoid, it would also mitigate potential oxidative stress from UV radiation. Spermidine synthase, the enzyme responsible for converting putrescine to spermidine, was also stimulated in the -10°C condition (Table 2.2). Spermidine is one of the most widely distributed polyamines in bacteria and can act as a free radical scavenger helping to reduce DNA strand breakage by ROS (Ha et al. 1998). As with other polyamines, it is also involved in stabilizing nucleic acids, and regulating intracellular pH and membrane potential (Shah and Swiatlo 2008). There is a significant presence at -10°C and 23C-salt of several glyoxalase family enzymes (>10-fold) (Table 2.1), as well as AldA, an aldehyde dehydrogenase (>3-fold) (Table 2.2), involved in the breakdown of toxic glyoxal (GO) and methylglyoxal (MGO), formed as by-products of several metabolic pathways, including glycolysis, as well as sugar, lipid and DNA oxidation from oxidative stress. The presence of these enzymes at -10°C attests to the importance of removing excess reactive electrophilic species (RES) under subzero conditions and recycling reactive carbonyls to a form (D- or L-lactate) which may be reused for cellular metabolism (Lee and Park 2017). At -10°C, *P. halocryophilus* also increases abundance (~2.5-fold) of rhodanese (thiosulfate/3-mercaptopyruvate sulfurtransferase) (Table 2.2), theorized to play a role in cyanide detoxification, a toxic metabolic by-product, as well as contributing to sulfur metabolism and cycling (also increased at -10°C in *P. halocryophilus*), in cysteine metabolism, and acting as an antioxidant (Bordo and Bork 2002; Nakajima 2015).

2.3.10 Osmotolerance and compatible solutes

Planococcus halocryophilus can tolerate up to 19% salt in solution (NaCl, aw = ~0.85) (Mykytczuk et al. 2012). In its terrestrial permafrost-associated environment, consisting of thin films of salty water surrounding soil particles (Gilichinsky et al. 2003; Steven et al. 2006), *P. halocryophilus* is believed to experience high salt and low water activity (aw), in addition to cold temperatures. Salt concentrations in Eureka permafrost have been measure at ~14.6 g/kg (Steven et al. 2007a) and water activity in permafrost has been measured at about 0.85 (Gilichinsky et al. 1993; Vishnivetskaya 2009). Although the absolute aw limit of *Planococcus halocryophilus* has not been determined, we expect that it would be able to tolerate at least 0.85. Accumulation of compatible solutes, both chaotropic (disorder-making) and kosmotropic (order-making), is important for osmoregulation in cold-adapted microbes, in addition to roles in increasing stability and preserving flexibility of macromolecular structures (Yancey 2005). Compatible solutes have been shown to reduce aw limits in certain organisms (Stevenson et al. 2015) and chaotropic substrances can extend activity windows for organisms in suboptimal cold environments (Chin et al. 2010). Although our previous genomic analyses on *P. halocryophilus* identified several genes with potential roles in osmoregulation through the use of compatible solutes, such as glycine betaine, upregulation in the transcriptomic data was very minor to non-existent (Mykytczuk et al. 2013, 2015). In this study, spectral counts for three proteins involved in compatible solute uptake, glycine betaine ABC transport system binding protein OpuAC (gi|495772060), L-proline glycine betaine binding ABC transporter protein ProX (gi|495773900), and L-proline glycine betaine ABC transport system permease protein ProV (gi|495774180) were detected in one of the triplicate conditions at -10°C (data not shown), but not in duplicate or triplicate, and were therefore not included in downstream analyses. One protein, the glycine betaine ABC transport system ATP-binding protein OpuAA (gi|495772057|) was detected at -10 °C in duplicate but did not pass the Benjamini Hochberg (BH) statistical test ($q < 0.05$) to be included in further analyses. Similar results were found in the 23C-salt condition, with one exception, OpuAC was detected in triplicate and strongly increased vs 23°C (> 20-fold). This, in combination with the detection of OpuAC and OpuAA at -10°C, points to the Opu glycine betaine ABC transport system as likely being at least partially important for osmotolerance in

Planococcus halocryophilus, although further studies would be needed to conclusively demonstrate this.

The significant increase in abundance of fructokinase at -10°C and 23C-salt (Table 2.2), the first enzyme in the utilization of fructose, and potentially mannitol, sorbitol, and sucrose (Gardiol et al. 1980), suggests the potential for accumulation of fructose inside the cell. While this would need to be investigated further, it is intriguing considering the known role of fructose as a chaotropic substance (Chin et al. 2010). Fructose has been shown to be an important metabolite for cellular tolerance and growth at subzero temperatures (Chin et al. 2010).

2.3.11 Energy metabolism

Expression of proteins involved in energy metabolism has been shown to decrease at low temperatures in several psychrophilic/psychrotrophic organisms (Gao et al. 2006; Bergholz et al. 2009; Campanaro et al. 2011; Tribelli et al. 2015) but increases in others (Kawamoto et al. 2007; Cacace et al. 2010), especially in 'true psychrophiles' (stenopsychrophiles), which grow faster and are more active at lower temperatures (Mock and Hoch 2005; Mock et al. 2005; Hwang et al. 2008; Chong et al. 2011). *P. halocryophilus* exhibits a mixed response (Figure 2.2), decreasing the abundance of certain enzymes involved in glycolysis (phosphoglycerate mutase), glucose metabolism (UTP--glucose-1-phosphate uridylyltransferase), and ATP synthesis (ATP synthase) (Table S2.1), while increasing others. Those with increased abundances at -10°C include an FMN reductase and glutamate-1-semialdehyde aminotransferase, highest at -10°C compared to both 23°C and 23C-salt, and ferredoxin, strongly increased at both -10°C and 23C-salt (Tables 2.1 & 2.2). In addition, NADPH dehydrogenase, naphthoate synthase, cytochrome c-type biogenesis protein Ccs1/ResB, and the beta subunit of an electron transfer flavoprotein were also increased in abundance. Emphasis on the breakdown of alternative simple sugars was seen at -10°C and 23C-salt with an increase in fructokinase (Table 2.2). Sulfur cycling and iron-sulfur cluster biogenesis is important under stress, as underlined by noticeable increases at -10°C and 23C-salt (~2.5-fold; Table 2.2) of cysteine desulfurase (CsdA/SufS), a primary intracellular source of sulfur due to its ability to act as a sulfur donor in sulfur cycling and is involved in the generation of iron-sulfur clusters through the recruitment of cluster assembly

proteins (Suf). Two of the Suf proteins, SufB and SufC, were close to 2-fold increased, in addition to the scaffold protein for cluster assembly (Table 2.2).

2.3.12 Cell growth and cell cycle control

P. halocryophilus cells at -10°C differentially express several cellular regulators of cell cycle and growth. Expression of a PemK/MazF family transcriptional regulator was unique to the -10°C condition (Table 2.1). Additional proteins which showed strong increases at -10°C include GTP-binding protein Era (> 5-fold), cell fate regulator YaaT (> 3.5), cell division protein FtsH (2-fold) and the ATP-binding subunit ClpX of the ATP dependent protease Clp (1.8 fold), although these proteins were also increased at 23C-salt, suggesting they are associated with a general stress response (Table 2.2). MazF, an endoribonuclease, is the toxin component of a bacterial toxin-antitoxin system (MazEF), which upon it's activation can lead to a number of altered phenotypes, including growth, persistence, the stress response, and starvation (Donegan et al. 2010; Wang and Wood 2011). MazF was recently identified as an important stress regulator in *Escherichia coli*, activating a broad cellular response to harsh stress, leading to persistence, through translation reprogramming, activation of 'stress-ribosomes', and selective translation (Sauert et al. 2016). Several proteins processed by MazF in the Sauert et al. study also increased in abundance in this study including, but not limited to, uridine kinase, multidrug efflux system, ATP-dependent RNA helicase, pseudouridine synthase, and FMN reductase. It is worthwhile to ponder whether a similar global regulatory role is at play here and further studies should be carried out to investigate the potential effect of MazF on the cell response and translation processes at subzero temperatures in this organism. The Clp protease is known to have an important role in regulation of the toxin-antitoxin system in both *E. coli* and *Staphylococcus aureus* (Gerdes et al. 2005; Donegan et al. 2010). FtsH, a cytoplasmic protease with roles in membrane protein quality control, cell division, and cell cycle regulation in several bacteria (Tomoyasu et al. 1993; Lin et al. 2014) was also detected in the surface proteome of study of *P. halocryophilus* at low temperatures, as was another cell division protein, FtsZ (Ronholm et al. 2015). GTP-binding protein Era is a highly conserved GTPase with described roles in cell cycle regulation, ribosome assembly, fatty acid metabolism, and energy metabolism

(Verstraeten et al. 2011; Voshol et al. 2015). In *B. subtilis* deletion of the era genes severely impairs spore formation (Minkovsky et al. 2002). While the exact role of Era in *P. halocryphilus* is unknown, given its widespread conserved role in cell growth and cell cycle regulation (Verstraeten et al. 2011), we would expect a similar involvement here, along with roles in ribosomal processes and fatty acid metabolism, both of which are strongly stimulated at -10°C. YaaT is involved in a complex that regulates sporulation, competence, and biofilm formation through activation of the sporulation initiation phosphotransferanse Spo0F (Carabetta et al. 2013). While common in Firmicutes, our previous analysis did not find evidence for sporulation in this organism (Mykytczuk et al. 2012), although the analysis was performed under optimal growth conditions. The heightened presence of YaaT and Era would suggest some role of sporulation in *Planococcus halocryophilus* when under significant cold or salt stress. Further studies would be needed to ascertain the exact role of these proteins and extent of sporulation under these conditions. The significant changes in abundance observed in cell cycle and growth proteins is indicative of a complex and tightly controlled cell cycle response by *P. halocryophilus* at -10°C, whereby the organism induces several stress responses, uses resources efficiently, and limits its growth to only those instances where it is energetically favorable to do so in order to maximize long term survival.

2.3.13 Genomic redundancy

Previous genomic and transcriptomic analysis of *Planococcus halocryophilus* revealed evidence for genomic redundancy and possible isozyme exchange (Mykytczuk et al. 2013) and it was hoped that this study might reveal the extent to which this mechanism is utilized for differential gene expression at different temperatures or growth conditions. *Planococcus halocryophilus* was found to have differentially abundant isozymes of a number of proteins in the different conditions (Table 2.3), including one copy each of FMN reductase and N-acetylmuramoyl-L-alanine amidase only detected at -10°C and 23C-salt and one copy of Glutamate-1-semialdehyde aminotransferase detected only at -10°C, while additional copies are only detected in the 23°C conditions. This is likely to offer a temperature growth range advantage,

whereby cold or hot adapted isozymes can catalyze similar reactions at different temperatures (Maki et al. 2006).

2.4 Conclusion

An overview of the -10°C-specific response, highlighting important processes and mechanisms present or increased in this condition, is provided in Figure 2.5. Growth of *Planococcus halocryophilus* at -10°C involves a series of complex responses and overall significant changes in abundance of proteins involved in many processes and functions rather than simply an increase or decrease in a specific category. Large changes were observed in translation and ribosome processes, amino sugar and nucleotide sugar metabolism, transporters, amino acid biosynthesis, and pyrimidine, purine and pyruvate metabolisms. Nonetheless, certain pathways and processes saw significant upregulation, with large increases in abundances of proteins mapping to those pathways and with only minor decreases, including peptidoglycan biosynthesis, DNA repair and replication, sulfur cycling, mRNA and ribosome biogenesis, nucleotide and RNA turnover, nicotinamide metabolism and fructose/mannose metabolism. We identified proteins (Figure 2.5) with potentially important roles in fatty acid and membrane metabolism, transport of metabolites, in the breakdown of ROS and RES, in ribosomal processes, nucleotide turnover, and regulating the translational response. The strong increase of MazF/PemK is intriguing and highlights its potentially central role in regulating the subzero response in *P. halocryophilus*, through translational reprogramming and selective translation, emphasized by the strong changes observed in most translational and ribosomal processes. This study also raises interesting questions with regards to the mechanisms by which *P. halocryophilus* modules membrane fluidity at subzero temperatures, which warrants further investigation. We found that *P. halocryophilus* has differential abundances of a number of isozymes in the different growth conditions, likely important for supporting the broad growth range and high salt tolerance observed in this organism.

While COG and KEGG annotations and BLAST results serve as useful indicators of potential function, it should be noted that they are still only able to provide predictions of function and

activity based on homology. Further studies targeting individual or subsets of genes for functional characterization at -10°C are needed to fully elucidate their roles and more thoroughly explore the specific functions and processes important for subzero growth. It is also important to highlight that this *in vitro* study does not replicate the harsher permafrost conditions which *P. halocryophilus* would be expected to encounter in its native environment and therefore these results should be weighed in that context. Nevertheless, it remains an ideal method for observing cellular and molecular responses of isolates in a controlled setting and provides important clues that forms a baseline for further research. The present study identified potentially novel and significant mechanisms involved in subzero growth in *Planococcus halocryophilus* and increases our global understanding of growth in cold-adapted microorganisms.

2.5 Materials and Methods

2.5.1 Growth Conditions

Planococcus halocryophilus Or1 was cultured on Tryptic Soy Agar (BD Difco) media at 23°C (Room Temperature). Cultures for protein extraction were grown in liquid Tryptic Soy Broth (BD Difco), supplemented with 12% w/v NaCl and 5% v/v glycerol, at 23°C or -10°C without shaking. NaCl and glycerol serve as freezing point depressants to allow the media to remain liquid at -10°C. Control cultures with no added NaCl or glycerol added were grown in Tryptic Soy Broth (TSB) at 23°C.

2.5.2 Protein Extraction

Triplicate cultures were grown and used for protein extraction for each of the following growth condition: 23°C TSB no added NaCl (23°C), 23°C TSB with 12% w/v NaCl (23C-salt), and -10°C TSB with 12% w/v NaCl (-10°C). Cultures for extraction were inoculated from overnight liquid cultures grown at 23°C at a starting optical density (OD_{600}) of 0.1. To maximize biomass, -10°C and 23C-salt cultures were grown for 4 months and 45 days, respectively, to late exponential phase, OD_{600} 0.35-0.4. Since 23°C cultures (no added NaCl) have higher cell densities at late

exponential phase (OD_{600} 1.0), they were diluted to achieve similar concentrations as the cultures under -10°C and 23C-salt conditions prior to extraction. It is challenging to grow this organism at -10°C. It is time consuming due to slow generation times (40 days) (Mykytczuk et al. 2013) and since excessive shaking or perturbation of the cultures is detrimental to growth, likely due to the disruption of the cell aggregates and encrustations that occur at subzero temperatures (Mykytczuk et al. 2013, 2015), care was taken to disturb the cultures as little as possible. Proteins were extracted as described in Chourey et al. (2010) with some modifications. Cultures were centrifuged (Thermo IEC 21000R) at 3000g for 10min to collect cells. 10 ml of SDS-based lysis buffer was added [5% SDS, 50 mM Tris-HCl, pH 8.5; 0.15M NaCl; 0.1mM EDTA; 1mM $MgCl_2$; 50 mM Dithiothretiol (DTT)] and the solution vortexed. Following brief sonication (3 x 10s), the tubes were transferred to a hot water bath for 15 min, and then cooled for 10 min. The solution was transferred to a sterile falcon tube and centrifuged at 21,000 g for 15min to collect cell debris. The supernatant was transferred to multiple Eppendorf tubes and 100% chilled TCA (Trichloroacetic acid) was added to a final concentration of 25%. Tubes were mixed by vortexing and incubated overnight at -20°C. Proteins were precipitated by centrifugation at 21,000 g for 15 min, the supernatant discarded, and the pellet washed 3 times with chilled acetone with centrifugation between each wash (21,000 g x 10 min). The washed pellet was air dried to remove all traces of acetone and stored at -20°C.

2.5.3 Sample preparation for Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

The dried protein pellet was solubilized in 6 M guanidine buffer (6 M guanidine; 10 mM dithiothreitol [DTT] in Tris-$CaCl_2$ buffer (50mM Tris; 10mM $CaCl_2$, pH 7.8) and incubated at 60°C for three hours. An aliquot of this mix was retained for protein estimation measurements using the RC/DC protein estimation kit (Bio-Rad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. The solubilized protein sample was subjected to trypsin (Promega, Madison, WI), digestion and overnight incubation at 37°C as described earlier (Thompson et al. 2007). Following digestion, the peptide mix was amended with 10 mM DTT (final concentration) and peptides stored at 80°C until MS analysis. For this study, we chose a semi-quantitative mass

spectrometry approach which is one of the best methods to gauge the relative abundance of proteins in and across the samples. This approach also assures that a rare or abundant peptide gets an equal chance at being detected (Hettich et al. 2013). Use of this technique is well established and widely accepted to study proteomes, especially for deep proteome measurements (Hettich et al. 2013; Bagnoud et al. 2016; North et al. 2016).

2.5.4 NanoLC-MS/MS Analysis

For MS analysis, 75ug of peptide mix was loaded onto a biphasic resin packed column as described earlier (Brown 2006; Thompson et al. 2007) and the loaded column subjected to 45 min offline wash and connected to the C18 packed nanospray tip (New Objective, Woburn, MA) as described by Bagnoud et al. (2016) and Sharma et al. (2012). Peptides were chromatographically separated using a 11-step separation method controlled via Ultimate 3000 HPLC system (Dionex, USA) connected to an LTQ-Orbitrap- Elite mass spectrometer (Thermo Fisher Scientific, Germany). Peptide fragmentation and measurements were carried out as described earlier (Sharma et al. 2012). The mass spectrometer was operated in data dependent mode, using Thermo Xcalibur software V2.1.0 as described earlier (Sharma et al. 2012; Bagnoud et al. 2016). Mass spectrometry data has been added to the online database MassIVE (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp), a member of ProteomeXchange, under ID MSV000081159.

2.5.5 Protein identification and Data analysis

For protein identifications, the raw spectra were searched against *Planococcus halocryophilus* database, obtained from the Joint Genome Institute Integrated Microbial Genomes website (JGI IMG/MER; https://img.jgi.doe.gov/cgi-bin/mer/main.cgi). The searches were carried out using Myrimatch v2.1 algorithm (Tabb et al. 2008) set to parameters described by Xiong *et al.* (2015) with no peptide modifications. Identification of two unique peptides per protein sequence was set as a prerequisite for protein identification. Common contaminant peptide sequences from trypsin and keratin were concatenated to the database. False discovery rate (FDR) was calculated using reverse database sequences as decoy and cutoff for peptide identification was

maintained at < 1%. Spectral counts of identified proteins were normalized as described before (Paoletti et al. 2006) to obtain the normalized spectral counts (nSpc). Only proteins identified in at least two of the three biological replicates in each growth condition were considered for further downstream analysis. Clusters of Orthologous Groups (COG) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations, as well as updated genome annotations, for *Planococcus halocryophilus* were obtained from JGI IMG/MER and used to match and assign function to the subset of proteins identified in each of the three conditions. Manual annotation of the *P. halocryophilus* genome was carried out after initial sequencing and is described in Mykytczuk *et al.* (2013) and checked against updated annotations by JGI. Mapping of proteins with KEGG annotations to KEGG pathways and KEGG Orthology was achieved using the KEGG pathway mapper (http://www.genome.jp/kegg/pathway.html). Care was taken to manually cross-reference predicted functions for proteins discussed in the text against function assignments in available literature studies. Only proteins with a fold change (FC) of ≥ 1.5 and *q* value of < 0.05 were included in KEGG and COG analyses. Heat maps were generated via Perseus software (Tyanova et al. 2016) using $\log_2 2$ transformed nSpc values. The cell model was constructed using Adobe Illustrator CS6.

2.6 Acknowledgements

Thanks to Jennifer Ronholm for experiment support and feedback. Funding for this research was provided by the Natural Sciences and Engineering Research Council (NSERC) through an NSERC CREATE PhD fellowship and an NSERC Canadian Graduate Scholarship to IRB, and through NSERC Discovery and Northern Research Supplement grants, and a Polar Continental Shelf Project grant, to LGW. KC and RH acknowledge support from the U.S. Department of Energy's Genomic Science Program.

Figure 2.1. Venn diagrams showing: **A**) Unique and shared proteins identified at -10°C, 23°C with NaCl (RTsalt), and 23°C (RT); **B**) Total and shared differentially abundant proteins between the three conditions, 23°C with salt (RTsalt) as compared to 23°C (RT), -10°C as compared to 23°C (RT), and -10°C as compared to 23°C with salt (RTsalt).

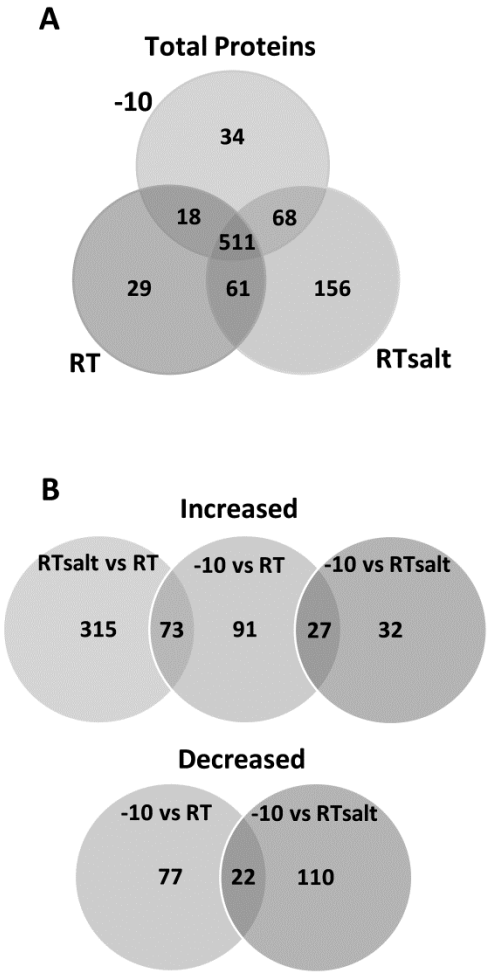


Figure 2.2. Predicted COG categories for total differentially abundant proteins (≥ 1.5-fold change) at -10°C compared to 23°C and 23°C with NaCl.

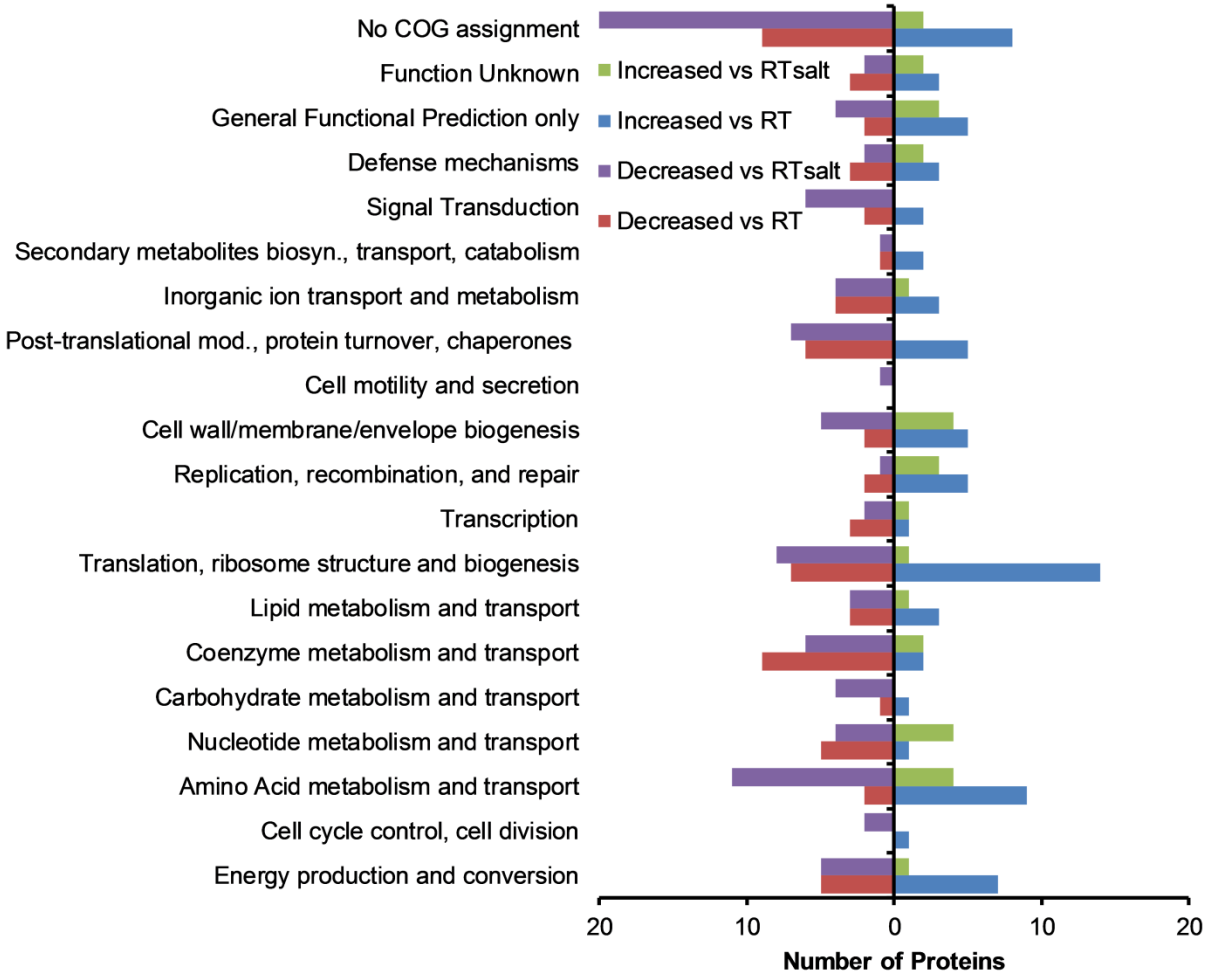


Figure 2.3. KEGG pathways and modules for proteins with significant differential abundances at -10°C compared to 23°C. Selected pathways include those for which at least a 3-fold increase or decrease or was observed or which contain at least 3 proteins with ≥ 1.5-fold change (FC).

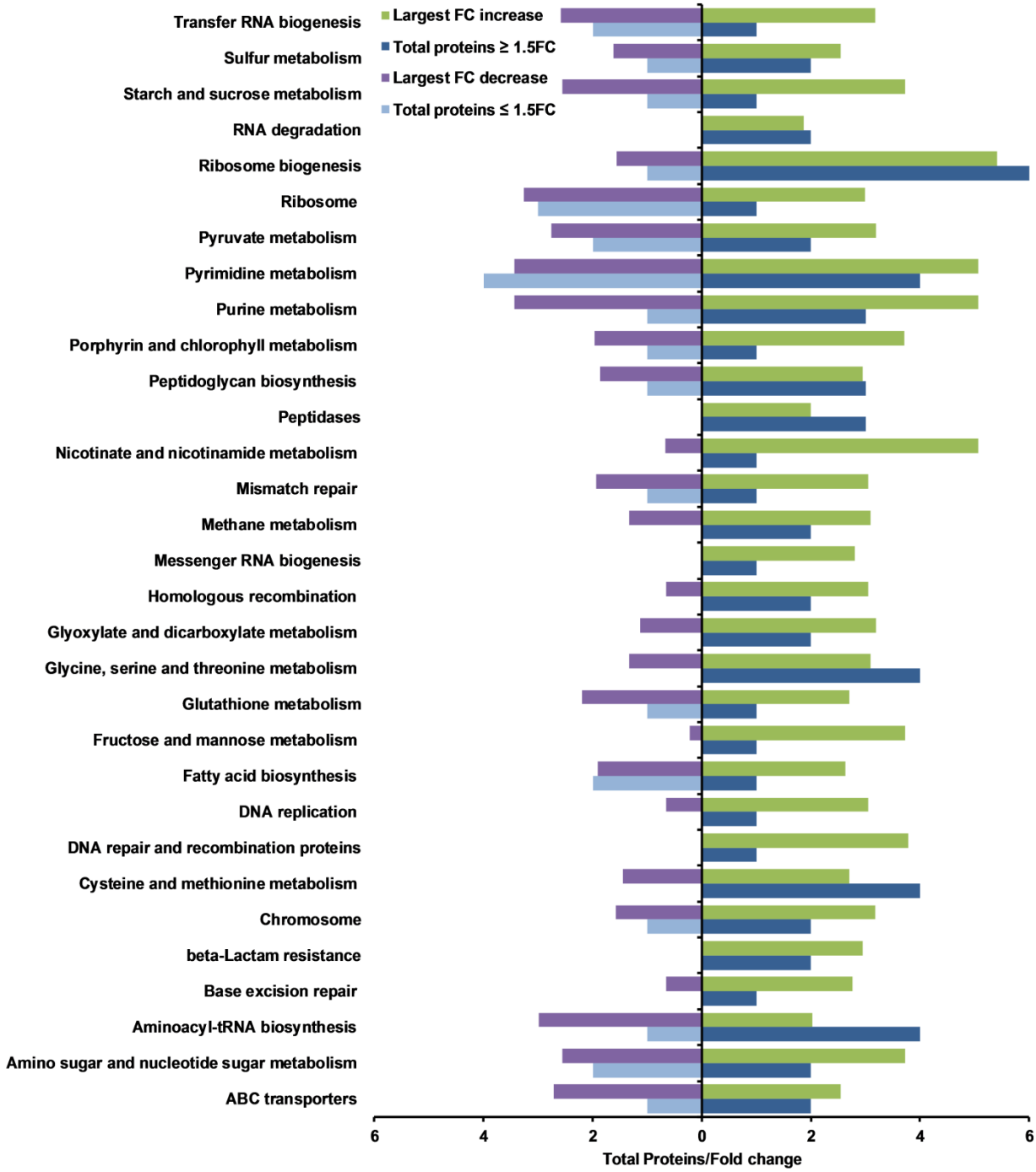


Figure 2.4. KEGG pathways and modules for proteins with significant differential abundances at -10°C compared to 23°C with NaCl. Selected pathways include those for which at least a 2-fold increase or decrease or was observed or which contain at least 2 proteins with ≥ 1.5-fold change (FC).

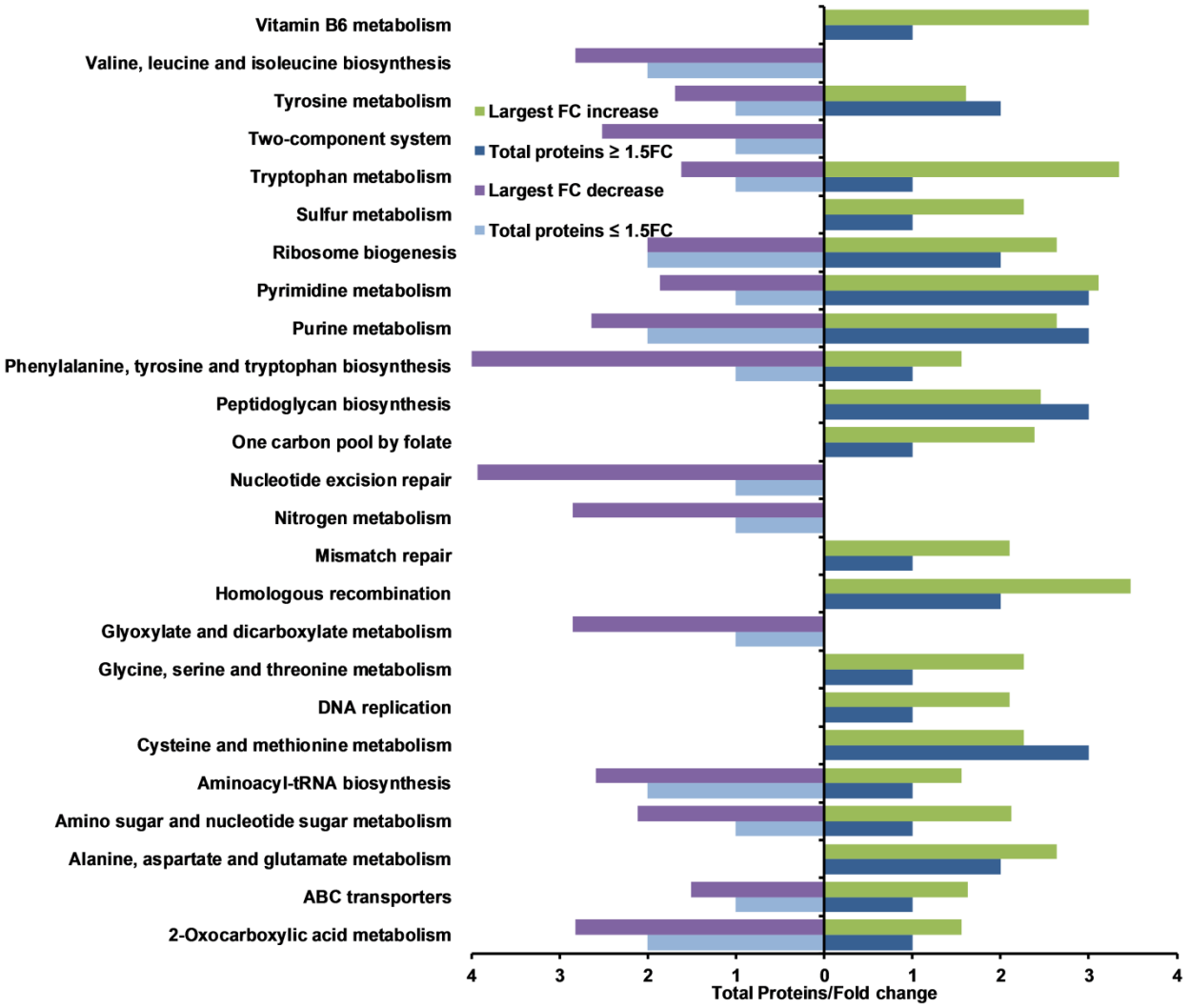


Figure 2.5. Cell model depicting active, important mechanisms and pathways at -10°C and 12% NaCl in *Planococcus halocryophilus*. Refer to Tables 2.1&2.2 for corresponding full protein name of abbreviations used in this figure. Colored boxes are used to distinguish between distinct categories/functional pathways and their associated proteins. Yellow: general functional category/pathway; Grey: specific protein function; White: products/intermediates of enzymatic pathway. Boxes with thick colored borders highlight relevant proteins that are increased in abundance at -10°C.

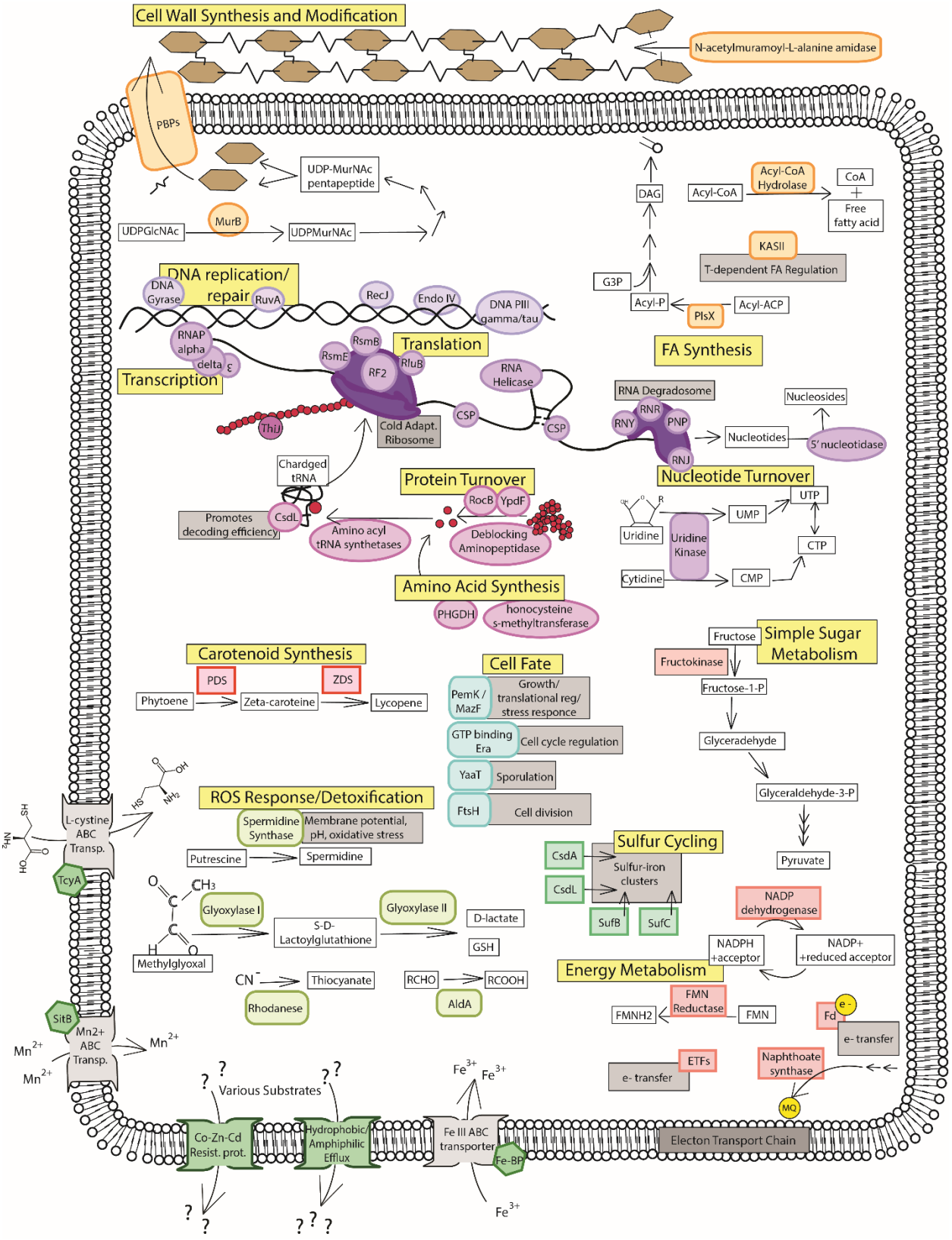

Cell Wall Synthesis and Modification
N-acetylmuramoyl-L-alanine amidase
PBPs
UDP-MurNAc pentapeptide
UDPGlcNAc
MurB
UDPMurNAc
DNA replication/ repair
DNA Gyrase
RuvA
RecJ
Endo IV
DNA PIII gamma/tau
RNAP alpha
delta
ε
Transcription
RsmB
RsmE
RF2
RluB
Translation
RNA Helicase
Cold Adapt. Ribosome
CSP
ThiJ
Chardged tRNA
Promotes decoding efficiency
CsdL
Protein Turnover
RocB
YpdF
Amino acyl tRNA synthetases
Deblocking Aminopeptidase
Amino Acid Synthesis
PHGDH
honocysteine s-methyltransferase
DAG
Acyl-CoA
Acyl-CoA Hydrolase
CoA
Free fatty acid
KASII
T-dependent FA Regulation
G3P
Acyl-P
PlsX
Acyl-ACP
FA Synthesis
RNA Degradosome
RNR
RNY
PNP
RNJ
Nucleosides
Nucleotides
5' nucleotidase
Nucleotide Turnover
UTP
UMP
Uridine
Uridine Kinase
CTP
Cytidine
CMP
Carotenoid Synthesis
PDS
ZDS
Phytoene
Zeta-caroteine
Lycopene
Cell Fate
PemK / MazF
Growth/ translational reg/ stress responce
GTP binding Era
Cell cycle regulation
YaaT
Sporulation
FtsH
Cell division
Fructose
Simple Sugar Metabolism
Fructokinase
Fructose-1-P
Glyceradehyde
Glyceraldehyde-3-P
Pyruvate
L-cystine ABC Transp.
TcyA
ROS Response/Detoxification
Spermidine Synthase
Membrane potential, pH, oxidative stress
Putrescine
Spermidine
Sulfur Cycling
CsdA
CsdL
Sulfur-iron clusters
SufB
SufC
Glyoxylase I
Glyoxylase II
S-D-Lactoylglutathione
D-lactate
GSH
Methylglyoxal
NADP dehydrogenase
NADPH +acceptor
NADP+ +reduced acceptor
Energy Metabolism
FMN Reductase
FMNH2
FMN
Fd
e-
e- transfer
ETFs
e- transfer
Naphthoate synthase
MQ
Electon Transport Chain
CN-
Thiocyanate
Rhodanese
RCHO
RCOOH
AldA
SitB
Mn2+ ABC Transp.
Mn2+
Various Substrates
Co-Zn-Cd Resist. prot.
Hydrophobic/ Amphiphilic Efflux
Fe III ABC transporter
Fe-BP
Fe3+

Table 2.1. Proteins with normalized spectral counts (nSpc) at -10°C and no corresponding counts observed in the 23°C condition (Benjamini Hochberg test, $q < 0.05$). Starred proteins showed no spectral counts at 23°C or 23°C with NaCl (23salt).

Gene ID	Protein	Short name	Predicted Function or KEGG Pathway	Fold change	
				vs 23	vs 23salt
gi|495773085|	D-alanyl-D-alanine carboxypeptidase	PBP	Peptidoglycan biosynthesis	9.98	1.54
gi|495774369|	N-acetylmuramoyl-L-alanine amidase		Peptidoglycan biosynthesis/Cationic antimicrobial peptide resistance	9.56	N/A
gi|495773390|*	Phosphate:acyl-ACP acyltransferase	PlsX	Fatty acid metabolism /Glycerophospholipid metabolism	9.51	22.80
gi|495773523|*	Hydrophobic/amphiphilic exporter-1, HAE1 family		Transporters	8.81	22.09
gi|495773697|	Cobalt-zinc-cadmium resistance protein; Multidrug efflux pump subunit	AcrB	Transporters	7.48	-1.13
gi|495774373|	tRNA A37 threonylcarbamoyladenosine dehydratase	CsdL/TcdA	Translation and ribosomal processes	12.10	0.60
gi|495773585|	Peptide chain release factor 2	RF2	Translation factors	12.87	N/A
gi|495774380|*	Uridine kinase		Nucleotide and amino acid metabolism	9.98	23.27
gi|495772785|	homocysteine S-methyltransferase		Cysteine and methionine metabolism	7.64	1.65
gi|495773309|*	DnaD and phage-associated domain-containing protein	DnaD	DNA replication and repair	8.92	22.21
gi|495705273|	RNA polymerase auxiliary subunit epsilon	RNAP ε	Transcription	8.39	N/A
gi|495774457|	Glyoxalase, beta-lactamase superfamily II		Glyoxal and methylglyoxal metabolism	13.68	N/A
gi|495774211|	Glyoxalase family protein		Glyoxal and methylglyoxal metabolism	11.07	0.81
gi|495707836|*	PemK/MazF family translational regulator	PemK/MazF	Translational reprogramming/Cell growth regulation	11.08	24.37
gi|493883633|	Ferredoxin		Energy Metabolism	13.00	-0.83

Table 2.2. Select proteins with significantly increased abundances (Benjamini Hochberg test, $q < 0.05$; fold change ≥ 1.5) at -10°C compared to 23°C and 23°C with NaCl (23salt). N/A = no significant changes in normalized spectral counts.

Gene ID	Protein	Short name	Fold change at -10 vs 23	Fold change at -10 vs 23salt
Cold shock proteins and helicases				
gi|495774437|	ATP-dependent RNA helicase YqfR	YqfR	2.81	2.54
gi|495774356|	Holliday junction DNA helicase RuvA	RuvA	1.61	3.47
gi|495703805|	Cold shock protein	CSP	N/A	N/A
gi|493883890|	Cold shock protein	CSP	N/A	N/A
Cell wall biosynthesis and remodeling				
gi|495772622|	Multimodular transpeptidase-transglycosylase		2.95	2.46
gi|495774200|	UDP-N-acetylenolpyruvoylglucosamine reductase	MurB	2.91	2.12
gi|495772357|	N-acetylmuramoyl-L-alanine amidase		0.99	2.17
gi|495773182|	Multimodular transpeptidase-transglycosylase		1.64	N/A
Fatty acid and lipid biosynthesis and modifications				
gi|495773772|	Acyl-CoA hydrolase		2.77	N/A
gi|495773567|	3-oxoacyl-[acyl-carrier-protein] synthase, KASII	KASII	2.64	N/A
gi|495774486|	Branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit		1.94	N/A
Transporters				
gi|495772257|	L-Cystine ABC transporter, periplasmic cystine-binding protein	TcyA	2.54	N/A
gi|495773646|	Ferric iron ABC transporter, iron-binding protein		2.19	N/A
gi|495774601|	Manganese ABC transporter, ATP-binding protein SitB	SitB	1.31	1.63
Translation and ribosomal processes				
gi|495706397|	Ribosomal large subunit pseudouridine synthase B	RluB	5.42	N/A
gi|495773401|	Ribosomal RNA small subunit methyltransferase B	RsmB	3.89	N/A
gi|495773072|	tRNA uridine 5-carboxymethylaminomethyl modification enzyme	GidA/MnmG	3.18	N/A
gi|493882693|	50S ribosomal protein L23		2.99	N/A
gi|495707932|	30S ribosomal protein S17		2.52	N/A
gi|495774376|	Alanyl-tRNA synthetase		2.03	N/A
gi|495774410|	Ribosomal RNA small subunit methyltransferase E	RsmE	2.00	N/A
gi|495773473|	GTP-binding protein TypA/BipA	TypA	1.93	N/A
gi|495772828|	Glutamyl-tRNA synthetase		1.92	N/A
gi|495773353|	Zn-dependent hydrolase, RNA-metabolising	RNaseJ/RNJ	1.86	N/A
gi|495774353|	GTP-binding protein Obg	Obg	1.74	N/A
gi|495773340|	Hydrolase (HAD superfamily)	RNaseY/RNY	1.53	N/A
Nucleotide and amino acid metabolism				
gi|495772362|	5-nucleotidase		5.07	0.62
gi|495773526|	Deblocking aminopeptidase		3.23	0.02
gi|495772350|	Arginine utilization protein RocB	RocB	3.22	1.63

gi|495773843|	D-3-phosphoglycerate dehydrogenase	PHGDH	3.09	N/A
gi|495774471|	Aminopeptidase YpdF	YpdF	2.00	N/A
gi|495773392|	L-serine dehydratase, alpha subunit		1.87	-0.57
gi|495773214|	Chorismate synthase		1.69	N/A
DNA replication, repair, and transcription				
gi|495771684|	DNA gyrase subunit B	GyrB	3.79	N/A
gi|495773076|	DNA polymerase III subunits gamma and tau	DNAP	3.04	2.10
gi|495774438|	Endonuclease IV	EndoIV	2.77	N/A
gi|495707950|	RNA polymerase subunit alpha	RpoA	1.56	N/A
Oxidative and Carbonyl stress and cryoprotectants				
gi|495771985|	ThiJ protein; Putative intracellular protease/amidase/deglycase	ThiJ/YajL	3.22	N/A
gi|495774143|	Aldehyde dehydrogenase A	AldA	3.19	N/A
gi|495772641|	Glyoxalase, beta-lactamase superfamily II		2.93	N/A
gi|495772274|	Spermidine synthase		2.71	N/A
gi|495773745|	Dehydrosqualene desaturase/phytoene desaturase		2.61	N/A
gi|495773175|	Thiosulfate/3-mercaptopyruvate sulfurtransferase; rhodanese		2.55	N/A
gi|495774225|	Pro-zeta-carotene desaturase, prolycopene producing		2.36	0.92
gi|495771542|	S-(hydroxymethyl)glutathione dehydrogenase		1.87	1.61
Energy Metabolism				
gi|495772243|	FMN reductase		2.87	3.35
gi|495772699|	Naphthoate synthase		2.48	N/A
gi|495772548|	Glutamate-1-semialdehyde aminotransferase		3.71	1.85
gi|495773783|	Fructokinase		3.73	N/A
gi|495774497|	NADPH dehydrogenase		2.87	N/A
gi|495773128|	Cysteine desulfurase/CsdA	CsdA/SufS	2.69	N/A
gi|495771922|	Electron transfer flavoprotein, beta subunit		2.29	N/A
gi|495773127|	Iron-sulfur cluster assembly protein SufB	SufB	1.93	N/A
gi|495773129|	Iron-sulfur cluster assembly ATPase protein SufC	SufC	1.60	N/A
gi|495772873|	Scaffold protein for [4Fe-4S] cluster assembly		1.67	N/A
gi|495773237|	Cytochrome c-type biogenesis protein Ccs1/ResB	Ccs1/ResB	1.93	N/A
Cell growth and cell cycle				
gi|495774420|	GTP-binding protein Era	Era	5.25	N/A
gi|495708509|	Cell fate regulator YaaT, sporulation protein	YaaT	3.73	N/A
gi|495773979|	Cell division protein FtsH	FtsH	1.97	N/A
gi|495771933|	ATP-dependent Clp protease ATP-binding subunit ClpX	ClpX	1.80	N/A

Table 2.3. Redundant proteins with differential abundances between the three growth conditions, -10°C, 23°C, and 23°C with NaCl (23salt).

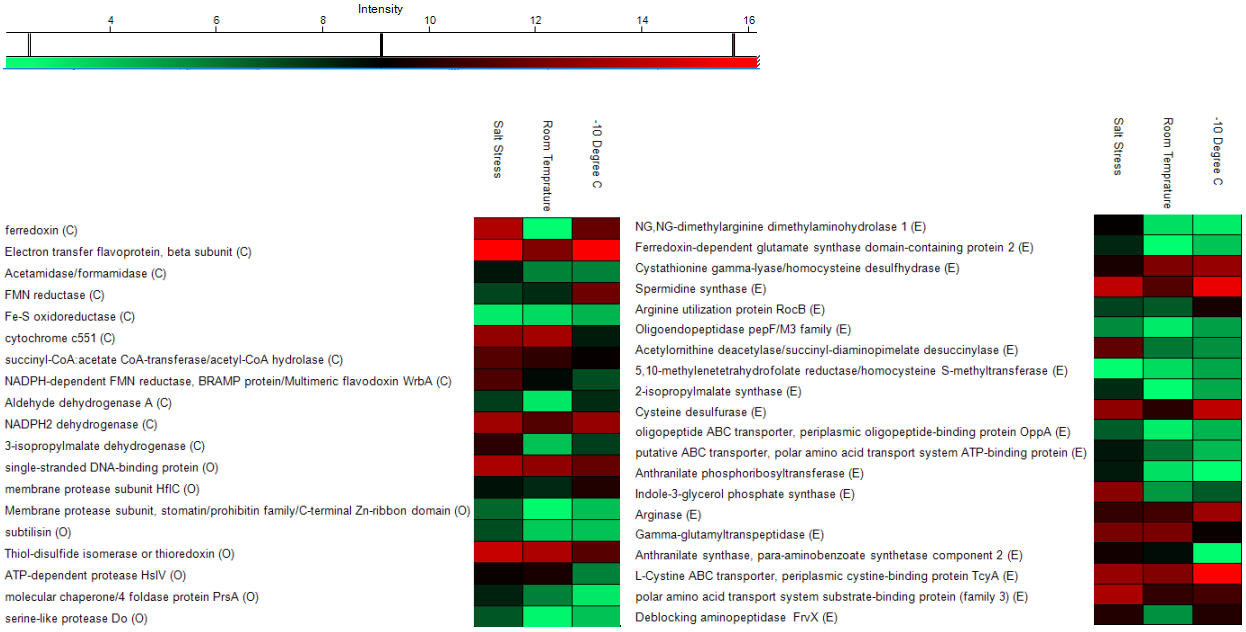
Protein	Gene ID	Conditions detected			Fold change at -10	
		-10	23salt	23	vs 23	vs 23salt
FMN reductase	gi|495772243|	Yes	Yes	No	2.87	3.35
	gi|495773816|	No	Yes	Yes	-1.25	-2.87
Acyl-CoA hydrolase	gi|495773772|	Yes	Yes	Yes	2.77	N/A
	gi|495771623|	Yes	Yes	Yes	-0.52	1.29
N-acetylmuramoyl-L-alanine amidase	gi|495774369|	Yes	Yes	No	9.56	N/A
	gi|495772357|	Yes	Yes	Yes	0.99	2.17
	gi|495772506|	No	Yes	No	N/A	-22.65
Heat shock protein	gi|495774056|	Yes	Yes	Yes	0.35	N/A
	gi|495773602|	Yes	Yes	Yes	N/A	-2.01
Oligopeptide transport system substrate- binding protein	gi|495773559|	Yes	Yes	Yes	0.69	N/A
	gi|495773563|	No	Yes	No	N/A	-22.27
Phytoene desaturase	gi|495773744|	Yes	Yes	Yes	-1.74	N/A
	gi|495773745|	Yes	Yes	Yes	2.61	N/A
Excinuclease ABC subunit A	gi|495771462|	Yes	Yes	Yes	0.08	N/A
	gi|495771968|	No	Yes	No	N/A	-3.93
Oligoendopeptidase F	gi|495773552|	Yes	No	Yes	1.64	N/A
	gi|495772582|	No	Yes	No	N/A	-21.43
Aldo/keto reductase	gi|495772427|	Yes	Yes	Yes	2.02	N/A
	gi|495771400|	Yes	Yes	Yes	0.40	N/A
	gi|495771963|	No	Yes	No	N/A	-23.11
Glutamate-1-semialdehyde aminotransferase	gi|495772548|	Yes	No	No	N/A	N/A
	gi|495771944|	Yes	Yes	Yes	N/A	N/A

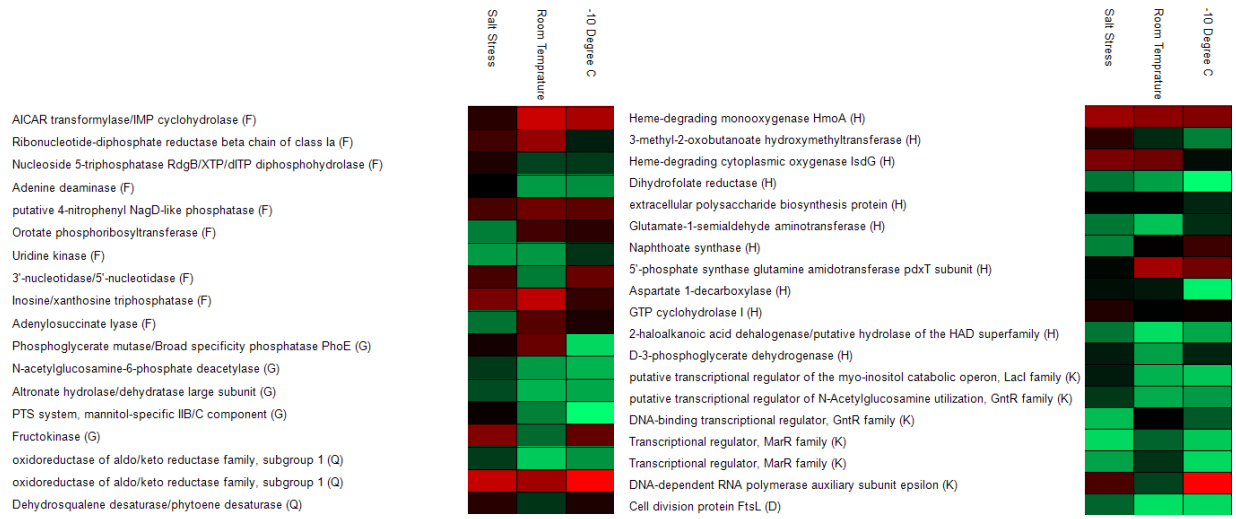
2.7 Supplementary Materials

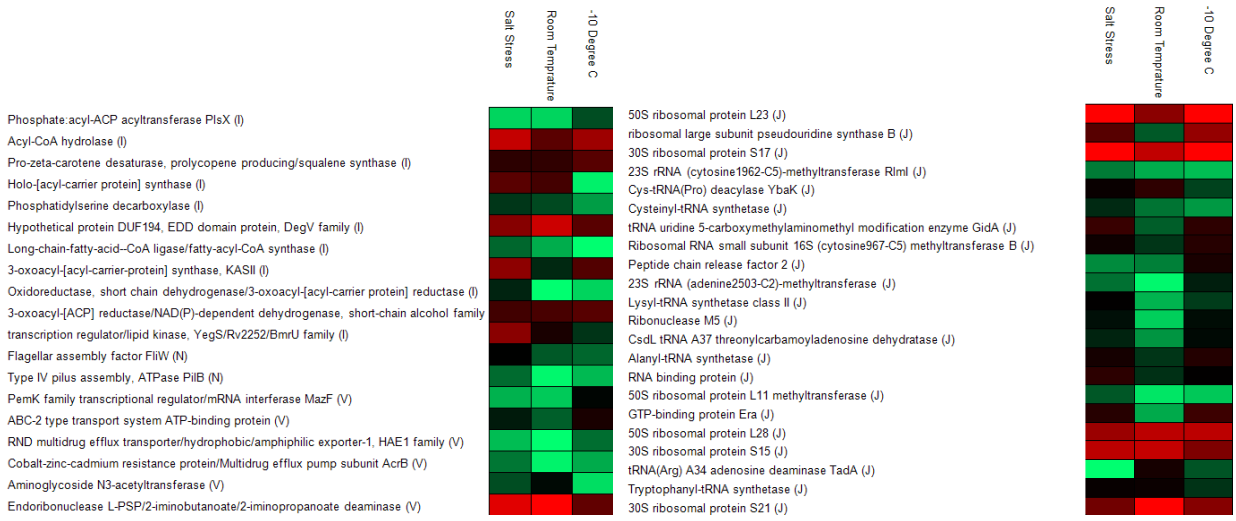
Supplementary Figure 1

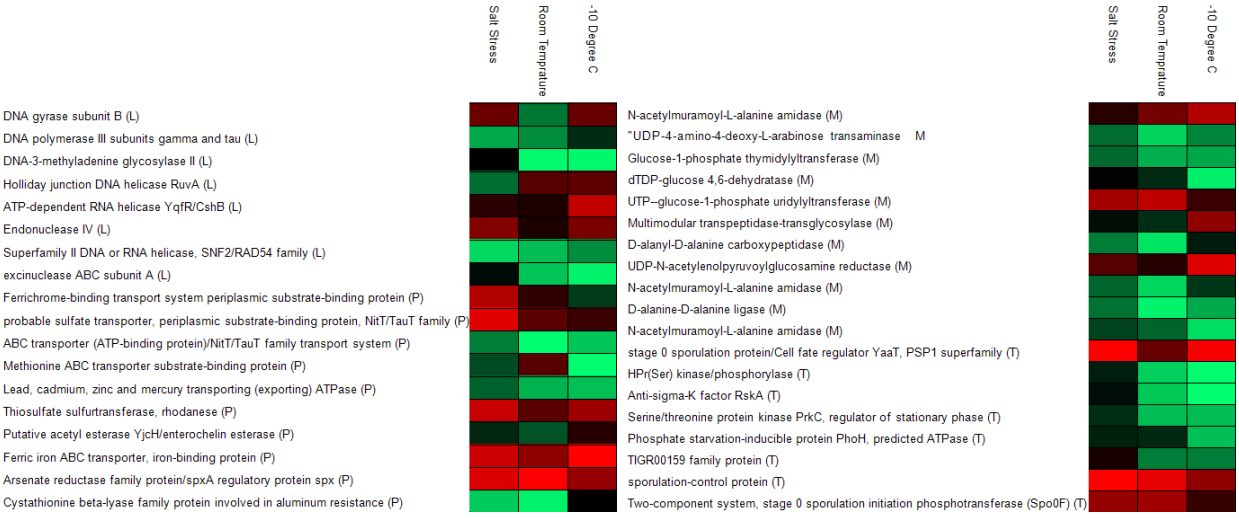
Supplementary Table 1

Figure S2.1 Heat maps showing average spectral counts (nSpc) for proteins differentially abundant at -10°C, 23°C with NaCl (Salt Stress), and 23°C (Room Temperature). Proteins are grouped by COG category. **C**: Energy production and conversion; **D**: Cell cycle control, cell division, chromosome partitioning; **E**: Amino Acid metabolism and transport; **F**: Nucleotide metabolism and transport; **G**: Carbohydrate metabolism and transport; **H**: Coenzyme metabolism and transport; **I**: Lipid metabolism and transport; **J**: Translation, ribosome structure and biogenesis; **K**: Transcription; **L**: Replication, recombination, and repair; **M**: Cell wall/membrane/envelope biogenesis; **N**: Cell motility and secretion; **O**: Post-translational modification, protein turnover, chaperone functions; **P**: Inorganic ion transport and metabolism; **Q**: Secondary metabolites biosynthesis, transport, and catabolism; **T**: Signal Transduction; **V**: Defense mechanisms; **R**: General Functional Prediction only




Salt Stress
Room Temprature
-10 Degree C
AICAR transformylase/IMP cyclohydrolase (F)
Ribonucleotide-diphosphate reductase beta chain of class Ia (F)
Nucleoside 5-triphosphatase RdgB/XTP/dITP diphosphohydrolase (F)
Adenine deaminase (F)
putative 4-nitrophenyl NagD-like phosphatase (F)
Orotate phosphoribosyltransferase (F)
Uridine kinase (F)
3'-nucleotidase/5'-nucleotidase (F)
Inosine/xanthosine triphosphatase (F)
Adenylosuccinate lyase (F)
Phosphoglycerate mutase/Broad specificity phosphatase PhoE (G)
N-acetylglucosamine-6-phosphate deacetylase (G)
Altronate hydrolase/dehydratase large subunit (G)
PTS system, mannitol-specific IIB/C component (G)
Fructokinase (G)
oxidoreductase of aldo/keto reductase family, subgroup 1 (Q)
oxidoreductase of aldo/keto reductase family, subgroup 1 (Q)
Dehydrosqualene desaturase/phytoene desaturase (Q)
Heme-degrading monooxygenase HmoA (H)
3-methyl-2-oxobutanoate hydroxymethyltransferase (H)
Heme-degrading cytoplasmic oxygenase IsdG (H)
Dihydrofolate reductase (H)
extracellular polysaccharide biosynthesis protein (H)
Glutamate-1-semialdehyde aminotransferase (H)
Naphthoate synthase (H)
5'-phosphate synthase glutamine amidotransferase pdxT subunit (H)
Aspartate 1-decarboxylase (H)
GTP cyclohydrolase I (H)
2-haloalkanoic acid dehalogenase/putative hydrolase of the HAD superfamily (H)
D-3-phosphoglycerate dehydrogenase (H)
putative transcriptional regulator of the myo-inositol catabolic operon, LacI family (K)
putative transcriptional regulator of N-Acetylglucosamine utilization, GntR family (K)
DNA-binding transcriptional regulator, GntR family (K)
Transcriptional regulator, MarR family (K)
Transcriptional regulator, MarR family (K)
DNA-dependent RNA polymerase auxiliary subunit epsilon (K)
Cell division protein FtsL (D)


Salt Stress
Room Temprature
-10 Degree C
Phosphate:acyl-ACP acyltransferase PlsX (I)
Acyl-CoA hydrolase (I)
Pro-zeta-carotene desaturase, prolycopene producing/squalene synthase (I)
Holo-[acyl-carrier protein] synthase (I)
Phosphatidylserine decarboxylase (I)
Hypothetical protein DUF194, EDD domain protein, DegV family (I)
Long-chain-fatty-acid--CoA ligase/fatty-acyl-CoA synthase (I)
3-oxoacyl-[acyl-carrier-protein] synthase, KASII (I)
Oxidoreductase, short chain dehydrogenase/3-oxoacyl-[acyl-carrier protein] reductase (I)
3-oxoacyl-[ACP] reductase/NAD(P)-dependent dehydrogenase, short-chain alcohol family
transcription regulator/lipid kinase, YegS/Rv2252/BmrU family (I)
Flagellar assembly factor FliW (N)
Type IV pilus assembly, ATPase PilB (N)
PemK family transcriptional regulator/mRNA interferase MazF (V)
ABC-2 type transport system ATP-binding protein (V)
RND multidrug efflux transporter/hydrophobic/amphiphilic exporter-1, HAE1 family (V)
Cobalt-zinc-cadmium resistance protein/Multidrug efflux pump subunit AcrB (V)
Aminoglycoside N3-acetyltransferase (V)
Endoribonuclease L-PSP/2-iminobutanoate/2-iminopropanoate deaminase (V)
50S ribosomal protein L23 (J)
ribosomal large subunit pseudouridine synthase B (J)
30S ribosomal protein S17 (J)
23S rRNA (cytosine1962-C5)-methyltransferase RlmI (J)
Cys-tRNA(Pro) deacylase YbaK (J)
Cysteinyl-tRNA synthetase (J)
tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA (J)
Ribosomal RNA small subunit 16S (cytosine967-C5) methyltransferase B (J)
Peptide chain release factor 2 (J)
23S rRNA (adenine2503-C2)-methyltransferase (J)
Lysyl-tRNA synthetase class II (J)
Ribonuclease M5 (J)
CsdL tRNA A37 threonylcarbamoyladenosine dehydratase (J)
Alanyl-tRNA synthetase (J)
RNA binding protein (J)
50S ribosomal protein L11 methyltransferase (J)
GTP-binding protein Era (J)
50S ribosomal protein L28 (J)
30S ribosomal protein S15 (J)
tRNA(Arg) A34 adenosine deaminase TadA (J)
Tryptophanyl-tRNA synthetase (J)
30S ribosomal protein S21 (J)


Salt Stress
Room Temprature
-10 Degree C
DNA gyrase subunit B (L)
DNA polymerase III subunits gamma and tau (L)
DNA-3-methyladenine glycosylase II (L)
Holliday junction DNA helicase RuvA (L)
ATP-dependent RNA helicase YqfR/CshB (L)
Endonuclease IV (L)
Superfamily II DNA or RNA helicase, SNF2/RAD54 family (L)
excinuclease ABC subunit A (L)
Ferrichrome-binding transport system periplasmic substrate-binding protein (P)
probable sulfate transporter, periplasmic substrate-binding protein, NitT/TauT family (P)
ABC transporter (ATP-binding protein)/NitT/TauT family transport system (P)
Methionine ABC transporter substrate-binding protein (P)
Lead, cadmium, zinc and mercury transporting (exporting) ATPase (P)
Thiosulfate sulfurtransferase, rhodanese (P)
Putative acetyl esterase YjcH/enterochelin esterase (P)
Ferric iron ABC transporter, iron-binding protein (P)
Arsenate reductase family protein/spxA regulatory protein spx (P)
Cystathionine beta-lyase family protein involved in aluminum resistance (P)
N-acetylmuramoyl-L-alanine amidase (M)
"UDP-4-amino-4-deoxy-L-arabinose transaminase M
Glucose-1-phosphate thymidylyltransferase (M)
dTDP-glucose 4,6-dehydratase (M)
UTP--glucose-1-phosphate uridylyltransferase (M)
Multimodular transpeptidase-transglycosylase (M)
D-alanyl-D-alanine carboxypeptidase (M)
UDP-N-acetylenolpyruvoylglucosamine reductase (M)
N-acetylmuramoyl-L-alanine amidase (M)
D-alanine-D-alanine ligase (M)
N-acetylmuramoyl-L-alanine amidase (M)
stage 0 sporulation protein/Cell fate regulator YaaT, PSP1 superfamily (T)
HPr(Ser) kinase/phosphorylase (T)
Anti-sigma-K factor RskA (T)
Serine/threonine protein kinase PrkC, regulator of stationary phase (T)
Phosphate starvation-inducible protein PhoH, predicted ATPase (T)
TIGR00159 family protein (T)
sporulation-control protein (T)
Two-component system, stage 0 sporulation initiation phosphotransferase (Spo0F) (T)

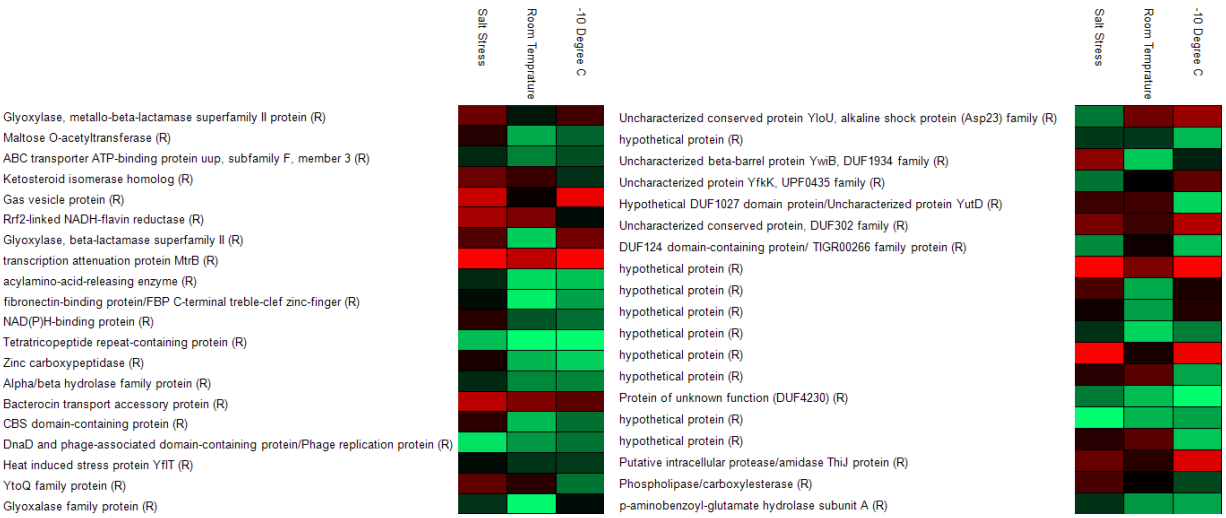

Salt Stress
Room Temprature
-10 Degree C
Glyoxylase, metallo-beta-lactamase superfamily II protein (R)
Maltose O-acetyltransferase (R)
ABC transporter ATP-binding protein uup, subfamily F, member 3 (R)
Ketosteroid isomerase homolog (R)
Gas vesicle protein (R)
Rrf2-linked NADH-flavin reductase (R)
Glyoxylase, beta-lactamase superfamily II (R)
transcription attenuation protein MtrB (R)
acylamino-acid-releasing enzyme (R)
fibronectin-binding protein/FBP C-terminal treble-clef zinc-finger (R)
NAD(P)H-binding protein (R)
Tetratricopeptide repeat-containing protein (R)
Zinc carboxypeptidase (R)
Alpha/beta hydrolase family protein (R)
Bacterocin transport accessory protein (R)
CBS domain-containing protein (R)
DnaD and phage-associated domain-containing protein/Phage replication protein (R)
Heat induced stress protein YflT (R)
YtoQ family protein (R)
Glyoxalase family protein (R)
Salt Stress
Room Temprature
-10 Degree C
Uncharacterized conserved protein YloU, alkaline shock protein (Asp23) family (R)
hypothetical protein (R)
Uncharacterized beta-barrel protein YwiB, DUF1934 family (R)
Uncharacterized protein YfkK, UPF0435 family (R)
Hypothetical DUF1027 domain protein/Uncharacterized protein YutD (R)
Uncharacterized conserved protein, DUF302 family (R)
DUF124 domain-containing protein/ TIGR00266 family protein (R)
hypothetical protein (R)
hypothetical protein (R)
hypothetical protein (R)
hypothetical protein (R)
hypothetical protein (R)
hypothetical protein (R)
Protein of unknown function (DUF4230) (R)
hypothetical protein (R)
hypothetical protein (R)
Putative intracellular protease/amidase ThiJ protein (R)
Phospholipase/carboxylesterase (R)
p-aminobenzoyl-glutamate hydrolase subunit A (R)

Table S2.1. Select proteins with differentially decreased abundances (fold change ≤ 1.5) and proteins with no normalized spectral counts detected at -10°C compared to 23°C and 23°C with NaCl.

Gene ID	Protein	Short name	Fold change -10 vs 23	Fold change -10 vs 23salt
Cold shock proteins and helicases				
gi|495773823|	Post-translocation molecular chaperone		-9.22	-24.29
Cell wall biosynthesis and remodeling				
gi|495772330|	UDP-N-acetylglucosamine 1-carboxyvinyltransferase		-1.86	N/A
CoA biosynthesis				
gi|495773707|	acetyl-CoA hydrolase/transferase family protein		-2.75	N/A
gi|495771888|	Dephospho-CoA kinase		-1.99	-1.67
gi|495771636|	3-methyl-2-oxobutanoate hydroxymethyltransferase		-2.13	N/A
Fatty acid and lipid biosynthesis and modifications				
gi|495774388|	Phosphatidylserine decarboxylase		N/A	-23.30
gi|495773176|	5-keto-D-gluconate 5-reductase		-1.91	N/A
Transporters				
gi|495772195|	Methionine ABC transporter substrate-binding protein		-12.58	-23.10
gi|495771661|	Ferrichrome-binding periplasmic protein precursor		-2.71	N/A
Translation and ribosomal processes				
gi|495773374|	ATP-dependent protease HslV	HslV	-2.86	-2.59
gi|495706306|	30S ribosomal protein S15		-3.27	N/A
gi|493882454|	30S ribosomal protein S21		-3.09	N/A
gi|495773562|	Tryptophanyl-tRNA synthetase		-2.98	N/A
gi|495772247|	Cys-tRNA(Pro) deacylase YbaK	YbaK	-2.58	-1.71
gi|493882545|	50S ribosomal protein L28		-2.46	N/A
gi|495771691|	methyltransferase RlmI	RlmI	N/A	-22.77
gi|495772831|	Cysteinyl-tRNA synthetase		N/A	-2.55
Nucleotide and amino acid metabolism				
gi|495773226|	Cytidylate kinase		-1.63	N/A
gi|495773418|	Orotidine 5-phosphate decarboxylase		-1.61	N/A
gi|495771930|	Nucleoside 5-triphosphatase RdgB	RdgB	N/A	-24.70
gi|495772119|	Adenine deaminase		N/A	-2.64
gi|495772849|	2-isopropylmalate synthase		N/A	-2.25
gi|495772850|	3-isopropylmalate dehydrogenase		N/A	-2.82
gi|495774611|	Arginase		-2.09	-1.84
gi|495773192|	Aspartate 1-decarboxylase		N/A	-25.69
gi|495773892|	Gamma-glutamyltranspeptidase		-2.19	N/A
gi|495774188|	Indole-3-glycerol phosphate synthase		N/A	-4.08

DNA replication, repair, and transcription				
gi|495772422|	Transcriptional regulator MarR	MarR	-11.48	N/A
gi|495771752|	Transcriptional regulator MarR	MarR	-10.26	N/A
gi|495771621|	Ribonucleotide reductase of class Ia		-3.44	-1.86
gi|495771681|	Chromosomal replication initiator protein DnaA		-1.76	-1.35
gi|495705396|	exodeoxyribonuclease VII small subunit		-1.94	N/A
gi|495771968|	excinuclease ABC subunit A		N/A	-3.93
Cell growth and cell cycle				
gi|495772307|	Sporulation initiation phosphotransferase (Spo0F)	Spo0F	-2.68	-2.52
gi|495706487|	Cell division protein GpsB	GpsB	-1.80	N/A
gi|495772633|/gi|495772632|	Cell division protein FtsK	FtsK	-1.55	-1.83
gi|495771886|	HflC protein	HflC	-2.90	-24.57
Energy Metabolism				
gi|495771656|	Phosphoglycerate mutase		-12.82	-24.55
gi|495773583|	cytochrome c551		-3.57	-3.24
gi|495772402|	UTP--glucose-1-phosphate uridylyltransferase		-2.56	-2.11
gi|495772636|	Thioredoxin		-1.60	-2.08
gi|495774058|	Rrf2-linked NADH-flavin reductase		-2.79	N/A
gi|495774197|	FMN-dependent NADH-azoreductase		-1.87	-1.36
gi|495772323|	ATP synthase B chain		-1.59	N/A
gi|495772327|	ATP synthase epsilon chain		-1.60	N/A

Connecting Text:

In order to: 1) increase the availability of genomes from cryophilic and permafrost isolates, 2) investigate the genome-wide features of cold adaptation in subzero growing permafrost bacteria, and 3) identify amino acid modifications with potentially important roles in cold adaptation, we selected subzero growing permafrost strains across several genera for genome sequencing and subsequent genomic and amino acid analyses targeting features of cold adaptation. I was also a collaborator on another study investigating traits of cold adaptation in the Antarctic cryophilic permafrost bacterium *Rhodococcus* sp. JG3, which is briefly outlined in Appendix 2.

Contributions of authors: The authors that contributed to this work are Isabelle Raymond-Bouchard[1], Jacqueline Goordial[1,2], Yevgen Zolotarov[1], Jennifer Ronholm[1], Martina Stromvik[1], Corien Bakermans[3], Lyle G. Whyte[1]. I.R.-B. wrote the manuscript, designed the experimental and bioinformatics pipeline, and performed all growth, phylogenic, genomic, and amino acid analyses. J.G. helped with design of the experimental and bioinformatics pipeline, and provided critical feedback and editing. Y.Z. wrote the python scripts and created the bioinformatics pipeline. J.R. helped with initial culturing and extracted isolate DNA for genome sequencing. M.S. provided critical feedback. C.B. was the lead PI on the genome sequencing project with the Joint Genome Institute, and provided experimental guidance and feedback. L.G.W. provided experimental guidance and critical feedback and editing.

[1]McGill University, Macdonald Campus, 21,111 Lakeshore Rd, Ste.-Anne-de-Bellevue, QC, Canada, H9X 3V9
[2]Bigelow laboratory for Ocean Sciences, East Boothbay, Maine, USA, 04544
[3]Altoona College, Pennsylvania State University, 3000 Ivyside Park, Altoona, PA, USA, 16601

This manuscript has been submitted for publication and is currently in review with the journal

FEMS Microbiology Ecology: FEMSEC-17-08-0399

Chapter 3. Conserved genomic and amino acid traits of cold adaptation in subzero-growing Arctic permafrost bacteria

3.1 Abstract

Permafrost accounts for 27% of all soil ecosystems on earth and is home to diverse and unique microbial communities. Our understanding of microbial life in these environments, their growth, activities, and adaptations, remains limited. We examined features of cold adaptation in bacteria capable of subzero growth (cryophilic), isolated from permafrost, through comparative genomic analyses with closely related mesophiles. Characteristics of cold adaptation such as presence/absence/redundancy of genes known to be important for growth at cold temperatures, and differences in protein flexibility as evidenced by amino acid modifications were investigated. For our analyses, we used 5 microorganisms isolated in the Canadian high Arctic, which had previously demonstrated growth at -5 °C. Genome analyses were carried out using in-house python scripts, bioinformatics programs, and Joint Genome Institute online database tools. The cryophiles have many genes typically associated with cold adaptation, including cold shock proteins, RNA helicases, osmotic and oxidative stress proteins, and carotenoid synthesis enzymes, as well as cell wall and membrane modifications. Higher abundances of genes associated with compatible solute and sodium transport, traits important for osmoregulation, were observed and likely necessary for survival in the pockets of salty liquid water of permafrost. Most of our cryophiles have a higher transposase copy number than their respective mesophilic relatives. Overall, for most of the amino acid (AA) substitutions we looked at, indicative of increased flexibility at cold temperatures, we found few significant differences between the cryophiles and mesophiles. However, there were exceptions, and we found significantly higher proportions of AA changes that would confer increased flexibility at cold temperature in several cryophiles, namely differences in proline, serine, glycine, and aromaticity. Cold/hot AA ratios of > 1, used previously in the literature to classify AA changes in psychrophiles as cold adapted, were also seen in most of the mesophiles we studied, and are inadequate to indicate cold adaptation on their own. Comparing the average of all cryophiles to

all mesophiles, we found that overall cryophiles had a much higher ratio of cold adapted proteins for serine (higher serine content), and to a lesser extent, proline and acidic residues (fewer prolines/acidic residues).

3.2 Introduction

Permafrost accounts for 27% of all soil ecosystems on earth and is home to active and diverse microbial communities, in spite of extreme conditions characterized by permanent sub-zero temperatures, low water activity, and often oligotrophic conditions (Goordial et al. 2012). The identification of active and viable microbial communities in permafrost has fueled interest in understanding the adaptations that allow these organisms to sustain their growth and survival in such harsh environments. Reduced enzyme activity, protein denaturation and misfolding, inhibitory secondary RNA/DNA structures, and increases in membrane rigidity, are challenges that must be overcome at low temperatures (Bakermans et al. 2012; Mykytczuk et al. 2013; De Maayer et al. 2014). Noted adaptations to cold found in psychrophiles include, but are not limited to, using cold shock proteins and helicases to stabilize DNA/RNA processes, membrane modifications to increase unsaturated and branched chain fatty acids, synthesis of carotenoids and antifreeze proteins, stimulation of osmotic and oxidative responses, differential expression of isozymes with different temperature optima, and broad changes in translational and ribosomal processes (Bakermans et al. 2012).

Ensuring that adequate flexibility is retained is a crucial adaptation of cold active proteins to preserve function at low temperatures. Cold adapted proteins have been shown to possess a number of amino acid changes that impart increased flexibility, including fewer salt bridges, a lower content of proline residues, fewer hydrogen bonds, a reduced Arg/(Arg+Lys) ratio, and increased serine and glycine content (Russell et al. 1998; Aghajari et al. 1998; Georlette et al. 2000; Huston et al. 2004; Collins et al. 2005; Ræder et al. 2008; Metpally and Reddy 2009). For example, the elongation factor 2 protein of the archaea *Methanococcoides burtonii* exhibits greater structural flexibility as a result of fewer salt bridges, less densely packed hydrophobic cores and a reduction in proline residues in surface loops (Thomas and Cavicchioli 2000). A

lipase from the Antarctic bacterium *Psychrobacter immobilis* B10 also contains a low number of salt bridges and proline residues, along with a reduced proportion of arginine to lysine residues, a small hydrophobic core and a very small number of aromatic-aromatic interactions (Arpigny et al. 1997). Modifications in k_{cat} and K_m values, to increase catalytic efficiency at lower temperatures, has been noted in certain psychrophilic enzymes when compared to their meso- or thermophilic homologs (Feller et al. 1992; Georlette et al. 2000; Thomas et al. 2001).

Several studies have carried out comparative genomic analyses between psychrophile and mesophile and/or thermophiles in order to examine differences in amino acid composition, which may impart greater flexibility in cold adapted microorganisms. Of these, only four focused on microorganisms that exhibit growth at subzero temperatures and in each case focused on a single psychrophile (Methé et al. 2005; Ayala-del-Río et al. 2010; Mykytczuk et al. 2013; Goordial et al. 2016b). Previous genomic analyses include those focused on psychrophilic protein comparisons against large nonredundant protein databases and comparisons against select specific mesophiles (Saunders et al. 2003; Rabus et al. 2004; Methé et al. 2005; Metpally and Reddy 2009; Zhao et al. 2010; Ayala-del-Río et al. 2010; Mykytczuk et al. 2013; Goordial et al. 2016b). Methods for comparison have included those that compare total amino acid counts or composition across an entire genome between mesophiles and psychrophiles, to those that compare amino acid counts only between previously matched homologous proteins. Results from these studies have been inconsistent. While some identified a number of changes in amino acid composition or proportions in psychrophilic genomes, consistent with those identified in psychrophilic proteins (Saunders et al. 2003; Zhao et al. 2010; Ayala-del-Río et al. 2010), others have found limited changes (Methé et al. 2005; Mykytczuk et al. 2013; Goordial et al. 2016b), and in some cases, none (Rabus et al. 2004; Yang et al. 2015). It remains unclear whether these amino acid substitutions are universal adaptations that can be conclusively identified on a large scale across genomes in psychrophiles. It is also of interest to determine if there are different adaptations associated with cryophiles capable of growth at sub-zero temperatures, compared with psychrophiles which may have temperature optima at low temperatures (4 – 10 °C) and that may not be adapted to the challenges associated with subfreezing temperatures. To our knowledge, very few studies on amino acid cold adaptive

traits in psychrophiles have looked specifically at cryophiles. Furthermore, in those studies that perform comparisons using distantly related organisms, it is not always apparent if the differences observed are the result of cold adaptation or simply phylogenic divergence. Comparisons using organisms in the same genus, or neighbor genus, provides a more stringent analysis, as we expect these members to share close common ancestry, and therefore observed amino acid differences between psychrophiles and their close mesophilic relatives are more likely to be the result cold adaptation.

In the present study, we did comparative analyses of 5 subzero-growing (cryophilic) strains (Table 3.1) previously isolated from the Canadian high Arctic that span several phyla (Actinobacteria, Proteobacteria, Firmicutes) (Steven et al. 2007a, 2008; Lacelle et al. 2011). These organisms were isolated from permafrost, which has an ambient temperature of ~-17 °C. In permafrost, these microbes are hypothesized to inhabit very thin brine veins. We compared the genomes of these organisms to closely related mesophilic relatives in the same genus. With these new genome sequences, we aim to increase our potential for understanding and identifying the amino acid basis of cold adaptation in sub-zero growing organisms, including potentially conserved or important adaptive traits. In addition, we also investigate the presence of known cold adaptation genes and genomic redundancy in each cryophile.

3.3 Results and Discussion

3.3.1 Genome and strain properties

High quality permanent drafts were obtained for all five cryophilic genomes with genome sizes ranging from 2.8 Mbp for *Kocuria* sp. KROCY2 to 7.2 Mbp for *Methylobacterium* sp. EUR3 AL-11 (Table 3.2). Accordingly, *Methylobacterium* sp. EUR3 AL-11 had the highest number of predicted genes with 7001, while *Kocuria* sp. KROCY2 had the lowest, 2480. The GC content also varied greatly from 38% in *Paenisporosarcina* sp. Eur1 9.01.10 to 72% for *Actinotalea* sp. KRMCY2. Close to 80% of all protein coding genes could be assigned function predictions (Table 3.2). While GC rich regions have been noted in some psychrophiles (De Maayer et al. 2014),

overall we found GC content in our cryophiles to be consistent with other members of their respective genera (Table S3.1).

We attempted to determine subzero growth rates for the cryophilic strains, however although chosen because of their ability to grow on solid R2A agar supplement with 7% sucrose at -5°C (Steven et al. 2007a, 2008; Lacelle et al. 2011), we struggled to find liquid media adequate for growth at low temperatures in several strains. R2A media supplemented with 7% sucrose is susceptible to freezing in liquid form. *Paenisporosarcina* sp. Eur1 9.01.10 grew well at -5°C with a generation time of 14.6 days, although it grew optimally at 25°C (Table S3.2). This growth rate is similar to that observed for other cryophiles at -5°C (Mykytczuk et al. 2013; Goordial et al. 2016b). Only *Polaromonas* sp. Eur3 1.2.1. exhibited characteristics of a true psychrophile (stenopsychrophile) with a faster generation time at 10°C (3.1 days) than at 22°C (3.4 days). It also showed similar generation times at 0°C (3.6 days) and was incapable of growth above 22°C (Table S3.2). Several members of the genus *Sporosarcina* and *Polaromonas* are known to be psychrophilic, having been isolated from numerous polar environments (Irgens et al. 1996; Yu et al. 2008; Margesin et al. 2012; Wang et al. 2015, 2016; Yan et al. 2016).

3.3.2 Genome comparisons and cold adaptation genes

All five organisms possess genes known to be important in cold adaptation and growth including cold shock proteins, RNA helicases, osmotic and oxidative stress, carotenoid synthesis, translation factors, and membrane and peptidoglycan modifications (Table 3.3). Although the presence of these genes is not unique to psychrophiles, we also found them to be present in mesophiles, their abundance was higher in the cryophiles in many cases. *Polaromonas* Eur3 1.2.1 possesses 13 copies of universal stress protein (UspA family) genes, produced in response to numerous environmental stressors (Kvint et al. 2003), and 12 copies of Na+/H+ antiporters. While also present in mesophilic *Polaromonas* strains, we found the MnhD subunit of Na+/H+ antiporters to be higher in copy number in *Polaromonas* sp. Eur3 1.2.1 (6) than in mesophilic relatives (1-2) (Figure 3.1A). Similarly, a Na+/melibiose symporter was higher in gene abundance in *Paenisporosarcina* sp. Eur1 9.01.10 than mesophiles (Figure 3.1C). *Kocuria* sp.

KROCY2, able to grow in up to 19% salt, possesses 9 copies of choline-glycine betaine transporter (Table 3.3), for uptake of the compatible solute glycine betaine, several more than the mesophiles we used in comparative analyses (Figure 3.1B). Four of the five cryophiles also possess the genes for synthesis of trehalose, another well-established compatible solute; 10 copies of the first enzyme in trehalose synthesis, trehalose-6-phosphate synthase, are found in the genome of *Actinotalea* sp. KRMCY2 (Table 3.3). Trehalose and glycine betaine are important compatible solutes in cold adapted organisms, and along with sodium transporters, have roles in osmoregulation and stability (Thomas et al. 2001; Doyle et al. 2012). Salt tolerance would be necessary for survival in permafrost habitats consisting of thin films of salty water. The permafrost cryophilic and halotolerant *Planococcus halocryophilus* is also known to have multiple copies of genes involved in glycine betaine transport (Mykytczuk et al. 2013).

All five strains have multiple copies of superfamily II DNA/RNA helicases, which includes the DEAD box helicases, shown to be crucial for stabilizing RNA at lower temperatures (Kuhn 2012). In addition, most cryophiles possessed toxin/antitoxin (TA) modules, with both *Actinotalea* sp. KRMCY2 and *Methylobacterium* sp. EUR3 AL-11 having copies of genes for TA systems not seen in their mesophilic counterparts (Figure 3.1D and 3.1E). TA systems are important regulators of persistence and stress responses in certain bacteria, including *Mycobacterium tuberculosis* and *Escherichia coli* (Tiwari et al. 2015; Sauert et al. 2016), and one such system was found to be strongly induced at -10°C in *Planococcus halocryophilus* (Raymond-Bouchard et al. 2017). *Paenisporosarcina* sp. Eur1 9.01.10 and *Actinotalea* sp. KRMCY2 both have higher copy numbers of the DNA binding transcriptional regulators of the LacI/PurR family than the mesophiles we analyzed (Figure 3.1C and 3.1E). This family contains several members and includes repressors of the lactose operon, purine nucleotide synthesis, and trehalose operon. Interestingly, transposase gene numbers were higher in abundance in four of the five strains, *Polaromonas* sp. Eur3 1.2.1, *Kocuria* sp. KROCY2, *Actinotalea* sp. KRMCY2, and *Methylobacterium* sp. EUR3 AL-11 compared to their respective mesophilic relatives (Figure 3.1A, B, D, and E). Transposase activity has been shown to be linked to the stress response, and therefore, may be a mechanism in which these organisms gain genomic plasticity and a selective advantage necessary to survive and colonize extreme cold environments (Seckbach et

al. 2013; Shen et al. 2014). Increases in gene copy numbers have also been theorized to be an adaptive feature in and of itself by offering an advantage through selective expression of cold or hot adapted gene copies at different temperatures (Maki et al. 2006; Mykytczuk et al. 2013). This is believed to confer advantages during cold growth given that selectively upregulated gene copies are likely to function better at their induced temperature, and thus allows organisms to have an expanded growth temperature range, such as *P. halocryophilus*, which grows from -15 to 37 °C.

3.3.3 Amino acid adaptations

To examine amino acid substitutions that could favor increased flexibility, we performed genome wide comparative amino acid analyses using the predicted protein sequences for each cryophile. We determined the number of significantly cold or hot adapted genes in each cryophilic genome for specific amino acid indices associated with cold adaptation (Figure 3.2). Previous studies have used the ratio of cold/hot proteins as a standard to determine cold adaptation for a given trait and deemed those with cold/hot ratios > 1, signifying enrichment of cold adapted proteins, as 'cold adapted' (Ayala-del-Río et al. 2010; Mykytczuk et al. 2013). For comparative purposes and as a control to check if these cold/hot ratios are specific to cold adaptation in cryophiles, we also determined the number of hot and cold adapted genes and cold/hot ratios for two mesophiles for each cryophile (Figures 3.2 and S3.1). As these results highlight, cold/hot ratios of > 1 can be seen in all of the mesophiles for certain traits and we therefore argue that using a ratio cutoff of 1 is not enough on its own to indicate cold adaptation for a given trait in psychrophilic genomes. We instead focused on determining significant differences in proportions of hot and cold genes in the cryophiles vs the mesophiles as determined by chi square analysis with Bonferroni correction ($P \leq 0.0167$) used to correct for multiple comparisons (Figures 3.2, S3.1, and S3.2). Of note, most of the proteins in both cryophile and mesophile genomes were classified as neutral with no significant changes in amino acid content (Figure S3.2). Cold/hot ratios were therefore determined for the remaining proteins which showed significant changes and could be classified as hot or cold adapted.

While we did not find the proportions of cold and hot adapted genes in the cryophilic genomes to be significantly different from the mesophiles for many of the amino acid traits, there were noteworthy exceptions. *Polaromonas* sp. Eur3 1.2.1 had significantly higher and lower proportions of cold and hot adapted genes, respectively, for the index proline than the mesophiles, with an overall much higher cold/hot ratio (Figure 3.2A), indicative that more cryophilic proteins possess overall fewer proline residues than their mesophilic counterparts. *Actinotalea* sp. KRMCY2 was also significantly cold adapted for proline (Figure 3.2C). Since prolines are covalently bonded to the nitrogen atom of the peptide group, they impose constraint on rotations of the peptide backbone and create rigid kinks in peptide chains, thus contributing to overall reduced flexibility of proteins. A reduction in proline residues has also been observed, when compared to mesophiles, in the genomes of the cold marine bacteria *Shewanella halifaxensis* and *Shewanella sediminis* (Zhao et al. 2010) and in a number of cold adapted enzymes, including the psychrophilic α-amylase from *Alteromonas haloplanctis* and the 3-isopropylmalate dehydrogenase from the eurypsychrophile bacterium *Vibrio* sp. 15 (Wallon et al. 1997; Aghajari et al. 1998).

Both *Kocuria* sp. KROCY2 and *Actinotalea* sp. KRMCY2 had higher proportions of cold adapted proteins for serine (i.e. more serine residues) than the mesophiles, while *Actinotalea* sp. KRMCY2 and *Methylobacterium* sp. EUR3 AL-11 had significantly higher proportions of cold adapted proteins for glycine (more glycine) (Figures 3.2B, 3.2C, & 3.2D). Due to its small size, glycine provides more conformational freedom, thus increasing flexibility, and serine, as a polar uncharged amino acid, may compensate partly for the general reduction in charged residues in psychrophilic proteins (Saunders et al. 2003). Serine and glycine have been shown to be higher in a number of psychrophilic proteins, including an Antarctic citrate synthase (Russell et al. 1998) and a DEAD-box RNA helicase (Linding et al. 2003). Increases in serine residues is one of the more commonly observed traits and has also been found in a uracil-DNA-N-glycosylate (Ræder et al. 2008), a thermolysin (Adekoya et al. 2006), and the genomes of *Colwellia psychrerythraea* and marine *Glaciecola* species (Methé et al. 2005; Qin et al. 2013). Significant cold adaptation was also observed for aromaticity (fewer aromatic residues) in *Kocuria* sp. KROCY2 and *Methylobacterium* sp. EUR3 AL-11, and for R/K ratio, with an overall lower number

of arginines to lysines, in *Polaromonas* sp. Eur3 1.2.1. The bacterial lipase from the Antarctic psychrophile *Psychrobacter immobilis* B10 features a very small number of aromatic-aromatic interactions (Arpigny et al. 1997), and proteins present at the cell surface of *Planococcus halocryophilus* at -10°C have significantly fewer aromatic residues than surface proteins present at higher temperatures (Ronholm et al. 2015; Appendix 1).

In addition to serine and glycine, *Actinotalea* sp. KRMCY2 showed the greatest amount of significantly cold adapted indices with 6 out of the 10 being significantly higher for cold adaption from the mesophiles. However, for the other cryophiles, only a few of the indices showed evidence of cold adaptation, and in the case of *Paenisporosarcina* sp. Eur1 9.01.10, none showed significant cold adaptation (Figure 3.2E). It is worthwhile to note here that many members of the genera *Paenisporosarcina* and *Sporosarcina* are psychrophilic, as discussed above, and therefore it is possible that most members of these genera possess characteristics of cold adaption, even in those characterized as mesophiles.

By comparing the average cryophile ratios for each trait with the average of the mesophile ratios, we were able to highlight some differences in several indices. A comparison of all cryophiles with all mesophiles revealed higher average ratios of cold/hot adapted genes in the cryophiles for serine, and slightly higher for acidic, proline, polar, charged and R/K indices (Figure 3.3). We did not, however, find these results to be significantly different by t-test ($P \leq 0.05$), though the results were close for proline ($P = 0.07$), serine ($P = 0.1$) and acidic residues ($P = 0.1$). This lack of significant difference is in large part the result of noticeable variation in cold/hot ratios between the different genera and phyla for the specific amino acid indices we studied. Amino acid cold adaptive strategies may be taxa specific and vary greatly between individual proteins. Indeed, to date, not every amino acid feature discussed here has been found in a single genome or cold-active protein, rather most tend to possess only a few of these traits (Rodrigues and Tiedje 2008). Contradictory findings have also been reported with certain amino acid changes, such as glycine, appearing to be increased in some psychrophilic proteins (Russell et al. 1998; Linding et al. 2003) but decreased in certain psychrophilic genomes (Zhao et al. 2010). Thus, the adaptive strategies employed by psychrophiles may vary from organism

to organism. These adaptations may not be present in all psychrophiles, as is the case with *Desulfotalea psychrophila*, which did not show detectable amino acid substitutions indicative of cold adaptation in its genome (Rabus et al. 2004), and in our case with *Paenisporosarcina* sp. Eur1 9.01.10. The cold-active isocitrate dehydrogenase from psychrophilic *Colwellia maris* also did not appear to show any characteristic amino acid changes associated with psychrophilic proteins (Watanabe et al. 2005).

Proteins with only a single or very few amino acid changes, which is enough to have significant impacts on function, would not be as easily detectable in these large-scale analyses since a single a single change is less likely to result in a protein begin detected as significantly cold adapted when compared to mesophiles. In addition, changes may, in some cases, only occur in a subset of proteins and not result of large-scale changes across the entire genome/proteome. The overall large number of proteins in this study that were neither cold or hot adapted would add weight to this theory. In *Psychrobacter arcticus*, noted adaptations appeared to be more prevalent in proteins involved in cell growth and reproduction (Ayala-del-Río et al. 2010). Changes may also be conformational in nature, affecting secondary, tertiary and quaternary structure, but not overall amino acid sequences.

Lastly, and perhaps most importantly, the specific location of the amino acids, not simply total numbers, may be especially important. The citrate synthase from Antarctic strain DS2-3R showed a reduction in salt bridges and proline residues specifically in the loop regions of the protein, while increases in glycine and serine occurred close to catalytic sites (Russell et al. 1998). In the psychrophilic *Alteromonas haloplanctis* α-amylase, it is the core of the protein that shows reduced numbers of proline and arginine (Aghajari et al. 1998). Nevertheless, while we did not find significantly conserved traits across the cryophiles and observed noticeable differences in cold adaptation between cryophiles for many amino acid features, our results do highlight noteworthy trends in amino acid properties of cryophilic proteins, and did identify traits which are significantly different from mesophilic relatives for several of the cryophiles, especially with regards to glycine, proline, and serine. Changes in these amino acids is amongst

the most consistently observed cold adaptive traits in studies focusing on cold adaption of psychrophilic proteins, and these changes seem to span broad phyla.

3.4 Conclusion

We found evidence for presence of many cold adaptation genes in the cryophilic genomes, as well as genomic redundancy, theorized to be an important feature of cold adapted organisms. We identified significant cold adaptation, to maintain protein flexibility at low temperatures, in four of the five cryophiles for a subset of the amino acid traits we studied, including proline, glycine, serine, and aromaticity. Comparing all cryophiles to all mesophiles, we found trends favoring lower proline and acidic residues, and higher serine content in cryophilic proteins. It is possible that changes in content of these specific amino acids may be especially important to increase protein flexibility during subzero growth. Further comparisons between cryophiles, psychrophiles, and mesophiles would need to be done to determine if this is the case. On the whole, however, we could not easily detect cold adaptations on a genome-wide scale for many of the amino acid indices we investigated, and we found a significant degree of variability between the genera we studied. This may indicate that, while some overlap exists, overall, cold-adapted microbes use different approaches to adapt to low temperatures. In addition, since amino acid adaptations may be specific to certain subsets or categories of proteins, we believe that individual comparative analyses focusing on single/few proteins, and taking into account not only total amino acid numbers, but the location of specific amino acids within the sequence, the overall potential conformation of the protein, and, if possible, functional assays, are more likely to provide an accurate overall representation of cold adaption in psychrophilic proteins. Overall, this study has increased our understanding of the genetic basis of cold adaptation in permafrost microbes, and thus helps up better understand how these organisms behave and grow at subzero temperatures.

3.5 Materials and Methods

3.5.1 Organism selection and classification

Actinotalea sp. KRMCY2, *Paenisporosarcina* sp. Eur1 9.01.10, *Methylobacterium* sp. AL-11, *Kocuria* sp. KROCY2, and *Polaromonas* sp. Eur3 1.2.1. were isolated from permafrost cores from Eureka, Nunavut or from ice samples from the Yukon, as previously described (Steven et al. 2007a, 2008; Lacelle et al. 2011). These strains were selected for genomic sequencing due to their ability to grow at sub-zero temperatures under laboratory conditions (Table 3.1). *Kocuria* sp. KROCY2, *Paenisporosarcina* sp. Eur1 9.01.10, and *Actinotalea* sp. KRMCY2 were initially classified as *Microbacterium* sp. KROCY2, *Sporosarcina* sp. Eur1 9.01.10, and *Cellulomonas* sp. KRMCY2, respectively. However, our more recent and comprehensive 16s phylogenic analyses would place these strains within the *Kocuria*, *Paenisporosarcina*, and *Actinotalea*, respectively (Figure S3.3). The highest taxonomically classified blast matches for *Microbacterium* sp. KROCY2, *Sporosarcina* sp. Eur1 9.01.10, and *Cellulomonas* sp. KRMCY2 were *Kocuria palustris* strain IHBB 17003 (100% identity), *Paenisporosarcina indica* PN2 (99% identity), and *Actinotalea ferrariae* CF5-4 (98% identify), respectfully. As such, they were renamed accordingly. The highest taxonomically classified blast matches for *Polaromonas* sp. Eur3 1.2.1 and *Methylobacterium* sp. EUR3 AL-11 were *Polaromonas cryoconiti* Cr4-35 (99% identity) and *Methylobacterium radiotolerans*, respectfully.

3.5.2 Growth conditions

Cultures were maintained on R2A agar or liquid (BD Difco) for *Actinotalea* sp. KRMCY2, *Paenisporosarcina* sp. Eur1 9.01.10, *Methylobacterium* sp. AL-11, and *Kocuria* sp. KROCY2, and ½ R2A for *Polaromonas* sp. Eur3 1.2.1. Growth temperatures were described previously (Steven et al. 2007a, 2008; Lacelle et al. 2011) on agar only and tested in this study using liquid cultures at -5°C, 0°C, 5°C, 10°C, 22°C, and 25°C in tryptic soy broth (TSB; BD Difco), 1/10 TSB, ¼ TSB, R2A, or ½ R2A and supplemented with varying concentrations (2-7%) of sucrose, glycerol, or NaCl at subzero temperatures. Salinity tolerance was determined from liquid cultures grown on R2A

amended with NaCl in 1-2% increments from 0-20%. Previous phylogenic classification was verified by comparing the 16S rRNA gene sequence of each organism to the nucleotide collection database (nr/nt) using NCBI nucleotide BLAST and default parameters (Altschul et al. 1990). A phylogenic tree was constructed for each organism highlighting its position relative to select taxonomically classified strains. Phylogenic inferences were obtained using maximum-likelihood with bootstrapping (1,000) within MEGA6 (Tamura et al. 2013).

3.5.3 DNA extraction

Strains were grown to stationary state at room temperature (~23 °C) on Tryptic Soy Broth (BD Difco). Genomic DNA was isolated using the Epicentre MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, Wisconsin) as per the manufacturer's instructions. Purified DNA was checked with the NanoDrop 1000 (Thermoscientific, Wilmington, Delaware) and sent for whole genome sequencing according the DOE Join Genome Institute (JGI) standards.

3.5.4 Genome sequencing and assembly

The draft genomes of *Kocuria* sp. KROCY2, *Actinotalea* sp. KRMCY2, *Polaromonas sp.* EUR3 1.2.1, and *Methylobacterium* sp. EUR3 AL–11 were generated at the DOE Joint Genome Institute (JGI) using the Pacific Biosciences (PacBio) technology. Pacbio SMRTbell™ libraries were constructed for each isolate and sequenced on the PacBio RS platform, which generated 166,364 filtered subreads totaling 486.7 Mbp for *Kocuria* sp. KROCY2, 253,745 filtered subreads totaling 785.8 Mbp for *Actinotalea* sp. KRMCY2, 150,954 filtered subreads totaling 668.5 Mbp for *Polaromonas sp.* EUR3 1.2.1, and 369,217 filtered subreads totaling 928.2 Mbp for *Methylobacterium* sp. EUR3 Al-11. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. The raw reads were assembled using HGAP (version: 2.3.0) (Chin et al. 2013). The final draft assembly contained 1 contig in 1 scaffold for *Kocuria* sp. KROCY2, *Actinotalea* sp. KRMCY2, and *Polaromonas sp.* EUR3 1.2.1 and produced an average input read coverage of 186.4X, 179.6X, and 108.4X, respectively, and 7 contigs in 7 scaffolds for *Methylobacterium* sp. EUR3 AL-11 with input read coverage of 158.9X.

The draft genome of *Paenisporosarcina* sp. EUR1 9.01.10 was generated at the DOE Joint Genome Institute (JGI) using the Illumina technology. An Illumina shotgun library and long insert mate pair library was constructed and sequenced using the Illumina HiSeq 2000 platform (Bennett 2004). 22,752,872 reads totalling 3,412.9 Mb were generated from the shotgun and 40,430,662 reads totalling 3,679.2 Mb were generated from the long insert mate pair library. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. All raw Illumina sequence data was passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artifacts (Li et al. 2011). Filtered Illumina reads were assembled using AllpathsLG (PrepareAllpathsInputs: PHRED 64=1 PLOIDY=1 FRAG COVERAGE=150 JUMP COVERAGE=25; RunAllpathsLG: RUN=std pairs TARGETS=standard VAPI WARN ONLY=True OVERWRITE=True) (Gnerre et al. 2011). The final draft assembly contained 18 contigs in 5 scaffolds. The final assembly is based on 3,412.4 Mb of Illumina Std PE, 3,679.1 Mb of Illumina CLIP PE post filtered data, which provides an average 2026.1X Illumina coverage of the genome.

3.5.5 Genome annotation

Genes were identified using Prodigal (Hyatt et al. 2010), followed by a round of manual curation using GenePRIMP (Pati et al. 2010) for finished genomes and draft genomes in fewer than 10 scaffolds. The predicted CDSs were translated and used to search the National Center for Biotechnology Information (NCBI) nonredundant database, UniProt, TIGRFam, Pfam, KEGG, COG, and InterPro databases. The tRNAScanSE tool (Lowe and Eddy 1996) was used to find tRNA genes, whereas ribosomal RNA genes were found by searches against models of the ribosomal RNA genes built from SILVA (Pruesse et al. 2007). Other non–coding RNAs such as the RNA components of the protein secretion complex and the RNase P were identified by searching the genome for the corresponding Rfam profiles using INFERNAL (Nawrocki and Eddy 2013). Additional gene prediction analysis and manual functional annotation was performed within the Integrated Microbial Genomes (IMG) platform (http://img.jgi.doe.gov) developed by the Joint Genome Institute, Walnut Creek, CA, USA (Markowitz et al. 2009).

3.5.6 Genome and amino acid analyses

The whole genomes of close relatives (genus or neighboring genus) to the cryophilic strains, which have been annotated as mesophiles, were collected from publicly available databases, and were used for comparative analyses to the cryophilic genomes (Table S3.1). Functional gene assignments, including COG and KEGG categories and pathways, were obtained from the JGI website for all the genomes, cross-referenced and used for comparative analyses. Amino acid cold adaptation analyses was carried out as described previously (Goordial et al. 2016b), with some modifications. Briefly, custom databases were created using the mesophilic genomes, and each cryophilic genome was compared to the mesophilic databases using stand-alone Blastp (Camacho et al. 2009) with a cut-off e-value of 1e−15. The top blast match protein sequence from each mesophile genome for each cryophilic protein was used for further analyses. Only non-hypothetical proteins and their mesophilic matches (full proteins) were included and assessed for cold adaptation at the amino acid level for the following indices previously associated with cold adaptation: arginine to lysine ratio; frequency of acidic, polar uncharged, and charged residues; proline, glycine, serine residues; aromaticity; aliphacity; and grand average of hydropathicity (GRAVY). The results were averaged for mesophilic proteins and compared to the cryophilic protein. Cryophilic proteins that were found to be significantly different from the average of the mesophilic proteins ($P < 0.05$) for each index were then assigned as 'cold adapted' or 'hot adapted' depending on the direction of change. We considered proteins cold adapted for each index if the direction of change was significantly lower for proline, R/K (arginine/lysine) ratio, acidic, charged, aliphatic, aromatic and hydrophobicity (GRAVY) indices and significantly higher for serine, glycine and polar uncharged indices. This analysis was repeated using the mesophile genomes, in the place of the cryophile genome, as controls and for comparison purposes. The proportions, in the total genome, of cold and hot adapted genes for the cryophiles and their mesophile controls were compared using a chi-square test with Bonferroni correction ($P \leq 0.0167$) to determine if the differences seen were significant. All amino acid analyses were carried out using in-house python scripts publicly available at https://github.com/ColdAdaptationScripts.

3.6 Acknowledgements

Financial support was provided by fellowships from the Natural Sciences and Engineering Research Council (NSERC) CREATE Canadian Astrobiology Training Program (CATP) and Canadian Graduate Scholarship to IRB, and NSERC Discover Grant Program, Northern Research Supplement grant, and a Polar Continental Shelf Project grant to LGW. Sequencing was performed as part of a U.S. Department of Energy Joint Genome Institute Community Sequencing Program Project (Quartely; ID 1015712). The work conducted by JGI is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Thanks to Nicole Shapiro, Tanja Woyke, Marcel Huntemann, James Han, Amy Chen, Nikos Kyrpides, Victor Markowitz, Krishna Palaniappan, Natalia Ivanova, Natalia Mikhailova, Galina Ovchinnikova, Andrew Schaumberg, Amrita Pati, Dimitrios Stamatis, Tatiparthi Reddy, Henrik P. Nordberg, Michael N. Cantor, and Susan X. Hua of JGI.

Figure 3.1. COG categories and gene functions with higher abundances of copies in the cryophilic genomes when compared to mesophiles. **A**) Polaromonas sp. Eur3 1.2.1; **B**) Kocuria sp. KROCY2; **C**) Paenisporosarcina sp. Eur1 9.01.10; **D**) Methylobacterium sp. EUR3 AL-11; and **E**) Actinotalea sp. KRMCY2. The blue column closest to the Y-axis represents copy numbers for the cryophiles in each case. Letters for the COG categories are given in parenthesis after the gene description: C) Energy production and conversion; D) Cell cycle control, cell division, chromosome partitioning; E) Amino acid transport and metabolism; G) Carbohydrate transport and metabolism; H) Coenzyme transport and metabolism; I) Lipid transport and metabolism; J) Translation, ribosomal structure and biogenesis; K) Transcription; L) Replication, recombination and repair; M) Cell wall/membrane/envelope biogenesis; N) Cell motility O) Post-translational modification, protein turnover, and chaperones; P) Inorganic ion transport and metabolism; R) General function prediction only; S) Function unknown T) Signal transduction mechanisms; U) Intracellular trafficking, secretion, and vesicular transport; V) Defense mechanisms; X) Mobilome: prophages, transposons.

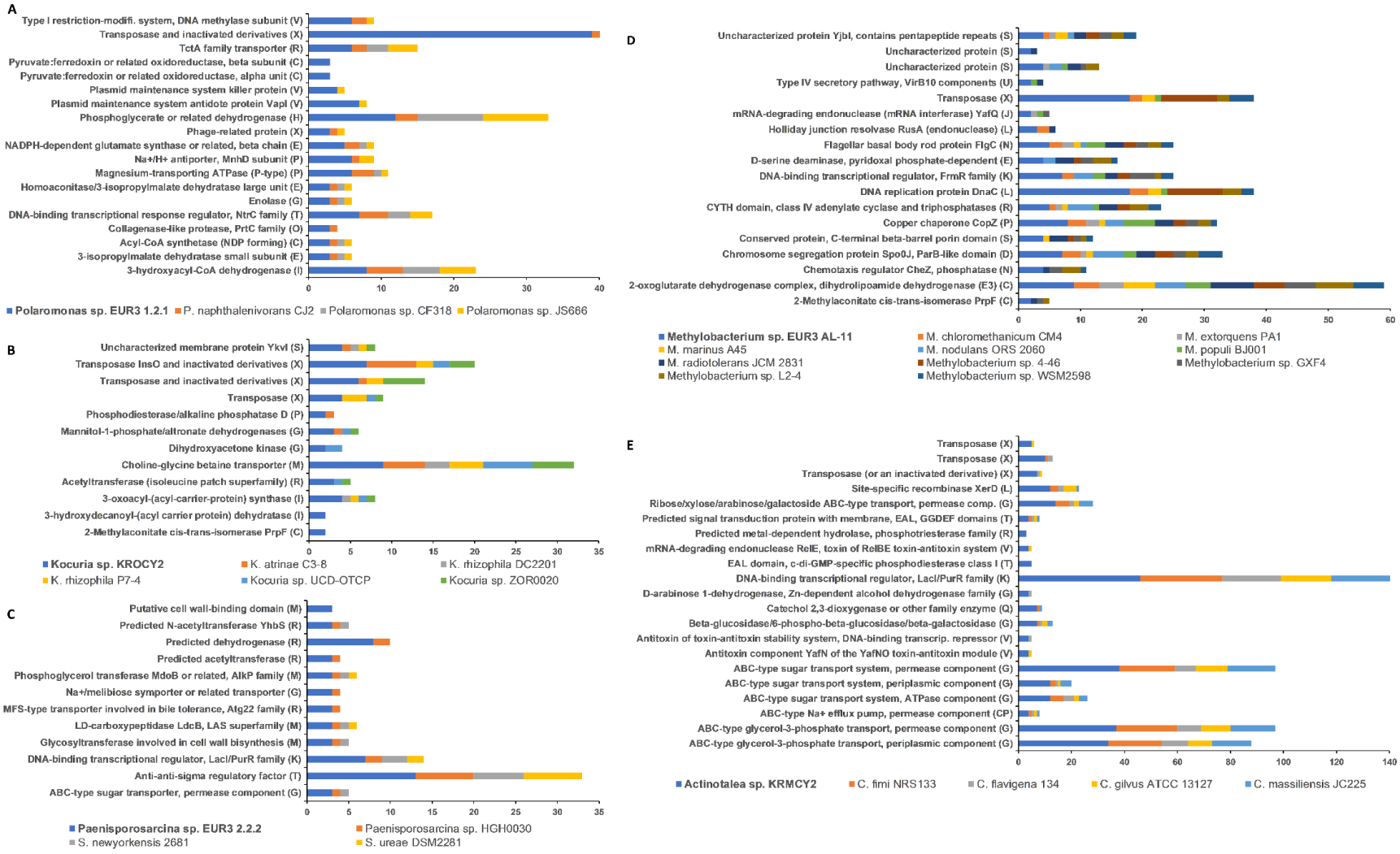

A
Type I restriction-modifi. system, DNA methylase subunit (V)
Transposase and inactivated derivatives (X)
TctA family transporter (R)
Pyruvate:ferredoxin or related oxidoreductase, beta subunit (C)
Pyruvate:ferredoxin or related oxidoreductase, alpha unit (C)
Plasmid maintenance system killer protein (V)
Plasmid maintenance system antidote protein VapI (V)
Phosphoglycerate or related dehydrogenase (H)
Phage-related protein (X)
NADPH-dependent glutamate synthase or related, beta chain (E)
Na+/H+ antiporter, MnhD subunit (P)
Magnesium-transporting ATPase (P-type) (P)
Homoaconitase/3-isopropylmalate dehydratase large unit (E)
Enolase (G)
DNA-binding transcriptional response regulator, NtrC family (T)
Collagenase-like protease, PrtC family (O)
Acyl-CoA synthetase (NDP forming) (C)
3-isopropylmalate dehydratase small subunit (E)
3-hydroxyacyl-CoA dehydrogenase (I)
0 10 20 30 40
Polaromonas sp. EUR3 1.2.1
P. naphthalenivorans CJ2
Polaromonas sp. CF318
Polaromonas sp. JS666
B
Uncharacterized membrane protein YkvI (S)
Transposase InsO and inactivated derivatives (X)
Transposase and inactivated derivatives (X)
Transposase (X)
Phosphodiesterase/alkaline phosphatase D (P)
Mannitol-1-phosphate/altronate dehydrogenases (G)
Dihydroxyacetone kinase (G)
Choline-glycine betaine transporter (M)
Acetyltransferase (isoleucine patch superfamily) (R)
3-oxoacyl-(acyl-carrier-protein) synthase (I)
3-hydroxydecanoyl-(acyl carrier protein) dehydratase (I)
2-Methylaconitate cis-trans-isomerase PrpF (C)
0 5 10 15 20 25 30 35
Kocuria sp. KROCY2
K. atrinae C3-8
K. rhizophila DC2201
K. rhizophila P7-4
Kocuria sp. UCD-OTCP
Kocuria sp. ZOR0020
C
Putative cell wall-binding domain (M)
Predicted N-acetyltransferase YhbS (R)
Predicted dehydrogenase (R)
Predicted acetyltransferase (R)
Phosphoglycerol transferase MdoB or related, AlkP family (M)
Na+/melibiose symporter or related transporter (G)
MFS-type transporter involved in bile tolerance, Atg22 family (R)
LD-carboxypeptidase LdcB, LAS superfamily (M)
Glycosyltransferase involved in cell wall bisynthesis (M)
DNA-binding transcriptional regulator, LacI/PurR family (K)
Anti-anti-sigma regulatory factor (T)
ABC-type sugar transporter, permease component (G)
0 5 10 15 20 25 30 35
Paenisporosarcina sp. EUR3 2.2.2
Paenisporosarcina sp. HGH0030
S. newyorkensis 2681
S. ureae DSM2281
D
Uncharacterized protein YjbI, contains pentapeptide repeats (S)
Uncharacterized protein (S)
Uncharacterized protein (S)
Type IV secretory pathway, VirB10 components (U)
Transposase (X)
mRNA-degrading endonuclease (mRNA interferase) YafQ (J)
Holliday junction resolvase RusA (endonuclease) (L)
Flagellar basal body rod protein FlgC (N)
D-serine deaminase, pyridoxal phosphate-dependent (E)
DNA-binding transcriptional regulator, FrmR family (K)
DNA replication protein DnaC (L)
CYTH domain, class IV adenylate cyclase and triphosphatases (R)
Copper chaperone CopZ (P)
Conserved protein, C-terminal beta-barrel porin domain (S)
Chromosome segregation protein Spo0J, ParB-like domain (D)
Chemotaxis regulator CheZ, phosphatase (N)
2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) (C)
2-Methylaconitate cis-trans-isomerase PrpF (C)
0 10 20 30 40 50 60
Methylobacterium sp. EUR3 AL-11
M. chloromethanicum CM4
M. extorquens PA1
M. marinus A45
M. nodulans ORS 2060
M. populi BJ001
M. radiotolerans JCM 2831
Methylobacterium sp. 4-46
Methylobacterium sp. GXF4
Methylobacterium sp. L2-4
Methylobacterium sp. WSM2598
E
Transposase (X)
Transposase (X)
Transposase (or an inactivated derivative) (X)
Site-specific recombinase XerD (L)
Ribose/xylose/arabinose/galactoside ABC-type transport, permease comp. (G)
Predicted signal transduction protein with membrane, EAL, GGDEF domains (T)
Predicted metal-dependent hydrolase, phosphotriesterase family (R)
mRNA-degrading endonuclease RelE, toxin of RelBE toxin-antitoxin system (V)
EAL domain, c-di-GMP-specific phosphodiesterase class I (T)
DNA-binding transcriptional regulator, LacI/PurR family (K)
D-arabinose 1-dehydrogenase, Zn-dependent alcohol dehydrogenase family (G)
Catechol 2,3-dioxygenase or other family enzyme (Q)
Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (G)
Antitoxin of toxin-antitoxin stability system, DNA-binding transcrip. repressor (V)
Antitoxin component YafN of the YafNO toxin-antitoxin module (V)
ABC-type sugar transport system, permease component (G)
ABC-type sugar transport system, periplasmic component (G)
ABC-type sugar transport system, ATPase component (G)
ABC-type Na+ efflux pump, permease component (CP)
ABC-type glycerol-3-phosphate transport, permease component (G)
ABC-type glycerol-3-phosphate transport, periplasmic component (G)
0 20 40 60 80 100 120 140
Actinotalea sp. KRMCY2
C. fimi NRS133
C. flavigena 134
C. gilvus ATCC 13127
C. massiliensis JC225

Figure 3.2. Amino acid traits of cold adaptation in cryophilic proteins. Left side graphs: significantly ($P < 0.05$) hot and cold adapted proteins identified in each cryophile (A-E) for each amino acid trait measured: aliphatic index, R/K ratio, acidic residues, aromaticity, GRAVY, and proline, serine, glycine, polar and charged residues. Right side graphs: ratio of cold/hot adapted proteins identified in each cryophile (name bolded; black bar) and in two mesophilic relatives of each cryophile. Proportions of cold adapted proteins that were significantly different in the cryophile genome when compared to both mesophilic genomes are starred.

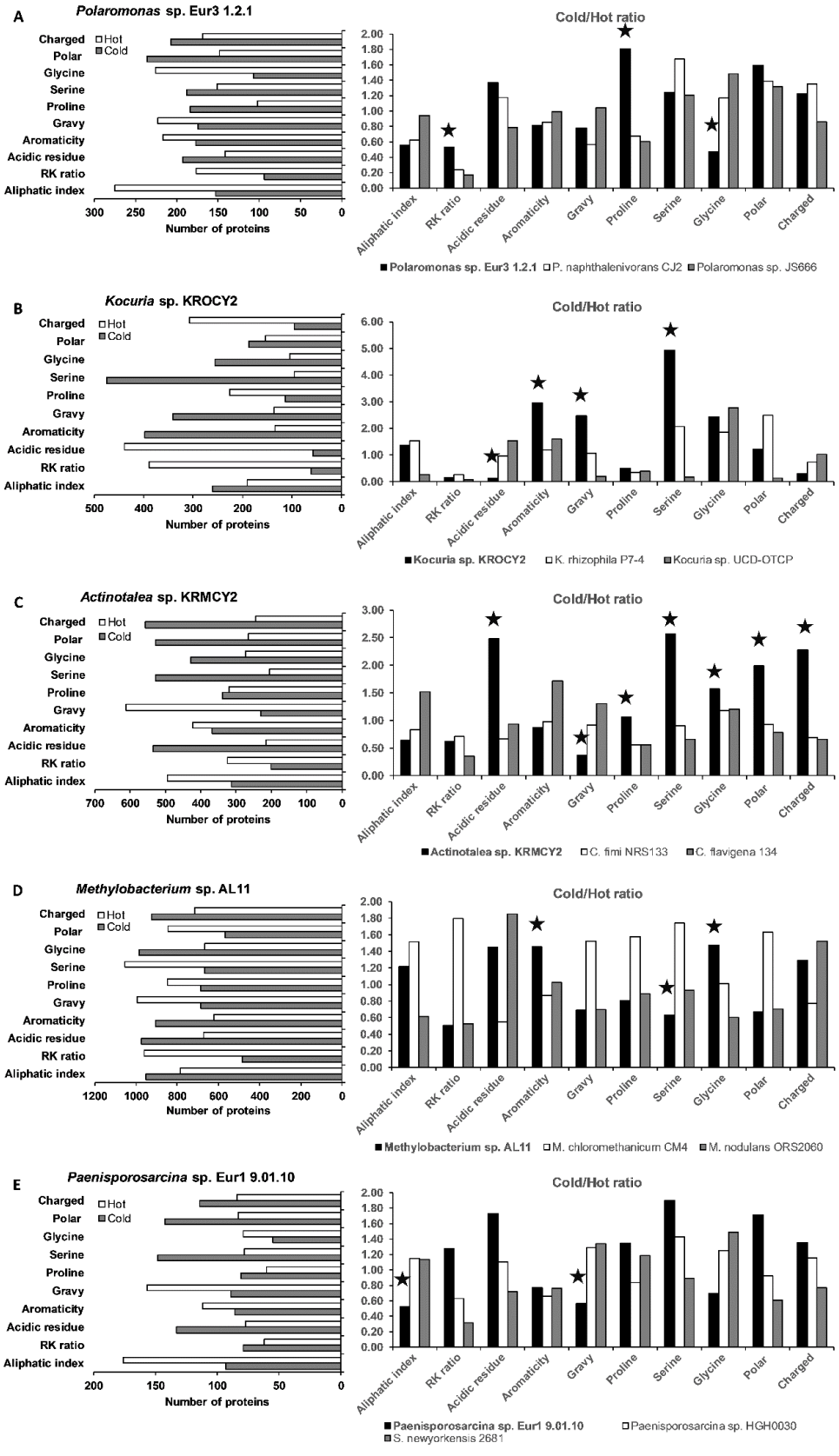

A
Polaromonas sp. Eur3 1.2.1
Hot
Cold
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
Number of proteins
Cold/Hot ratio
Aliphatic index
RK ratio
Acidic residue
Aromaticity
Gravy
Proline
Serine
Glycine
Polar
Charged
Polaromonas sp. Eur3 1.2.1
P. naphthalenivorans CJ2
Polaromonas sp. JS666
B
Kocuria sp. KROCY2
Kocuria sp. KROCY2
K. rhizophila P7-4
Kocuria sp. UCD-OTCP
C
Actinotalea sp. KRMCY2
Actinotalea sp. KRMCY2
C. fimi NRS133
C. flavigena 134
D
Methylobacterium sp. AL11
Methylobacterium sp. AL11
M. chloromethanicum CM4
M. nodulans ORS2060
E
Paenisporosarcina sp. Eur1 9.01.10
Paenisporosarcina sp. Eur1 9.01.10
Paenisporosarcina sp. HGH0030
S. newyorkensis 2681

Figure 3.3. Average ratio of cold/hot adapted proteins for all cryophiles and all mesophiles for each amino acid trait measured.

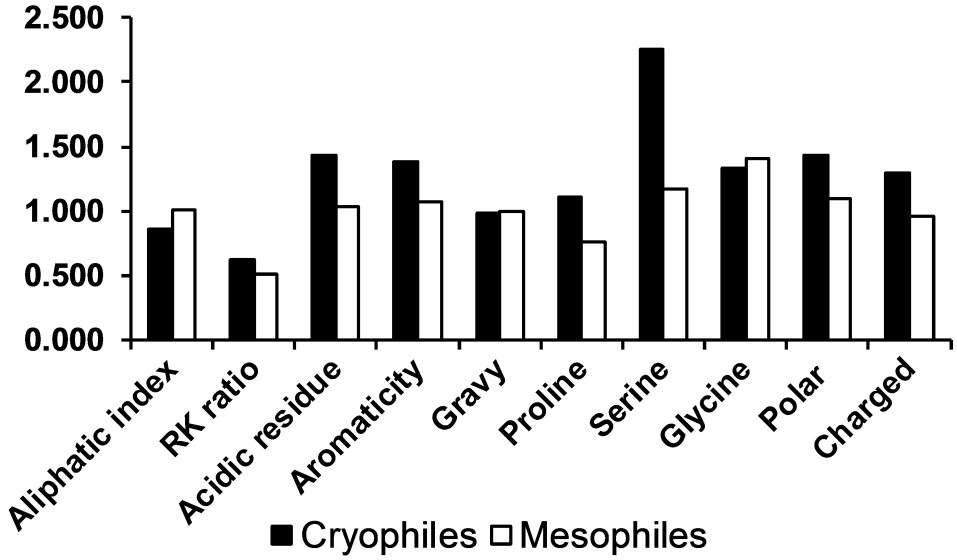


Table 3.1. Permafrost and ice wedge strains selected for sequencing and cold adaptation analysis.

Strain	Location isolated	Sample type	Temp °C	Salinity (%)	Phylum	Reference
Actinotalea sp. KRMCY2*	Moose Lake, Yukon	Ice Wedge	-5 – 25	0-8	Actinobacteria	This study & (Lacelle et al. 2011)
Polaromonas sp. Eur3 1.2.1	Eureka, Nunavut	Permafrost	-5 – 22	0-3	Proteobacteria	This study & (Steven et al. 2008)
Paenisporosarcina sp. Eur1 9.01.10*	Eureka, Nunavut	Permafrost	-5 – 25	0-8	Firmicute	This study & (Steven et al. 2007a)
Methylobacterium sp. AL-11	Eureka, Nunavut	Permafrost	-5 – 25	0-5	Proteobacteria	This study & (Steven et al. 2008)
Kocuria sp. KROCY2*	Old Crow, Yukon	Ice Wedge	-5 – 25	0-19	Actinobacteria	This study & (Lacelle et al. 2011)

* *Actinotalea* sp. KRMCY2, *Paenisporosarcina* sp. Eur1 9.01.10, and *Kocuria* sp. KROCY2 were originally classified as *Cellulomonas* sp. KRMCY2, *Sporosarcina* sp. Eur1 9.01.10, and *Microbacterium* sp. KROCY2, respectively, but were renamed following 16S phylogenic analysis (see text).

Table 3.2. Genome properties of sequenced permafrost strains

Organism	***Polaromonas* sp. Eur3 1.2.1**	***Actinotalea* sp. KRMCY2**	***Kocuria* sp. KROCY2**	***Methylobacterium* sp. EUR3 AL-11**	***Paenisporosarcina* sp. Eur1 9.01.10**
Genome name	Polaromonas sp. Eur3 1.2.1	Cellulomonas sp. KRMCY2	Microbacterium sp. KROCY2	Methylobacterium sp. EUR3 AL-11	Sporosarcina sp. Eur3 2.2.2
Genome size (Mbp)	4.4	4.5	2.8	7.2	3.5
%GC	60	72	71	71	38
Gene Count	4303	4207	2480	7001	3503
Protein coding genes	4238	4145	2415	6907	3438
16s rRNA	2	1	3	6	3
Protein coding genes with function prediction	3471	3205	1931	4913	2711
with enzymes	1234	1000	786	1393	905
with KEGG Pathways	1281	1121	817	1653	962
with KEGG Orthology	2235	1907	1305	2798	1821
with COG	2915	2687	1710	4206	2314
with Pfam	3582	3359	2014	5100	2850
Horizontally Transferred Count (percent %)	417 (9.69)	185 (4.4)	122 (1.74)	25 (1.01)	57 (1.63)
JGI Taxon ID	2619618817	2545824564	2540341240	2546826724	2528768230
NCBI taxon ID	1305734	1304865	1305732	1305730	1305836
Sequencing status	Permanent draft	Permanent draft	Permanent draft	Permanent draft	Permanent draft
Release date	30/06/2015	13/11/2013	24/09/2013	02/12/2013	08/08/2013
High quality	Yes	Yes	Yes	Yes	Yes
Bioproject accession	PRJNA195644	PRJNA195883	PRJNA195886	PRJNA195879	PRJNA195884

Table 3.3. Gene functions and COG categories present in psychrophilic genomes with known or predicted roles in cold adaptation and growth.

Category and Gene name	COG ID	*Polaromonas* Eur3 1.2.1	*Actinotalea* KRMCY2	*Kocuria* KROCY2	*Methylobacterium* EUR3 AL-11	*Paenisporosarcina* Eur1 9.01.10
Cold shock, stress and HSP proteins						
Cold shock protein, CspA family	COG1278	1	1	2	6	4
Universal stress protein, UspA family	COG0589	13	0	1	4	3
Co-chaperonin GroES (HSP10)	COG0234	1	2	1	1	1
Chaperonin GroEL (HSP60 family)	COG0459	2	1	2	1	1
Molecular chaperone DnaK (HSP70)	COG0443	3	0	1	2	1
Molecular chaperone IbpA, HSP20 family	COG0071	5	1	0	4	0
Molecular chaperone, HSP90 family	COG0326	1	1	0	1	0
Molecular chaperone GrpE (heat shock protein)	COG0576	1	3	1	1	1
Ribosomal 50S subunit-recycling heat shock protein	COG1188	1	2	1	1	1
DNA replication and repair						
DNA gyrase/topoisomerase IV, subunits A & B	COG0187; 0188	4	4	3	4	4
Recombinational DNA repair protein RecR	COG0353	1	0	1	1	1
RecA-superfamily ATPase, KaiC/GvpD/RAD55 family	COG0467	1	1	0	2	0
RecA/RadA recombinase	COG0468	1	2	1	1	1
DNA repair ATPase RecN	COG0497	1	6	1	1	1
Superfamily II DNA helicase RecQ	COG0514	1	0	2	2	2
Rad3-related DNA helicase	COG1199	3	4	1	0	2
Membrane and peptidoglycan alteration						
3-oxoacyl-(acyl-carrier-protein) synthase	COG0304	2	0	4	4	1
3-oxoacyl-[acyl-carrier-protein] synthase III (KASIII)	COG0332	1	1	3	2	1
3-oxoacyl-[acyl-carrier-protein] reductase	EC:1.1.1.100	12	3	3	6	5
Glycosyltransferase involved in cell wall biosynthesis	COG0438; 0463	17	10	19	39	7

3-hydroxyacyl-CoA dehydrogenase	COG1250	8	1	1	5	4
Fatty-acid desaturase	COG1398; 3239	1	1	1	0	1
D-alanyl-D-alanine carboxypeptidase	COG1686; 2027	2	1	1	4	3
Carotenoid biosynthesis						
Phytoene dehydrogenase-related protein	COG1233	0	2	3	4	1
Phytoene/squalene synthetase	COG1562	2	1	1	4	1
Polysaccharide capsule						
Capsular polysaccharide biosyn. protein EpsC	COG1086	1	0	1	1	1
Capsule polysaccharide export protein	COG3524	0	0	0	3	0
Capsular polysaccharide biosynthesis protein	COG3944; 4421	0	0	3	1	1
Exopolysaccharide biosynthesis protein	COG4632	0	1	0	0	0
Osmotic stress						
ABC proline/glycine betaine transport, ATPase component	COG1125; 4175	1	1	2	2	1
ABC proline/glycine betaine transport, permease	COG1174; 4176	2	0	4	3	1
Choline-glycine betaine transporter	COG1292	0	0	9	0	1
Osmoprotectant binding protein	COG1732	1	0	3	1	1
Choline dehydrogenase or related flavoprotein	COG2303	1	1	1	7	1
Trehalose-6-phosphate synthase	COG0380	2	10	1	1	0
Trehalose-6-phosphatase	COG1877	1	1	1	1	0
Maltooligosyltrehalose synthase	COG3280	0	1	1	1	0
Na+/proline symporter	COG0591	1	1	1	2	3
Na+/H+ antiporters	COGs: 3004; 1055; 0025; 0651; 1009; 1320	12	5	7	11	5
Oxidative stress						
Catalase	COG0753	2	0	2	1	3
Peroxiredoxin	COG0678; 1225	3	3	2	4	3
Glutathione peroxidase	COG0386	2	1	1	2	1
Spermidine synthase	COG0421	1	1	0	0	1
Thioredoxin reductase	COG0492	2	1	2	4	5
Glyoxylase or related hydrolase, β-lactamase	COG0491	6	4	4	9	7

superfam II						
Toxin/Antitoxin modules						
Ser/Thr kinase RdoA, MazF antagonist	COG2334	2	1	0	2	1
Antitoxin component of MazEF	COG2336	1	1	0	0	0
mRNA-interferase, toxin component of MazEF	COG2337	2	1	0	1	1
mRNA interferase YafQ, toxin component of YafQ-DinJ	COG3041	2	1	0	2	0
Antitoxin component of RelBE or YafQ-DinJ	COG3077	1	0	0	2	0
Translation and transcription factors						
tRNA-dihydrouridine synthase	COG0042	3	1	1	2	2
tRNA A37 threonylcarbamoyladenosine dehydratase	COG1179	1	0	0	0	1
Translation elongation factor EF-Tu, GTPase	COG0050	2	1	1	2	1
Translation elongation factor EF-G, GTPase	COG0480	3	1	1	1	2
Translation initiation factor IF-2, GTPase	COG0532	1	1	1	1	1
Translation initiation factor IF-3	COG0290	1	1	1	1	1
Transcription antitermination factor NusA	COG0195	1	1	1	1	1
Transcription termination factor NusB	COG0781	1	2	1	1	1
Superfamily II DNA and RNA helicase	COG0513; 1061	7	4	5	6	3
Superfamily II DNA or RNA helicase, SNF2	COG0553	0	0	1	0	2
Superfamily II RNA helicase	COG4581	0	2	1	0	0

3.7 Supplementary Materials

Supplementary Figures 1-3

Supplementary Tables 1-2

Figure S3.1. Significantly ($P < 0.05$) hot and cold adapted proteins in each mesophile for each amino acid trait measured: aliphatic index, R/K ratio, acidic residues, aromaticity, GRAVY, and proline, serine, glycine, polar, and charged residues.

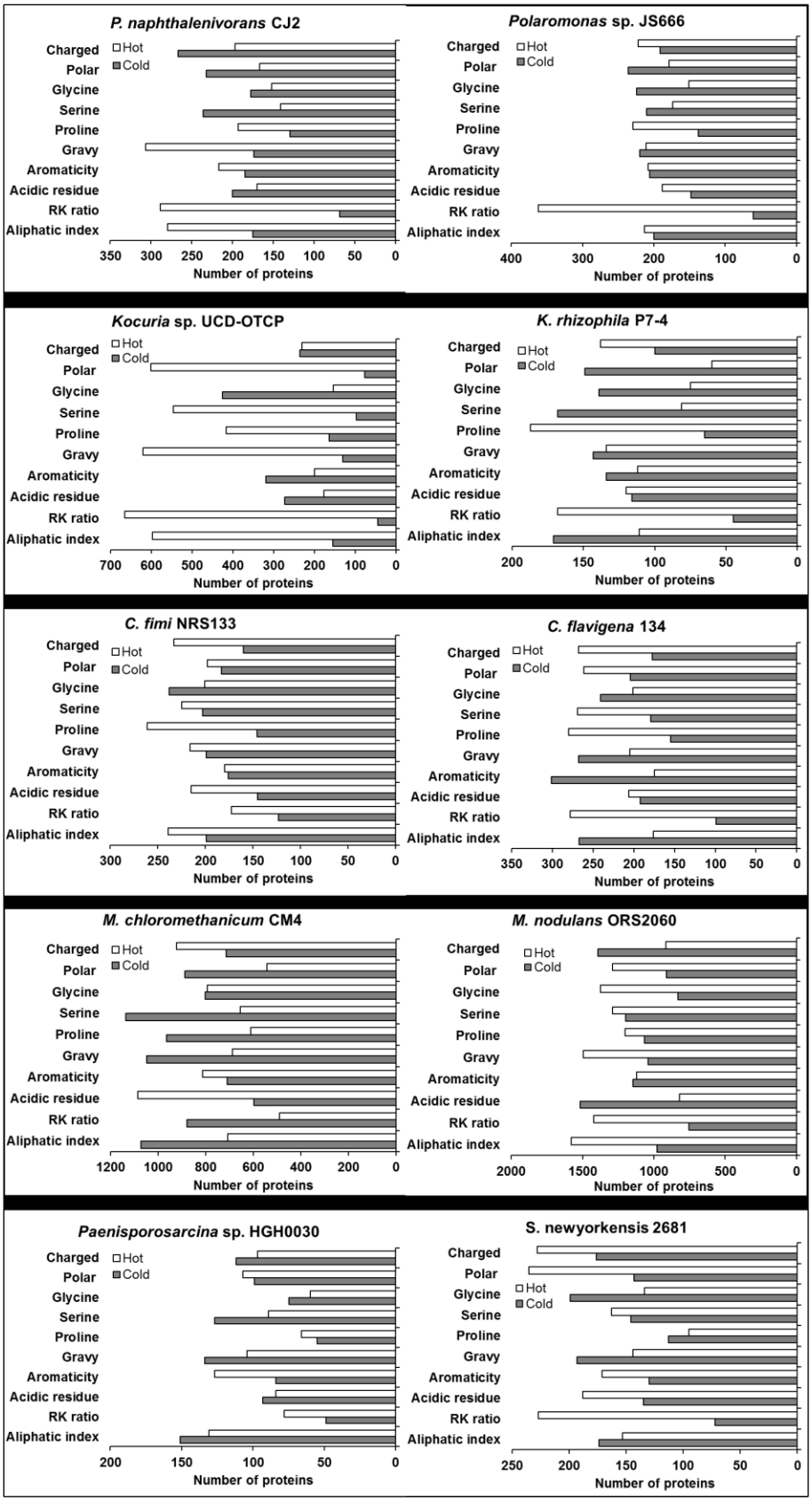

P. naphthalenivorans CJ2
Hot
Cold
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
350 300 250 200 150 100 50 0
Number of proteins
Polaromonas sp. JS666
400 300 200 100 0
Number of proteins
Kocuria sp. UCD-OTCP
700 600 500 400 300 200 100 0
Number of proteins
K. rhizophila P7-4
200 150 100 50 0
Number of proteins
C. fimi NRS133
300 250 200 150 100 50 0
Number of proteins
C. flavigena 134
350 300 250 200 150 100 50 0
Number of proteins
M. chloromethanicum CM4
1200 1000 800 600 400 200 0
Number of proteins
M. nodulans ORS2060
2000 1500 1000 500 0
Number of proteins
Paenisporosarcina sp. HGH0030
200 150 100 50 0
Number of proteins
S. newyorkensis 2681
250 200 150 100 50 0
Number of proteins

Figure S3.2. Neutral proteins not found to be significantly cold or hot adapted in each cryophile (A-E; bolded, black bar) and its mesophilic relatives for each amino acid trait measured.

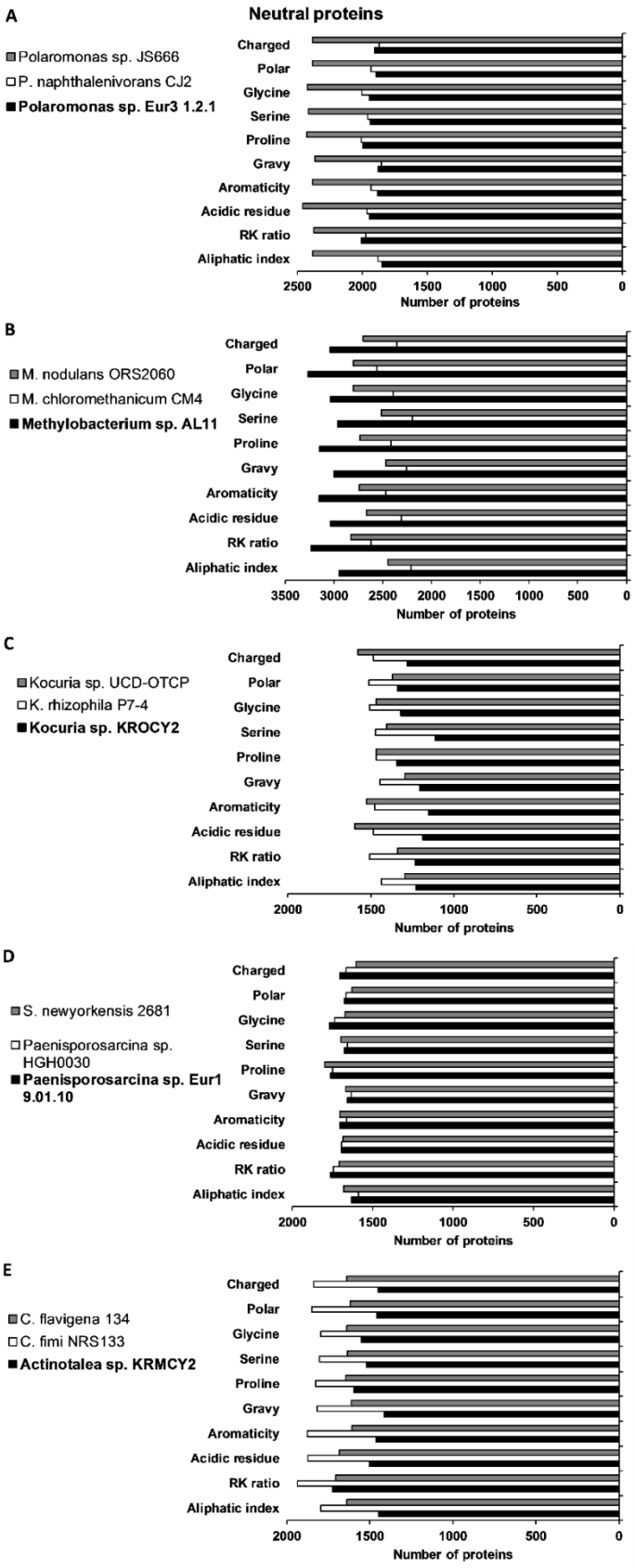
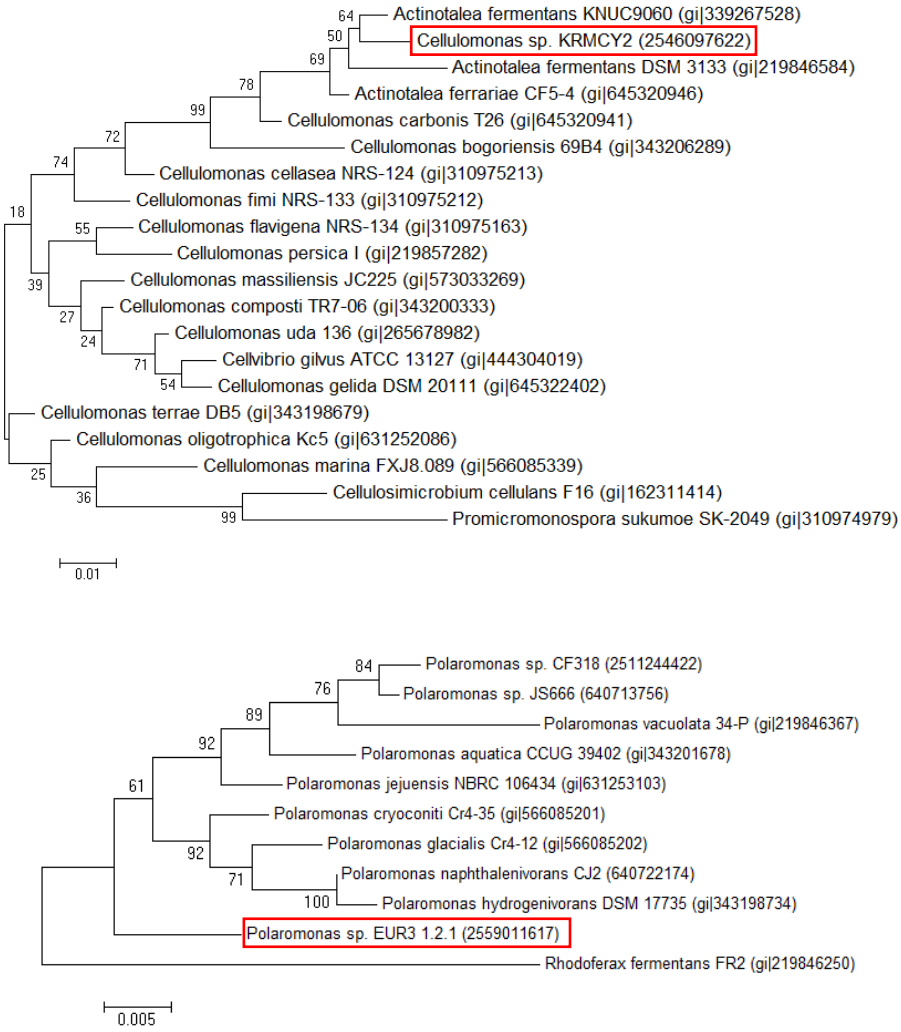
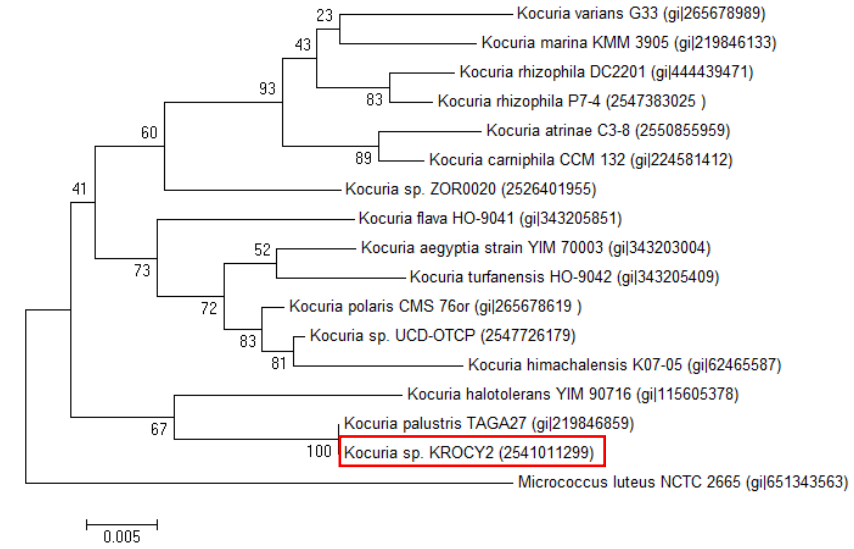
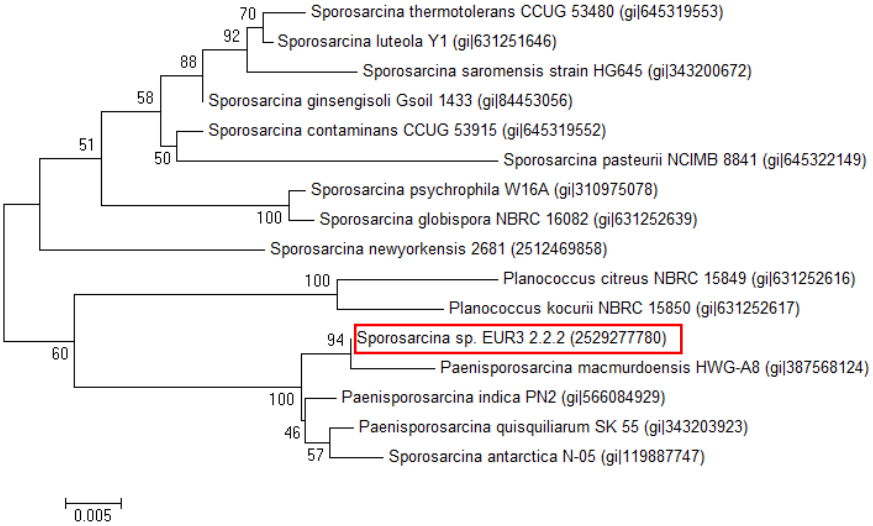

A
Neutral proteins
Polaromonas sp. JS666
P. naphthalenivorans CJ2
Polaromonas sp. Eur3 1.2.1
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
2500 2000 1500 1000 500 0
Number of proteins
B
M. nodulans ORS2060
M. chloromethanicum CM4
Methylobacterium sp. AL11
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
3500 3000 2500 2000 1500 1000 500 0
Number of proteins
C
Kocuria sp. UCD-OTCP
K. rhizophila P7-4
Kocuria sp. KROCY2
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
2000 1500 1000 500 0
Number of proteins
D
S. newyorkensis 2681
Paenisporosarcina sp. HGH0030
Paenisporosarcina sp. Eur1 9.01.10
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
2000 1500 1000 500 0
Number of proteins
E
C. flavigena 134
C. fimi NRS133
Actinotalea sp. KRMCY2
Charged
Polar
Glycine
Serine
Proline
Gravy
Aromaticity
Acidic residue
RK ratio
Aliphatic index
2000 1500 1000 500 0
Number of proteins

Figure S3.3. Phylogenic tree highlighting the position of each cryophile (in red) relative to selected taxonomically classified strains within their respective genera. The tree was constructed using the maximum likelihood model within MEGA6. Numbers at the nodes are bootstrapping percentages obtained by repeating the analysis 1,000.




Kocuria varians G33 (gi|265678989)
Kocuria marina KMM 3905 (gi|219846133)
Kocuria rhizophila DC2201 (gi|444439471)
Kocuria rhizophila P7-4 (2547383025)
Kocuria atrinae C3-8 (2550855959)
Kocuria carniphila CCM 132 (gi|224581412)
Kocuria sp. ZOR0020 (2526401955)
Kocuria flava HO-9041 (gi|343205851)
Kocuria aegyptia strain YIM 70003 (gi|343203004)
Kocuria turfanensis HO-9042 (gi|343205409)
Kocuria polaris CMS 76or (gi|265678619)
Kocuria sp. UCD-OTCP (2547726179)
Kocuria himachalensis K07-05 (gi|62465587)
Kocuria halotolerans YIM 90716 (gi|115605378)
Kocuria palustris TAGA27 (gi|219846859)
Kocuria sp. KROCY2 (2541011299)
Micrococcus luteus NCTC 2665 (gi|651343563)
23
43
93
83
60
89
41
52
73
72
83
81
67
100
0.005


Sporosarcina thermotolerans CCUG 53480 (gi|645319553)
Sporosarcina luteola Y1 (gi|631251646)
Sporosarcina saromensis strain HG645 (gi|343200672)
Sporosarcina ginsengisoli Gsoil 1433 (gi|84453056)
Sporosarcina contaminans CCUG 53915 (gi|645319552)
Sporosarcina pasteurii NCIMB 8841 (gi|645322149)
Sporosarcina psychrophila W16A (gi|310975078)
Sporosarcina globispora NBRC 16082 (gi|631252639)
Sporosarcina newyorkensis 2681 (2512469858)
Planococcus citreus NBRC 15849 (gi|631252616)
Planococcus kocurii NBRC 15850 (gi|631252617)
Sporosarcina sp. EUR3 2.2.2 (2529277780)
Paenisporosarcina macmurdoensis HWG-A8 (gi|387568124)
Paenisporosarcina indica PN2 (gi|566084929)
Paenisporosarcina quisquiliarum SK 55 (gi|343203923)
Sporosarcina antarctica N-05 (gi|119887747)
70
92
88
58
51
50
100
100
60
94
100
46
57
0.005

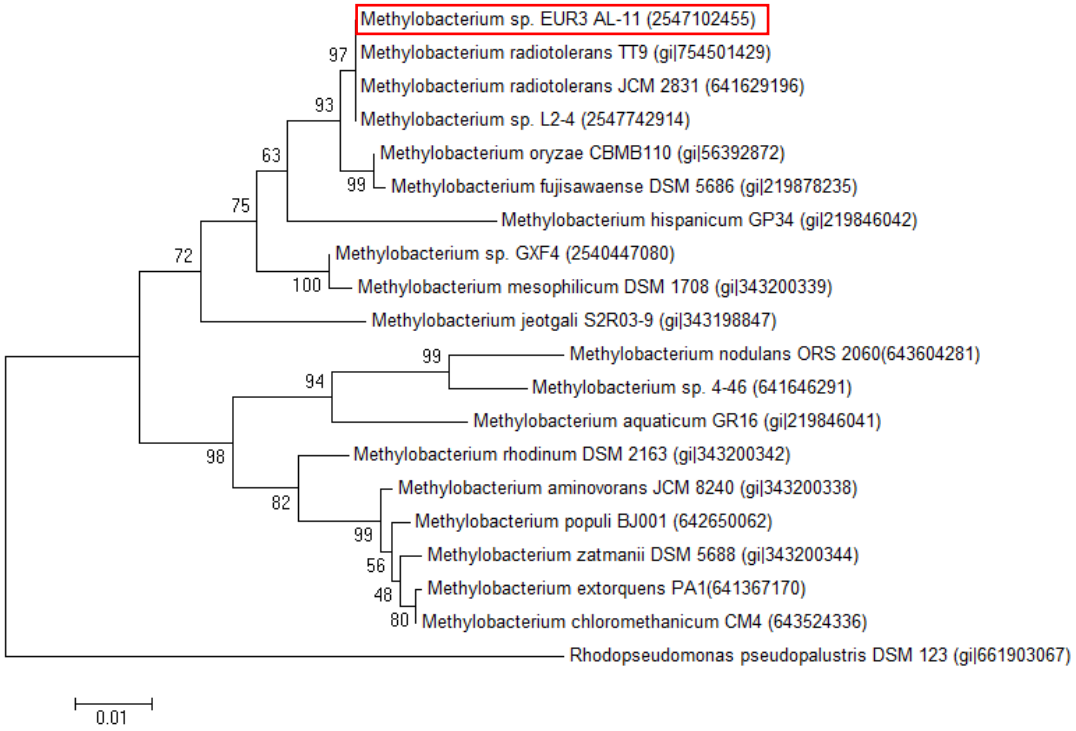

Methylobacterium sp. EUR3 AL-11 (2547102455)
97
Methylobacterium radiotolerans TT9 (gi|754501429)
Methylobacterium radiotolerans JCM 2831 (641629196)
93
Methylobacterium sp. L2-4 (2547742914)
63
Methylobacterium oryzae CBMB110 (gi|56392872)
99
Methylobacterium fujisawaense DSM 5686 (gi|219878235)
75
Methylobacterium hispanicum GP34 (gi|219846042)
72
Methylobacterium sp. GXF4 (2540447080)
100
Methylobacterium mesophilicum DSM 1708 (gi|343200339)
Methylobacterium jeotgali S2R03-9 (gi|343198847)
99
Methylobacterium nodulans ORS 2060(643604281)
94
Methylobacterium sp. 4-46 (641646291)
Methylobacterium aquaticum GR16 (gi|219846041)
98
Methylobacterium rhodinum DSM 2163 (gi|343200342)
82
Methylobacterium aminovorans JCM 8240 (gi|343200338)
99
Methylobacterium populi BJ001 (642650062)
56
Methylobacterium zatmanii DSM 5688 (gi|343200344)
48
Methylobacterium extorquens PA1(641367170)
80
Methylobacterium chloromethanicum CM4 (643524336)
Rhodopseudomonas pseudopalustris DSM 123 (gi|661903067)
0.01

Table S3.1. Genome properties of publicly available mesophilic strains used in genome and amino acid comparative analyses. Data was obtained from the JGI website.

Organism	Isolation location/source	Genome size (Mbp)	16s rRNA	%GC	Gene Count	Protein coding genes	Genes with COG	JGI Taxon ID	NCBI taxon ID
Polaromonas naphthalenivorans CJ2	Freshwater sediment, New York	5.4	2	62	5000	4929	3215	639633051	365044
Polaromonas sp. JS666	Freshwater sediment	5.9	1	62	5634	5569	3844	637000208	296591
Polaromonas sp. CF318	Soil Rhizosphere, Tennessee	5.0	1	65	4889	4828	3469	2511231002	52972
Methylobacterium chloromethanicum CM4	Soil at a petrochemical factory in Tatarstan Russia	6.2	5	68	5847	5771	3397	643348563	440085
Methylobacterium extorquens PA1	Arabidopsis	5.5	5	68	4939	4864	3116	641228497	419610
Methylobacterium marinus A45	Marine ecosystem	5.0	3	53	4610	4523	2786	2517287034	674036
Methylobacterium nodulans ORS 2060	Root nodules from the legume Crotalaria	8.8	7	68	8885	8791	4755	643348564	460265
Methylobacterium populi BJ001	Poplar plantlets	5.8	5	69	5538	5464	3333	642555139	441620
Methylobacterium radiotolerans JCM 2831	Unpolished rice	6.9	6	71	6510	6431	3952	641522638	426355
Methylobacterium sp. 4-46	Root nodule from Lotononis bainesii	7.7	6	72	7125	7043	4260	641522639	426117
Methylobacterium sp. GXF4	Grape xylem sap, New York, USA	6.1	1	70	5976	5927	3659	2540341035	1096546
Methylobacterium sp. L2-4	Jatropha surface sterilized leaves	6.8	1	71	6696	6618	3924	2547132193	1166158

Methylobacterium sp. WSM2598	Listia bainesii root nodule collected at Estcourt Research Station in South Africa	8.1	6	71	7667	7551	4463	2545555834	398261
Kocuria rhizophila DC2201	Waterfall, Sungai Gabai, Selangor, Malaysia	2.7	3	71	2413	2357	1647	642555133	378753
Kocuria rhizophila P7-4	Intestine of Siganus doliatus	2.8	1	71	2521	2463	1711	2547132105	1029824
Kocuria sp. ZOR0020	Zebrafish gastrointestinal tract	3.0	1	64	2975	2913	1728	2526164567	1339234
Kocuria atrinae C3-8	Isolated from Korean traditional fermented food	3.2	1	64	4126	4069	1360	2548877030	1179225
Kocuria sp. UCD-OTCP	Restaurant chair cushion, Davis, CA	3.8	1	73	3511	3453	2376	2547132190	1292021
Paenisporosarcina sp. HGH0030	Human gastrointestinal tract	3.5	1	39	3519	3449	2204	2541046999	1078085
Sporosarcina newyorkensis 2681	Human blood sample	3.6	1	42	3898	3825	2441	2512047070	1027292
Sporosarcina ureae DSM2281	Lab enrichment	3.3	8	41	3376	3287	2286	2523533529	1123290
Cellulomonas fimi NRS133	Soil	4.3	2	75	3875	3818	2498	2505679089	590998
Cellulomonas flavigena 134	Soil	4.1	2	74	3783	3730	2257	646564520	446466
Cellulomonas massiliensis JC225	Stool of healthy Senegalese patient	3.2	1	75	3262	3202	2003	2547132154	23739
Cellulomonas gilvus ATCC 13127	Bovine feces	3.5	2	74	3262	3209	2079	2505679016	593907

Table S3.2. Generation times (g; days) and growth rates (k) for Paenisporosarcina sp. Eur1 9.01.10 and Polaromonas sp. Eur3 1.2.1 grown on TSB supplemented with 5% salt and 5% sucrose, and R2A, respectively.

	-5 °C		0 °C		5 °C		10 °C		22 °C		25 °C	
Organisms	g (d)	k (d^{-1})	g (d)	k (d^{-1})	g (d)	k (d^{-1})	g (d)	k (d^{-1})	g (d)	k (d^{-1})	g (d)	k (d^{-1})
Paenisporosarcina sp. Eur1 9.01.10	14.6	0.057	-	-	4.5	0.155	-	-	-	-	1.73 (0.46[1])	0.4 (1.48[1])
Polaromonas sp. Eur3 1.2.1	on agar[2]		3.6	0.19	-	-	3.1	0.23	3.4	0.21	no growth	

[1]When grown on TSB with no added salt and sucrose.
[2]This study and Steven et al. 2008

Connecting Text:

In order to expand on the genomic analyses and look at actively transcribed genes important for cold growth, as well as compare and contrast the cold growth properties of psychrophiles with different growth profiles, we selected two permafrost isolates, a stenopsychrophile and a eurypsychrophile, for transcriptomic analysis at their coldest temperatures (-5 and 0 °C) and at warmer (20 and 25 °C) temperatures.

Contributions of authors: The authors who contributed to this work are Isabelle Raymond-Bouchard[1], Julien Tremblay[2], Ianina Altshuler[1], Charles Greer[2], Lyle G. Whyte[1]. I.R.-B. wrote the manuscript, performed all laboratory experiments, and all downstream analyses with the results of pipeline data. J.T. performed bioinformatic analysis of the raw RNAseq data using the transcriptomic pipeline, provided experimental guidance, and criticial feedback and editing. I.A. helped with creation of the cell figure and assisted with library preparation. C.G. and L.G.W. provided guidance, and critical feedback and editing of the manuscript.

[1]McGill University, Macdonald Campus, 21,111 Lakeshore Rd, Ste.-Anne-de-Bellevue, QC, Canada, H9X 3V9
[2]National Research Council Canada, Montreal, QC, Canada

This manuscript is in preparation to be submitted for publication.

Chapter 4. Comparative transcriptomics of cold growth and adaptive features of a eury- and steno-psychrophile

4.1 Abstract

We performed transcriptomic analyses on two cryophilic permafrost isolates with different growth profiles in order to characterize and compare their cold temperature growth and cold-adaptive strategies. The two organisms, *Rhodococcus* sp. JG3 (-5 to 30°C) and *Polaromonas* sp. Eur3 1.2.1 (-5 to 22°C), shared several common responses during low temperature growth, including induction of translation and ribosomal processes, upregulation of nutrient transport, increased oxidative and osmotic stress responses, and stimulation of polysaccharide capsule synthesis. Recombination appeared to be an important adaptive strategy for both isolates at low temperatures, likely as a mechanism to increase genetic diversity and the potential for survival in cold systems. While *Rhodococcus* sp. favored upregulating iron and amino acid transport, sustaining redox potential, and modulating fatty acid synthesis and composition, *Polaromonas* sp. increased transcription involved in primary energy metabolism and the electron transport chain, in addition to signal transduction and peptidoglycan synthesis. The increase in energy metabolism may explain why *Polaromonas* sp. is able to sustain growth rates at 0°C comparable to higher temperature growth. For *Rhodococcus* sp., flexibility in use of carbon sources, iron acquisition, control of membrane fatty acid composition, and modulating redox and co-factor potential may be ways in which this organism is able to sustain growth over a wider range of temperatures.

4.2 Introduction

The discovery of active, growing communities in polar habitats has given rise to much interest in studying the microbes that inhabit these regions and obtaining a greater understanding of the mechanisms and adaptive features that allow them to grow in cryoenvironments, generally defined as environments that exist continuously and predominantly at subzero temperatures. Microorganisms living in cryoenvironments must adapt to overcome numerous physiological and kinetic challenges, including decreased membrane fluidity, reduced diffusion and reaction rates, lower enzyme activity, stable secondary inhibitory DNA/RNA structures, and protein misfolding (Bakermans et al. 2012; De Maayer et al. 2014). Subzero environments, including permafrost and ice, are characterized by limited water availability, and liquid water in these habitats is believed to exist primarily in salty brine veins where increased solute and salt concentrations prevent freezing (Gilichinsky et al. 2005). As such, mechanisms must also exist to overcome osmotic stress and high salt concentrations.

Organisms isolated from these environments and capable of growth at low (≤0 °C) temperatures have generally been classified as eurypsychrophiles (formerly psychrotrophs/ psychrotolerant), as those organisms possess a broader growth range (Tmax >20 °C), and while capable of growth at very low temperatures (-15°C), they usually retain optimum growth rates at temperatures above 20°C. In comparison, stenopsychrophiles (formerly 'true psychrophiles'), exhibit narrower growth ranges (Tmax ~20°C), and usually grow optimally at temperatures ranging between 5-15°C. We will use the term cold-adapted bacteria here to refer to both steno- and eury-psychrophiles. While it is true that eurypsychrophiles generally grow optimally at >20°C, they often exhibit growth over a wide range of temperatures and their low temperature minimum is often lower than stenopsychrophiles; indeed, almost all of the organisms reported to date capable of growth at -5°C and below are eurypsychrophiles (Bakermans et al. 2007; Rodrigues et al. 2008; Bergholz et al. 2009; Mykytczuk et al. 2013; Koh et al. 2017). This is an interesting observation and raises questions about the cold adaptive properties and limitations of both types of cold-adapted bacteria.

The vast majority of studies into cold adaptation and growth, especially proteomes and transcriptomes, have focused heavily on eurypsychrophiles, and most of these, on above zero growth conditions (reviewed extensively in: Kawamoto et al. 2017 and Raymond-Bouchard and Whyte 2017). Comparatively little is known about stenopsychrophiles, especially in bacteria, whose members are more limited, and include *Colwellia psychrerythraea* and *Desulfotalea psychrophila* (Rabus et al. 2004; Methé et al. 2005). One characteristic of stenopsychrophiles that may allow for faster growth at lower temperatures is their ability to increase energy and carbon metabolism at these temperatures, when compared to higher suboptimal temperatures (Mock and Hoch 2005; Mock et al. 2005; Hwang et al. 2008; Chong et al. 2011). These mechanisms are often downregulated in eurypsychrophiles at lower temperatures (Bergholz et al. 2009; Mykytczuk et al. 2013; Tribelli et al. 2015).

While both types of organisms have been studied for their cold-adaptive properties, almost no studies have directly compared the two types of psychrophiles to elucidate their cold-adapted features and compare and contrast the different strategies that characterize their varied growth capabilities and optimums. We aim to increase our knowledge of cold and subzero growth in permafrost isolated eury- and steno-psychrophiles and gain a better understanding of their cold adaptive strategies for growth at these temperatures. *Polaromonas* sp. Eur3 1.2.1, isolated from permafrost in Eureka (Steven et al. 2008), Ellesmere Island, Nunavut, is a stenopsychrophile capable of growth from -5°C up to 22°C and exhibits a faster generation time (g) at 10°C (3.1 d) than 22°C (3.4 d), and only slightly lower at 0°C (3.6 d) (I. Raymond-Bouchard, in Review FEMS Microbiology Ecology). *Polaromonas* sp. Eur3 1.2.1 is a slow-growing, fastidious organism and while growth down to -5°C has been detected on ½ R2A agar supplemented with 7% sucrose, so far no liquid media adequate for subzero growth has been found. The eurypsychrophile *Rhodococcus* sp. JG3, isolated from permafrost (Goordial et al. 2016b) in the McMurdo Dry Valley, Antarctica, is capable of growth from at least -5°C to 30°C, and grows optimally at 20-25°C. In this study, our goal was to perform transcriptomic analyses of both organisms at their lower temperature limits of growth, 0°C and -5°C, in addition to higher ≥20°C temperatures, in order to characterize and compare their low temperature growth and cold-adaptive strategies.

4.3 Results and Discussion

Using RNAseq we carried out transcriptomic analyses on (triplicate) cultures of *Rhodococcus* sp. grown at -5°C and 25°C and *Polaromonas* sp. grown at 0°C and 20°C. Overall, 515 and 359 transcripts were found to be differentially expressed for *Rhodococcus* sp. and *Polaromonas* sp., respectively, between the higher and lower temperatures (Table 4.1 and Figure S4.1). Of these about 66% and 45% could be assigned to COG and KEGG categories for both organisms. The largest shifts in abundance of transcripts observed for *Rhodococcus* sp. between -5°C and 25°C, according to COG assignments, were in translation and ribosomal proteins, amino acid and inorganic ion transport and metabolism, lipid transport and metabolism, and energy production and conversion (Figure 4.1). Translation and amino acid and ion transport and metabolism processes saw significant upshifts in transcripts at -5°C, while transcripts involved in energy production and conversion, and lipid transport and metabolism were downregulated. Like *Rhodococcus* sp., *Polaromonas* sp. also showed upregulation of transcripts involved in translation and ribosomal proteins at low temperature (0°C), compared to 20°C (Figure 4.1). However, unlike *Rhodococcus* sp., *Polaromonas* sp. upregulated genes involved in energy production at low temperature and downregulated gene expression of amino acid transport and metabolism. In addition, noticeable increases in abundance of transcripts involved in signal transduction and carbohydrate transport and metabolism were also detected, along with a decrease in coenzyme transport and metabolism. Interestingly, at both 0°C and 20°C *Polaromonas* sp. upregulated a unique subset of transposon elements (discussed further below).

Overall, the response in *Polaromonas* sp. was more attenuated than in *Rhodococcus* sp. Fewer genes were found to be differentially regulated, with less than 40 genes upregulated >3-fold change (logFC) at 0°C, and no transcripts with logFC > 5.8, as compared to *Rhodococcus* sp. where ~150 genes were upregulated >3-fold and up to 8.7 logFC (Table 4.1 and Figure S4.1). It is possible, given the reduced response and the fairly consistent generation times (3.6 days at 0°C, 3.1 at 10°C, and 3.4 at 22°C) observed for *Polaromonas* sp. over its temperature range that

it remains somewhat constitutively adapted to the lower temperatures and does not need to shift processes to the extent that *Rhodococcus* sp. does at lower temperatures.

4.3.1 Translation and ribosomal proteins

The importance of sustaining appropriate and necessary translation and ribosomal processes at low temperatures is apparent in the responses of both *Rhodococcus* sp. and *Polaromonas* sp. At -5°C, *Rhodococcus* sp. shows upregulation of at least 24 genes with logFC > 1.5 involved in translation and ribosomal processes, including numerous ribosomal proteins, a GTPase Era, the methylase RsmH, and the translation elongation factor EF-Ts (Table 4.2). A queuine tRNA-ribosyltransferase and an O-acetyl-ADP-ribose deacetylase, which downregulates RNase III activity, as well as the ribosome binding factor A (*rbfA*) and a superfamily II DNA and RNA helicase (includes DEAD/DEAH-box family proteins) were upregulated > 3 logFC. Helicases of this family are a common feature of cold growth in numerous cold-adapted bacteria, owing to their importance in stabilizing translation processes (Piette et al. 2011b). RbfA, involved in ribosome maturation and translation initiation, binds to the 30S subunit and is important for cold tolerance in *Escherichia coli*, where overexpression of this protein at cold temperatures (15°C) suppresses a cold-sensitive mutation and increases protein synthesis and growth (Dammel and Noller 1995; Jones and Inouye 1996). Upregulation of RbfA has also been reported for the psychrophiles *Exiguobacterium sibiricum* at -2.5°C (Rodrigues et al. 2008) and *Psychrobacter arcticus* 273-4 at -6°C (Bergholz et al. 2009). A 5-fold upregulation of ADP-ribosylglycohydrolase, responsible for removal of ADP-ribose from ADP-ribosylated proteins was also observed in *Rhodococcus* sp. and may be important in the regulation of many cellular processes including DNA/protein repair, DNA recombination, signal transduction, and gene transcription (Liu and Yu 2015). Conversely, very few decreases were observed in this category (Table 4.2 and S4.2) and included mostly translation inhibitors, such as the gene for an mRNA degradation ribonuclease and the ribosome associated translation inhibitor RaiA, indicating the importance of keeping translation processes active at low temperatures.

Similarly, *Polaromonas* sp. increased expression of a number of ribosomal proteins, a superfamily II helicase, and the translation initiation factor IF-1, with concurrently small fold decreases in proteins in this category (Table 4.3 and S4.3). Two of the most upregulated genes in *Polaromonas* sp. were molecular chaperones, a disulfide isomerase subunit, *dsbG*, of the thiol:disulfide interchange protein, and a second thiol-disulfide isomerase. Another thiol:disulfide interchange subunit, *dsbD*, was also increased. The thiol:disulfide isomerases are required for proper folding of proteins through isomerization of incorrect disulfide bonds to the correct pattern (Shao et al. 2000; Gleiter and Bardwell 2008). They prevent misfolding and aggregation of proteins, which is believed to be more prevalent at lower temperatures (Doyle et al. 2012). *Polaromonas* sp. also downshifts expression of the Ribonuclease E in favor of its homologue Ribonuclease G, an enzyme with more limited enzymatic capabilities than RNase E (Deana and Belasco 2004). The importance of sustaining translation at low temperatures has been highlighted in studies of both eury- and steno-psychrophiles (Goodchild et al. 2004; Mock et al. 2005; Nunn et al. 2015; Koh et al. 2017) and has been postulated to be the rate-limiting step for protein synthesis in certain cold communities (Toseland et al. 2013). Shifts in ribosomal and translation proteins ultimately allow the ribosome to become 'cold-adapted' and preserve translation at cold temperatures, thus maintaining growth and metabolic activity.

4.3.2 Transposons, DNA recombination, and genomic redundancy

Polaromonas sp. appears to express a number of transposase elements, and at 0°C, shifts expression in favor of IS5 family transposases (Table 4.3). IS5 can mediate directed mutations and causes gene activation or inactivation through insertion upstream of targeted genes near the promoter (Zhang and Saier 2012). The competent protein, ComEA, important for free DNA uptake and horizontal gene transfer, was also increased slightly. While *Rhodococcus* sp. does not appear to differentially express transposon elements, several genes with roles in DNA recombination, including a single strand DNA binding protein (ssb), the Holliday junction helicase (RuvA), and the alpha subunit (RecD) of exonuclease V were all increased ~ 2-fold at -5°C (Table 4.2). Two transposons and several recombination proteins were expressed in the psychrophilic archaeon *Methanococcoides burtonii* at 4°C (Goodchild et al. 2004) and several

psychrophilic genomes, including the genome of subzero-growing *P. arcticus* 273-4, have been shown to possess large numbers of elements that contribute to genome plasticity such as plasmids, transposons and other mobile elements (Allen et al. 2009; Ayala-del-Río et al. 2010; Math et al. 2012). Recombination, both from transposition and site-specific recombination, may serve as a general adaptive strategy to increase genetic diversity and the potential for survival and growth in cold temperature systems.

Indeed, genomic redundancy and isozyme exchange has been shown to be a cold adaptive strategy in certain cold-adapted bacteria, including *E. sibiricum*, *P. arcticus*, *Planococcus halocryophilus*, and *Psychrobacter* sp. PAMC 21119, with different copies of genes being expressed at different temperatures (Rodrigues et al. 2008; Bergholz et al. 2009; Mykytczuk et al. 2013; Koh et al. 2017). Both *Rhodococcus* sp. and *Polaromonas* sp. show evidence of genomic redundancy with high copy numbers of certain genes known to have roles during cold temperature growth (Goordial et al. 2016; I. Raymond-Bouchard, manuscript in Review FEMS Microbiology Ecology). In this study, differential expression was observed for several gene copies including catalase, the iron uptake regulator fur, sigma-70 polymerase, universal stress protein, TRAP dicarboxylate permease, and ABC-type polar amino acid transporter (Table 4.4). This may confer an advantage during cold growth whereby cold adapted versions of a given gene product would be expressed and likely function better under that condition.

4.3.3 Iron acquisition

Fourteen genes with roles in iron acquisition and transport were found to be upregulated greater than 1.5-fold at -5°C in *Rhodococcus* sp., with 1/2 of these increased >5-fold (Table 4.2). This response may be one way to counteract the reduced solubility of iron at cold temperatures (Lide 2005). The most highly upregulated were those involved in the biosynthesis and transport of siderophores, including mycobactin (*mtbA, mtbB, mtbI*) and hydroxamate siderophores (*pvdA*), as well as a ferric siderophore reductase likely involved in reduction mediated release of the iron from the siderophore. Siderophores are low molecular weight molecules with very high affinity for ferric iron (Fe^{3+}). The gene for the transcriptional repressor Fur, which binds ferrous

iron (Fe^{2+}) and negatively regulates iron uptake genes, was downregulated over 4-fold (Table 4.2). Fe^{3+} is a crucial co-factor in numerous cellular processes and the concurrently large increases in transcripts for genes containing or synthesizing porphyrin and Fe-S cluster containing molecules, such as protoporphyrinogen IX oxidase, catalases, selenocysteine dehydrogenase, and flavoproteins, explains the greater need for iron in *Rhodococcus* sp. Genes for iron uptake were not differentially expressed in *Polaromonas* sp. at 0°C, though a few genes for Fe-S cluster formation were slightly increased (1.5-2 logFC) (Table 4.3). As with *Rhodococcus* sp., Fe-S clusters are important co-factors for a number of processes that are increased at cold temperature in *Polaromonas* sp. including tetrathionate reductase and cytochromes important for energy generation.

Large increases in iron acquisition mechanisms are not usually observed in cold-adapted bacteria during cold temp growth, and there is evidence that iron uptake genes and iron-associated proteins are suppressed in cold-adapted bacteria (Piette et al. 2011a; Ronholm et al. 2015; Tribelli et al. 2015). This suppression is theorized to perhaps contribute to alleviating oxidative stress by iron (Fenton reaction). However, as with *Polaromonas* sp., increases in Fe-S cluster generating proteins is one exception that has been reported (Goodchild et al. 2005; Bergholz et al. 2009) . While this strategy of reliance on additional iron during cold growth in *Rhodococcus* sp. can be partly explained within the context of its natural environment, the McMurdo Dry Valleys, which have relatively high soluble (bioavailable) iron content and noticeable ferric oxides (Tamppari et al. 2012; Bhattachan et al. 2015), it is intriguing and warrants further investigation.

4.3.4 Cell envelope and extracellular polysaccharides

Some psychrophiles, such as *P. halocryophilus* and *Colwellia psychrethrerya*, create unique extracellular cell envelope structures at low temperatures, believed to offer some level of cryoprotection and antifreeze properties (Mykytczuk et al. 2013, 2015; De Maayer et al. 2014; Carillo et al. 2015). In *C. psychrethrerya*, this structure is a polysaccharide capsule with similarities to antifreeze proteins and glycoproteins. Both *Polaromonas* sp. and *Rhodococcus* sp.

increase the abundance of proteins predicted to be in involved in exopolysaccharide and capsular polysaccharide biosynthesis at low temperatures, including colanic acid biosynthesis, UDP-glucose lipid carrier transferase, cellulose synthase, exopolysaccharide biosynthesis proteins, mannose-6-phosphate isomerase, and heparin binding hemagglutinin (Tables 4.2 & 4.3). The exopolysaccharide (EPS) biosynthesis protein is part of the EPS system in *Bacillus subtilis* (Roux et al. 2015), and heparin binding hemagglutinin induces aggregation of cells in mycobacteria (Menozzi et al. 1996). Mutational analyses to study impaired cold temperature growth in the deep-sea bacterium *Photobacterium profundum,* found the largest fraction of genes associated with temperature sensitivity to be those involved in the cell envelope formation, specifically extracellular polysaccharide biosynthesis (Lauro et al. 2008), implying that some level of EPS synthesis is especially relevant at cold temperatures. In addition, the capsule from *C. psychrethrerya* has been theorized to function as a protective mechanism at subzero temperatures (Carillo et al. 2015).

4.3.5 Cell wall/membrane

A common feature of many cold-adapted microorganisms is the presence of a system to increase or modulate membrane flexibility at lower temperatures (De Maayer et al. 2014). There is evidence for membrane modifications in *Rhodococcus* sp. at -5C with significant upregulation of an acyl-CoA thioesterase, an important regulator of lipid metabolism involved in both synthesis and degradation of fatty acids (Hunt and Alexson 2002), as well as an acyl dehydratase and a putative flippase (Table 4.2). Flippases are important for maintaining asymmetric distributions of phospholipids while acyl dehydratases can create double bonds in fatty acids. Increasing content of unsaturated fatty acids in the membrane is a common mechanism to increase flexibility in cold-adapted organisms. Similar increases were not observed in *Polaromonas* sp. However, emphasis on increased peptidoglycan synthesis and remodeling at 0°C was apparent in *Polaromonas* sp. with a ~2-fold upregulation of three enzymes, undecaprenyl diphosphate synthase and UDP-N-acetylmuramyl tripeptide synthase (*murE*), vital for peptidoglycan synthesis, and membrane-bound lytic murein transglycosylase B implicated in cell wall remodeling and cell growth (Hunt and Alexson 2002). *P. halocryophilus*

also increases synthesis of peptidoglycans at cold temperatures, and forms a protective envelope on its surface made up partly of peptidoglycan (Mykytczuk et al. 2013, 2015; Raymond-Bouchard et al. 2017).

4.3.6 Transporters

Overall, numerous transporters were strongly increased in both *Rhodococcus* sp. and *Polaromonas* sp. at the colder temperatures. In *Rhodococcus* sp., these were primarily branched-chain amino acid and peptide transporters, while *Polaromonas* sp. increased a polar amino acid transport system. Both organisms upregulated sulfate transport and MFS family efflux pumps, including multidrug transporters in *Rhodococcus* sp., which can recognize a variety of substrates and may also be important for toxin removal. A dicarboxylate symporter was strongly induced in *Rhodococcus* sp. (>7-fold; Table 4.2), while in *Polaromonas* sp. two tripartite tricarboxylate transporter (TTT) receptor components, *tctC*, and a TRAP-type dicarboxylate transport system were increased 2 to 5-fold (Table 4.3). Substrates for these transporters can include a variety of carboxylate containing molecules including malate, fumarate, 2-oxobutyrate, and pyruvate, as well as the compatible solutes ectoine (Mulligan et al. 2011), which may be relevant for osmoregulation (discussed below). The most upregulated (5.6 logFC) transport protein in *Polaromonas* sp. at 0°C is an *ompA-ompF* type porin, which forms a channel for uptake of a variety of hydrophilic solutes. These types of porins were also increased at 4°C in the Antarctic bacterium *Shewanella livingstonensis* (Kawamoto et al. 2007). Overall, increasing transporters at low temperatures is an important strategy for eury- and steno-psychrophiles (Mock et al. 2005; Campanaro et al. 2011; Koh et al. 2017), who must overcome reduced diffusion rates and ensure efficient nutrient uptake (Nedwell 1999).

4.3.7 Amino acid metabolism

In addition to the large increases in expression of amino acid transporters (discussed above), *Rhodococcus* sp. strongly upregulated two of the enzymes involved in methionine biosynthesis at -5°C, MetE and MetF, by 4 and 5-fold, respectfully (Table 4.2), likely due to this amino acid's importance in protein biosynthesis. In addition, methionine is a precursor to the major cellular

methyl donor in methylation reactions, S-Adenosyl methionine (Figge 2006), and is consistent with increases in several methyltransferases during growth at -5°C (Table 4.2 & S4.2). Smaller increases (<3-fold) in expression of enzymes involved in the synthesis of glutamine, cysteine, and branched-chain amino acids were also observed. Increases in amino acid synthesis was an important part of the response to cold growth in the marine bacterium *Sphingopyxis alaskensis* (Ting et al. 2010). Also of note is the induced presence of transcripts for two enzymes involved in the Kynurenine pathway, KynA and KynU, leading to the production of the co-enzyme nicotinamide adenine dinucleotide (NAD+) from the catabolism of tryptophan. NAD+ is an important co-factor in numerous cellular processes.

4.3.8 Carbon, energy, and co-enzyme metabolism

Numerous enzymes involved in glycolysis, D-mannose metabolism, the electron transport chain (ETC) and oxidative phosphorylation were increased in *Polaromonas* sp. at 0°C, including all subunits of NADH-quinone oxidoreductase, ATP synthase, cytochrome c553, triosephosphate isomerase, and mannose-6-phosphate isomerase (Table 4.3). The increased abundance of all subunits of tetrathionate reductase highlights the potential for *Polaromonas* sp. to utilize tetrathionate as a terminal electron acceptor and may be an important strategy for growth in permafrost at subzero temperatures by providing respiratory flexibility, including anaerobic respiration, for energy generation. The ability of stenopsychrophiles to have higher growth rates at cold temperatures has been attributed, at least in part, to the ability to increase energy acquisition. Photosynthesis in the stenopsychrophiles *Fragilariopsis cylindrus* and *Chlorella* sp. UMACC 234 was increased at -1°C and 4°C, respectively (Mock and Hoch 2005; Mock et al. 2005; Chong et al. 2011). The increase in expression of genes involved in oxidative phosphorylation may explain how *Polaromonas* sp. is able to maintain growth rates at 0°C (0.19 d^{-1}) that are very similar to >20°C (.21 d^{-1}), by keeping energy metabolism at a constant rate and offsetting reduced reaction rates at low temperatures.

These same increases were not seen in *Rhodococcus* sp. for ETC and glycolysis, and in fact multiple cytochromes were decreased at -5C (Table 4.2 & S4.2). However, there was a 7-fold

upregulation of l-lactate dehydrogenase, responsible for the conversion of l-lactate to pyruvate. There was also significant upregulation of genes involved in co-factor biosynthesis, biotin, NAD, porphyrin, and Fe-S clusters, and for maintaining redox potential. Amongst those most induced was 6 and 7-fold upregulation of two enzymes for protoporphyrinogen IX oxidase, menaquinone-dependent, required for porphyrin synthesis (Table 4.2). A pimeloyl-ACP methyl ester carboxylesterase, almost 5-fold upregulated, functions as a gatekeeper to remove the methyl group from pimeloyl-ACP methyl ester producing pimeloyl-ACP which stops further elongation of fatty acid synthesis and shuttles pimeloyl-ACP into the biosynthesis pathway for the co-factor biotin (Agarwal et al. 2012). Iron-sulfur cluster biosynthesis was also increased (discussed above). Maintenance of redox potential could be observed at -5°C in *Rhodococcus* sp. through the increased presence of numerous dehydrogenases and oxidoreductases (Table 4.2 & S4.2). Overall, these results indicate that *Rhodococcus* sp. places emphasis on preserving cellular activity through redox homeostasis and co-factor synthesis at subzero temperatures.

The ability of *Rhodococcus* to catabolize alcohols under subzero conditions was observed with large fold-change increases in homologues of a propanol-preferring alcohol dehydrogenase (7.21 logFC) and a secondary alcohol dehydrogenase (5.5 logFC). The large subunit of ethanolamine ammonia-lyase (*eutB*) was increased 6.5-fold, and is responsible, along with smaller subunit *eutC*, for cleaving ethanolamine to ammonia and ethanol, and subsequently, acetaldehyde (Tsoy et al. 2009). This suggests the potential for secondary alcohols, aromatic alcohols, and ethanolamine to serve as carbon sources and, in the case of ethanolamine, a nitrogen source as well (Roof and Roth 1988). Numerous members of the genus *Rhodococcus* are capable of breaking down secondary and aromatic alcohols (Ludwig et al. 1995; Peng et al. 2006).

Lastly, while strongly surpressed at -5°C, all of the genes involved in the four steps of the phenylacetic acid catabolism pathway for the breakdown of aromatic compounds (*paaA, paaB, paaC, paaD, paaE, paaG, paaI, paaZ, paaH*) were expressed in *Rhodococcus* sp. (Table 4.2). While this pathway does not appear to be important for growth of *Rhodococcus* sp. at -5°C, where it may be preferable to focus available energy on streamlining primary metabolism and

downregulate secondary metabolic processes, it is nevertheless worthwhile to note the catabolic potential of this organism.

4.3.9 Oxidative and universal stress responses

There is some evidence that oxidative stress is higher at colder temperatures due to increased gas solubility and higher rates of enzyme activity to compensate for reduced reaction rates (Chattopadhyay et al. 2011; De Maayer et al. 2014). This appears to be the case in *Rhodococcus* sp. and *Polaromonas* sp. given the increase in iron acquisition and redox potential observed at -5°C in *Rhodococcus* sp., and the emphasis on formation and activity of Fe-S cofactors by both *Rhodococcus* sp. and Polaromonas sp. at colder temperatures. Iron is involved in the production of oxygen radicals via the Fenton reaction (Fenton 1894; Troxell and Hassan 2013). To relieve oxidative stress, 2 catalases and a cytochrome peroxidase were increased almost 2-fold in *Polaromonas* sp. (Table 4.3). In *Rhodococcus* sp., a specific catalase gene was upregulated ~ 3-fold, replacing 3 other catalase genes, which were downregulated (Table 4.2). A superoxide dismutase, responsible for converting the superoxide radical to oxygen or hydrogen peroxide, was also increased in *Rhodococcus* sp.

Rhodococcus sp. and *Polaromonas* sp. possess 16 and 13 copies, respectively, of the universal stress protein (uspA) in their genome (Goordial et al. 2016; I.Raymond-Bouchard, in Review FEMS Microbiology Ecology). Four of these were strongly (3.5 to 6.5-fold) induced during subzero growth in *Rhodococcus* sp. (Table 4.2), while one copy of was upregulated about 3.5-fold in *Polaromonas* sp., and 2 copies were decreased (Table 4.3). While the exact function of this family of genes remains poorly elucidated, they are induced by a variety of environmental stressors such as nutrient starvation, extreme temperatures, high salinity, and drought. They are thought to protect the cell from stress and damage and in some cases are linked to protection against DNA-damaging agents (Kvint et al. 2003). Further studies looking into the function of uspA genes at low temperatures in *Rhodococcus* sp. and *Polaromonas* sp. would be interesting, given their selective upregulation and differential expression patterns.

4.3.10 Compatible solutes and osmoregulation

Compatible solutes, small water soluble organic compounds, are accumulated by cold-adapted microbes and play an important role in resisting osmotic pressure caused by high salinity and low water activity associated with cryoenvironments, such as the brine veins in permafrost and ice (Doyle et al. 2012). In addition, compatible solutes also play a role as cryoprotectants and increase the stability of macromolecules, membranes, and proteins, as well as enhancing folding and ligand binding in the latter (Thomas et al. 2001; Yancey 2005). Genes for the biosynthesis or transport of compatible solutes are induced at cold temperatures in several cold-adapted strains, including *E. sibiricum*, *M. burtonii*, and *P. arcticus* (Rodrigues et al. 2008; Bergholz et al. 2009; Campanaro et al. 2011), as well as this study. The choline transporter *betT* was increased by 3-fold, and components of the *opuABCD* system for uptake of the compatible solute glycine betaine were over 5-fold increased at -5°C in *Rhodococcus* sp., as was a putative osmotically induced protein (*osmC*) (Table 4.2). OsmC in *Mycobacterium* spp. functions as a hydroperoxide reductase and may therefore have a role as an antioxidant in *Rhodococcus* sp. (Saikolappan et al. 2011). However, *betT* was increased, and genes involved in the synthesis of glycine betaine, *betA* and *betB*, were more than 6 and 8-fold decreased in *Rhodococcus* sp. This might indicate preference for the uptake of available solutes from the environment, rather than energy-demanding de novo synthesis of solutes. It is also possible that the synthesis genes were induced during the initial acclimation period and were subsequently downregulated once optimal concentrations of compatible solutes had been achieved, and thus are not detected in these cultures which were harvested at mid-late exponential phase. A similar theory has been proposed for *P. halocryophilus* (Mykytczuk et al. 2013).

Polaromonas sp. does not appear to upregulate transport of compatible solutes to the extent that *Rhodococcus* sp. does, though this may be explained by the fact that the media used to culture *Polaromonas* sp. did not contain additional NaCl beyond what was present in the growth medium (R2A). Nevertheless, *Polaromonas* sp. also increases the abundance of transcripts for an osmotically inducible protein (*osmC*) by almost 4-fold. A >3-fold increase in a TRAP system capable of transporting ectoine suggests the potential for accumulation of

compatible solutes. Even under low salt conditions, cold growth appears to be linked at least partially with osmoregulation systems.

4.3.11 Transcription, signaling, and motility

Numerous transcription factors (TFs) were strongly upregulated during cold temperature growth in *Rhodoccocus* sp. and in *Polaromonas* sp., though slightly less so. The predicted regulatory functions of these TFs are consistent with many transcripts increased in abundance in both organisms. Ten transcriptional regulators were found to be >3-fold upregulated in *Rhodoccocus* sp., with 9 more than 5-fold upregulated (Table 4.2 & S4.2), including 2 *acrR* family regulators, known to be involved in modulating responses to osmotic stress, modification and elimination of toxic substances, and lipid metabolism (Deng et al. 2013), the primary sigma-70 factor required for transcription initiation (Paget and Helmann 2003), and the *whiB7* transcriptional regulator, which is highly conserved in actinomycetes, with characterized roles in redox homeostasis, cell metabolism, and antibiotic resistance (Burian et al. 2012).

NsrR is a nitric oxide (NO) sensitive transcription repressor which can upregulate the ResDE two-component system required for induction of nitrate respiration genes and NO detoxifying enzymes in the presence of NO (Yukl et al. 2008). The elevated transcription of *nsrR* and the flavohaemoglobin NO-detoxifying enzyme *hmp* suggests that *Rhodococcus* sp. may be under heightened NO stress at subzero temperatures. One of the genes involved in nitrate reduction, nitrite reductase, was detected, suggesting that *Rhodococcus* sp. may be capable of denitrification but it was >4.5-fold downregulated at -5°C. MarR (~6-fold increase) is important in the stress response and modification and export of toxic compounds, partly through the induction of efflux pumps (Grove 2013), and *ytrA* (5.5-fold increased) is involved in regulation of ATP-binding cassette transporters (Suvorova et al. 2015). These TFs may have roles in the induction of the MFS pumps and ABC transporters observed in *Rhodococcus* sp. J at -5°C (discussed above).

At cold temperatures both *Rhodococcus* sp. and *Polaromonas* sp. show increased levels of transcription factors of the *narL/fixJ/luxR* and *iclR* families (Tables 4.2 & 4.3). In addition,

Polaromonas sp. induces 2 *lysR* family TFs and a regulator of the *gntR* family, the same family as ytrA in *Rhodococcus* sp. Members of the *iclR* family are transcriptional activators and repressors often involved in regulating carbon metabolism, such as the glyoxylate bypass operon, degradation of aromatic compounds, quorum-sensing and multidrug resistance (Sunnarborg et al. 1990; Chao and Zhou 2013). *narL/fixJ/luxR* comprises a large family of TFs and, therefore, the exact role of these in *Polaromonas* sp. and *Rhodococcus* sp. is hard to pinpoint but potential roles could include activating the nitrate reductase operon and nitrogen-fixation genes and uptake of the compatible solute ectoine (Rabin and Stewart 1993; Galiniers et al. 1994; Rodríguez-Moya et al. 2010). The presence of the *nac/lysR* regulator, or nitrogen assimilation control protein, in *Polaromonas* sp. would suggest that at cold temperatures *Polaromonas* sp. experiences nitrogen limitation (Bender 2010). Signaling systems associated with these transcription factors were induced about 2-3-fold at 0°C in *Polaromonas* sp. (Tables 4.3 & S4.3) including the two-component system of *fixJ/luxR*, adenylate cyclase, and components of the cAMP receptor protein, a global transcriptional activator that regulates transcription of many genes including energy metabolism, consistent with increases in energy metabolism observed in the bacterium at 0°C. Overall, the transcription factors upregulated at cold temperatures suggests the induction of numerous pathways important during cold growth, including lipid metabolism, redox homeostasis, ABC transporters, and osmoregulation in *Rhodococcus* sp., and ABC transporters, carbon and energy metabolism, and export of toxic compounds in *Polaromonas* sp.

4.4 Conclusion

Overall, the transcriptomic responses to cold growth in *Rhodococcus* sp. and *Polaromonas* sp., shared many cold-adaptive features (Figure 4.2). Common responses included induction of translation and ribosomal processes, resulting in translationally active cold-adapted ribosomes, upregulation of nutrient transport, increased oxidative and osmotic stress responses, modulating cell wall/membrane features, induction of EPS synthesis, and accumulation of compatible solutes, though this last item was much more pronounced in *Rhodococcus* sp. Recombination and genomic redundancy also appeared to be a shared strategy at low

temperatures, though the mechanism used to achieve this was different in each organism. *Polaromonas* sp. utilized specific transposases, while *Rhodococcus* sp. induced recombination proteins. The presence of the above properties in most psychrophiles, whether eury- or steno-, suggests that these may be conserved adaptive features, necessary for growth at low temperatures in most organisms.

In *Rhodococcus* sp., the marked differences during cold growth featured increased abundance of transcripts involved in iron transport, amino acid transport and metabolism, modulating fatty acid synthesis and composition, catabolism of alcohols/ethanolamine, and sustaining redox potential (Figure 4.2). Conversely, *Polaromonas* sp. was found to induce energy metabolism relating to the electron transport chain, oxidative phosphorylation, and glycolysis, as well as global signal transduction mechanisms and transport and metabolism of carboxylates (Figure 4.2). In addition, enzymes vital to peptidoglycan synthesis and modulation were increased. Lastly, *Polaromonas* sp. differentially up/down regulated a specific subset of transposases at the two growth temperatures.

It is important to note one caveat to this study: comparison of a gram-positive organism (*Rhodococcus* sp.) and a gram-negative organism (*Polaromonas* sp.) from two different phyla. As such, some natural differences can be expected in terms of their cold adaptive strategies. Though we have yet to identify such candidates from permafrost, in the future, comparing a stenopsychrophile and a eurypsychrophile from the same genus would be worthwhile. Nevertheless, this study does provide us with valuable insight into the shared and unique cold adaptive strategies of eury- and steno-psychrophiles.

Increased activity of primary energy production and the electron transport chain in *Polaromonas* sp. and other stenopsychrophiles, is likely to be one major mechanism by which these organisms are able to sustain optimal growth at colder temperatures or consistent growth rates over their temperature range. The more attenuated transcriptomic response in *Polaromonas* sp. also suggests that the organism may be, at least partly, constitutively adapted to colder temperatures or for growth over its temperature range and does not need to

upregulate its response to the same levels as *Rhodococcus* sp. at lower temperatures. However, more research will need to be done to determine if this is true and to identify those features which are unique to these types of organisms, and differ from eurypsychrophiles. Certainly, it may simply be that stenopsychrophiles are adapted to grow at a narrow range and temperature changes, both above and below this range, are difficult for these organisms. In the same vein, it is difficult to pinpoint the mechanisms that favor eurypsychrophilic growth at subzero temperatures from this study alone, though in the case of *Rhodococcus* sp. increased co-factor formation, flexibility in use of carbon sources, control of fatty acid composition in the membrane, iron acquisition, and sustaining redox potential appear to be important factors. These may represent adaptive strategies that allow this organism to grow at subzero temperatures, in addition to sustaining growth over a wide range of temperatures (-5 to 30°C).

4.5 Material and Methods

4.5.1 Culturing and growth conditions

Polaromonas sp. Eur3 1.2.1 and *Rhodococcus* sp. JG3 were cultured on ½ R2A and R2A agar (BD Difco), respectively. For RNA extraction, liquid cultures of each strain were grown in biological triplicate to mid-late exponential phase on ½ R2A at 0°C and 20°C (OD_{600} 0.165) for *Polaromonas* sp., and in tryptic soy broth (TSB; BD Difco) supplemented with 5% salt and 5% sucrose at -5°C (OD_{600} 1.0) and 25°C (OD_{600} 2.0) for *Rhodococcus* sp. *Polaromonas* sp. is a slow-growing organism with low cell densities, and as a result, several cultures had to be combined into one for each triplicate condition to compensate for the reduced amount of biomass obtained.

4.5.2 RNA extraction and library preparation

RNA extraction was performed using the Direct-zol RNA MiniPrep Plus Kit from Zymo Research (#R2070) according to manufacturer's instructions. RNA quantity and purity were checked using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent

Technologies). Sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA LT Sample Prep Kit as outlined in the manufacturer's guide.

4.5.3 Illumina sequencing and bioinformatics analysis

Sequencing was performed by RNAseq on the Illumina MiSeq system using a Reagent Kit v3 150 cycles with a 2 x 75bp paired-end configuration. Sequencing raw data (728 Mega-bases) was processed through our metatranscriptomics bioinformatic pipelines. Read count summaries are provided for each sequencing library in Table S4.1. Sequencing adapters were removed from each read and bases at the end of reads having a quality score less than 30 were cut off (Trimmomatic v0.32) (Bolger et al. 2014) to generate quality controlled (QC) reads. QC-passed reads were mapped (BWA mem v0.7.10) (unpublished - http://bio-bwa.sourceforge.net) against *Polaromonas* sp. or *Rhodococcus* sp. genome references to obtain contig abundance profiles. *Polaromonas* sp. and *Rhodococcus* sp. genomes were obtained from the Joint Genome Institute (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi), ID 2619618817 and 2529292502, respectively. Alignment files in bam format were sorted by read coordinates using samtools v1.1 and only properly aligned read pairs were kept for downstream steps. Each bam file (containing properly aligned paired-reads only) was analyzed for coverage of genes and contigs using bedtools (v2.17.0) (Quinlan and Hall 2010) using corresponding gene models (gene coordinates on each contig for both genome references). Only paired reads both overlapping their contigs or genes were considered for gene counts. Coverage profiles of each sample were merged to generate an abundance matrix (rows = contig, columns = samples) for which a corresponding CPM (Counts Per Million) abundance matrix (edgeR v3.10.2) (Robinson et al. 2009) was generated. According to our experimental design, differentially expressed genes (DEGs) were assessed with edgeR (v3.10.2) using its GLM (Generalized Linear Model) approach detailed by the authors (https://www.bioconductor.org/packages/3.3/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf - section 3.2.3, page 29) with transcriptomics raw counts matrices as input. Genes having a logFC (log Fold-Change) ratio >= |1.5| and FDR (False Discovery Rate) < 0.05 were considered

as differentially expressed. Metadata for all samples reported in this study are available in Table S4.1.

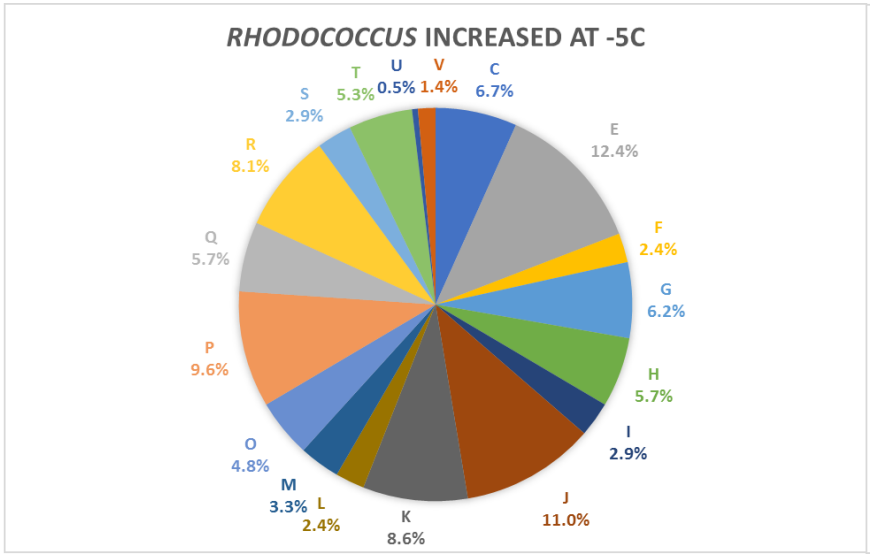
4.5.4 Data analysis

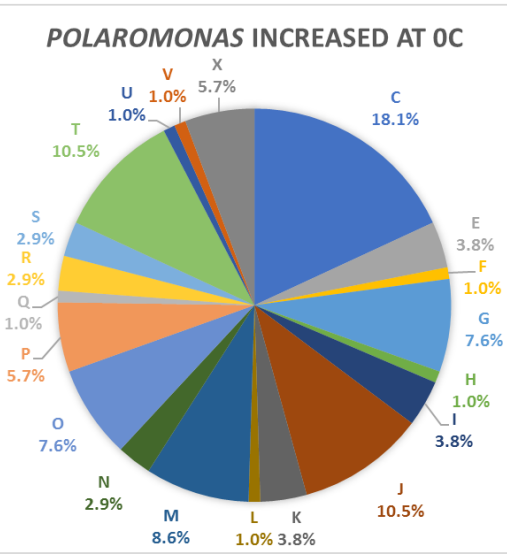
Predicted COG and KEGG assignments for *Rhodococcus* sp. (ID 2529292502) and *Polaromonas* sp. (ID 2619618817) were downloaded from the JGI IMG/ER website (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi), and used to obtain preliminary function and pathway assignments. These were then manually cross-referenced against available literature for the proteins discussed in the text to ensure function and pathway assignments are as accurate as possible. Focus was given to those genes with logFC ≥ 1.5 (FDR < 0.05) for which functional and pathway assignments could best be predicted based on homology assignments, conserved domains, and literature data. The volcano plot was created in R (v3.4.0) (R Core Team 2017).

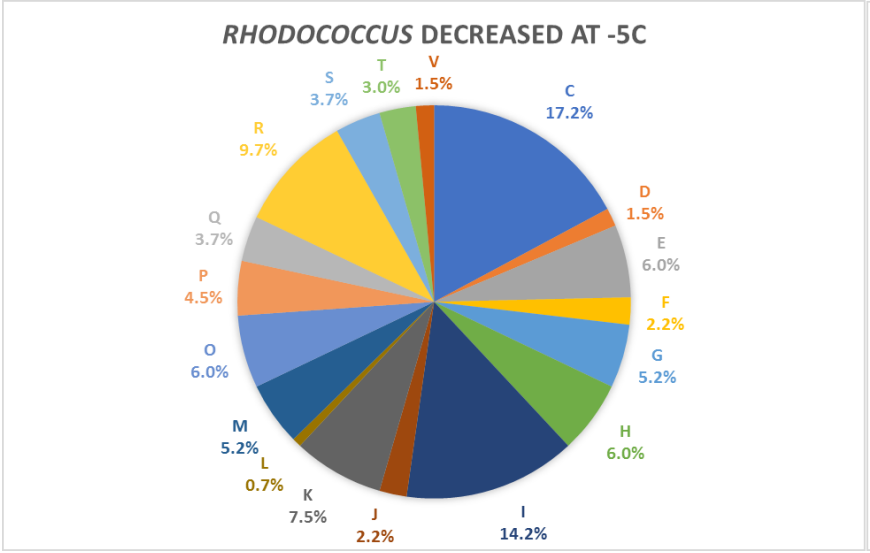
4.6 Acknowledgements

Funding for this research was provided by the Natural Sciences and Engineering Research Council (NSERC) through an NSERC CREATE PhD fellowship and an NSERC Canadian Graduate Scholarship to IRB, and through NSERC Discovery and Northern Research Supplement grants, and a Polar Continental Shelf Project grant, to LGW. We acknowledge the U.S. Department of Energy Joint Genome Institute (JGI) who sequenced both genomes under a Community Sequencing Program Project (Quartely; ID 1015712). We also wish to acknowledge Compute Canada for access to the McGill University High Performance Computing (HPC) infrastructure (Guillimin system).

Figure 4.1. Diagram of COG categories with differentially expressed genes in *Polaromonas* sp. Eur3 1.2.1 at 0°C and *Rhodococcus* sp. JG3 at -5°C.The number of differentially expressed genes in each category is expressed as a percent of total. Letters correspond to COG categories: **C**: Energy production and conversion; **D**: Cell cycle control, cell division, chromosome partitioning; **E**: Amino Acid metabolism and transport; **F**: Nucleotide metabolism and transport; **G**: Carbohydrate metabolism and transport; **H**: Coenzyme metabolism and transport; **I**: Lipid metabolism and transport; **J**: Translation, ribosome structure and biogenesis; **K**: Transcription; **L**: Replication, recombination, and repair; **M**: Cell wall/membrane/envelope biogenesis; **N**: Cell motility and secretion; **O**: Post-translational modification, protein turnover, chaperone functions; **P**: Inorganic ion transport and metabolism; **Q**: Secondary metabolites biosynthesis, transport, and catabolism; **T**: Signal Transduction; **V**: Defense mechanisms; **R**: General Functional Prediction only


RHODOCOCCUS INCREASED AT -5C
C 6.7%
E 12.4%
F 2.4%
G 6.2%
H 5.7%
I 2.9%
J 11.0%
K 8.6%
L 2.4%
M 3.3%
O 4.8%
P 9.6%
Q 5.7%
R 8.1%
S 2.9%
T 5.3%
U 0.5%
V 1.4%


POLAROMONAS INCREASED AT 0C
C 18.1%
E 3.8%
F 1.0%
G 7.6%
H 1.0%
I 3.8%
J 10.5%
K 3.8%
L 1.0%
M 8.6%
N 2.9%
O 7.6%
P 5.7%
Q 1.0%
R 2.9%
S 2.9%
T 10.5%
U 1.0%
V 1.0%
X 5.7%


RHODOCOCCUS DECREASED AT -5C
C 17.2%
D 1.5%
E 6.0%
F 2.2%
G 5.2%
H 6.0%
I 14.2%
J 2.2%
K 7.5%
L 0.7%
M 5.2%
O 6.0%
P 4.5%
Q 3.7%
R 9.7%
S 3.7%
T 3.0%
V 1.5%

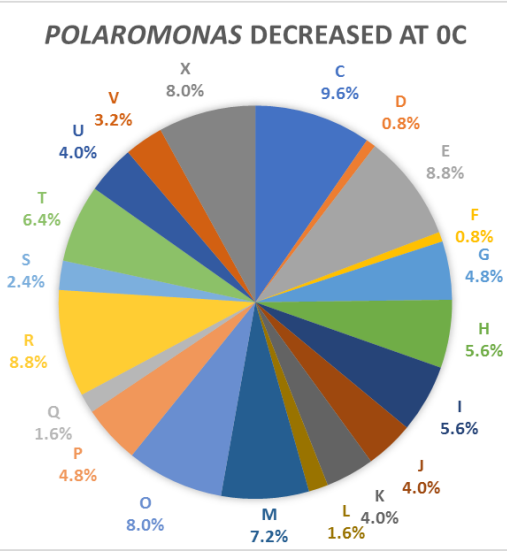

POLAROMONAS DECREASED AT 0C
C 9.6%
D 0.8%
E 8.8%
F 0.8%
G 4.8%
H 5.6%
I 5.6%
J 4.0%
K 4.0%
L 1.6%
M 7.2%
O 8.0%
P 4.8%
Q 1.6%
R 8.8%
S 2.4%
T 6.4%
U 4.0%
V 3.2%
X 8.0%

Figure 4.2. Cell diagram showing important processses and pathways increased during cold growth. Blue labels represent processes increased in *Polamonas* sp. Eur3 1.2.1 at 0°C, yellow labels represent processes increased in *Rhodococcus* sp. JG3 at -5°C and green labels represent shared processes increased in both organisms during cold growth. Full gene descriptions corresponding to the short protein names used in the figure can be found in Tables 3.2, S3.2, 3.3, and S3.3.

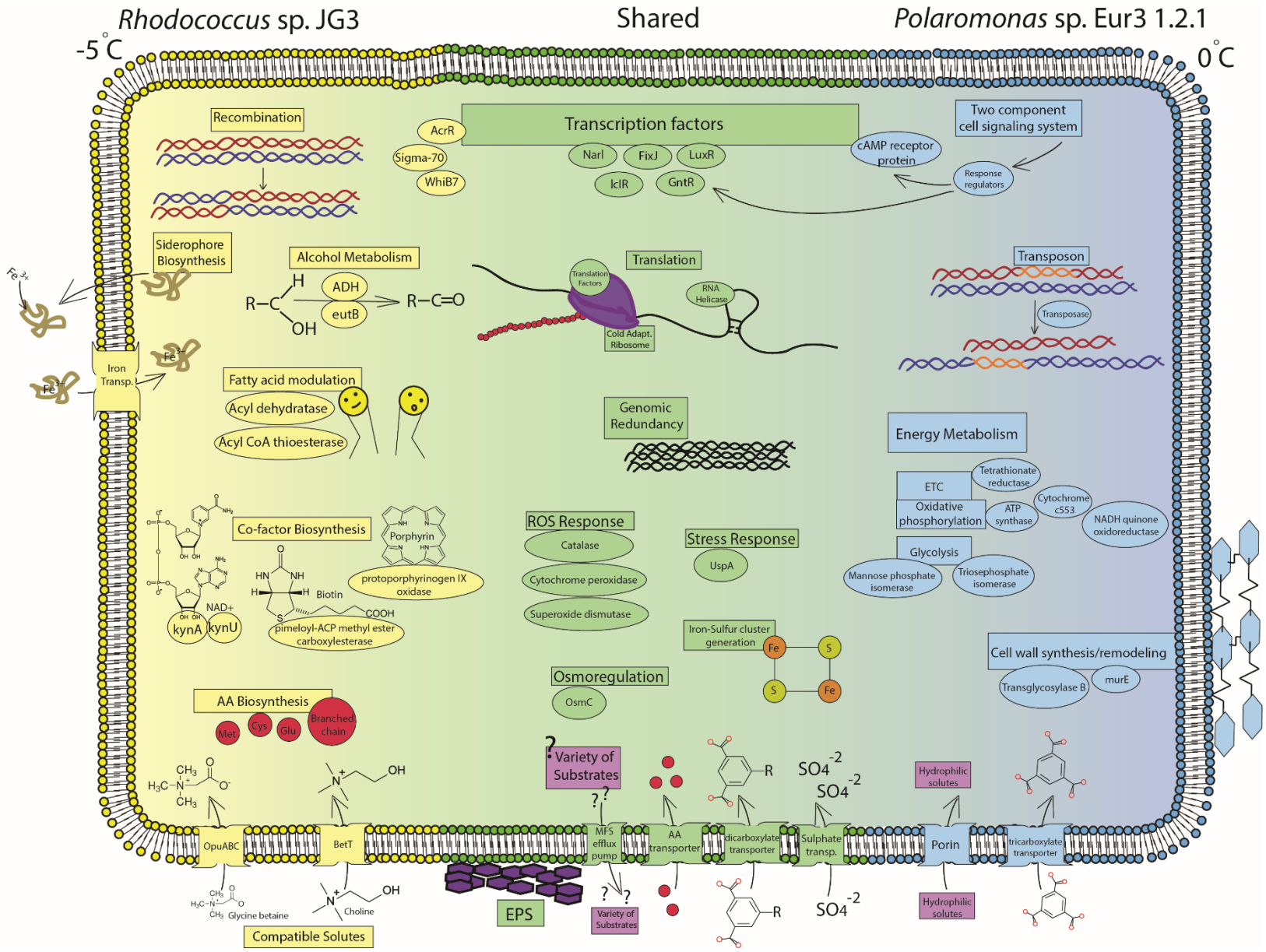

Rhodococcus sp. JG3
Shared
Polaromonas sp. Eur3 1.2.1
-5 °C
0 °C
Recombination
Transcription factors
AcrR
Sigma-70
WhiB7
NarI
FixJ
LuxR
IclR
GntR
Two component cell signaling system
cAMP receptor protein
Response regulators
Siderophore Biosynthesis
Alcohol Metabolism
ADH
eutB
Translation
Translation Factors
RNA Helicase
Cold Adapt. Ribosome
Transposon
Transposase
Iron Transp.
Fatty acid modulation
Acyl dehydratase
Acyl CoA thioesterase
Genomic Redundancy
Energy Metabolism
ETC
Tetrathionate reductase
Oxidative phosphorylation
ATP synthase
Cytochrome c553
NADH quinone oxidoreductase
Glycolysis
Mannose phosphate isomerase
Triosephosphate isomerase
Co-factor Biosynthesis
Porphyrin
protoporphyrinogen IX oxidase
Biotin
pimeloyl-ACP methyl ester carboxylesterase
NAD+
kynA
kynU
ROS Response
Catalase
Cytochrome peroxidase
Superoxide dismutase
Stress Response
UspA
Iron-Sulfur cluster generation
Fe
S
Cell wall synthesis/remodeling
Transglycosylase B
murE
Osmoregulation
OsmC
AA Biosynthesis
Met
Cys
Glu
Branched chain
Variety of Substrates
SO4-2
Hydrophilic solutes
OpuABC
BetT
MFS efflux pump
AA transporter
dicarboxylate transporter
Sulphate transp.
Porin
tricarboxylate transporter
Glycine betaine
Choline
Compatible Solutes
EPS

Table 4.1. Strain information and summary of transcriptomic results for *Rhodococcus* sp. JG3 and *Polaromonas* sp. Eur3 1.2.1.

	***Rhodococcus* sp. JG3**	***Polaromonas* sp. Eur3 1.2.1**
Phylum	Actinobacteria	Proteobacteria
Location isolated	Permafrost, University Valley, Antarctica	Permafrost, Eureka, Nunavut
Growth range	-5-30°C	-5*-22°C
Salt (NaCl) tolerance	0-7%	0-3%
Size of genome (Mbp)	5.3	4.4
Total number of genes	5067	4303
Transcriptomic results		
Total differentially expressed genes (≥1.5 FC)	515	359
Increased at low temperature	313	177
Decreased at low temperature	202	182
With COG	343	236
% COG	66	66
With KEGG	234	161
%KEGG	45	45

*-5°C on agar only (I. Raymond-Bouchard, in Review FEMS Microbiology Ecology)

Table 4.2. Select genes differentially expressed at -5°C compared to 25°C in *Rhodococcus* sp. JG3. logFC is log fold change at -5°C as compared to 25°C. Includes COG and KEGG Orthology (KO) assignments.

Gene	Gene Description	Shortname	logFC	COG	KO
Amino acid metabolism					
2529301904	5,10-methylenetetrahydrofolate reductase	metF	5.31	COG0685	K00297
2529300492	Methionine synthase II (cobalamin-independent)	metE	4.08	COG0620	K00549
Cell wall/membrane and EPS biosynthesis					
2529303100	Heparin binding hemagglutinin	hbhA	6.13		K16645
2529302855	Acyl-ACP thioesterase		5.94	COG3884	
2529302771	Exopolysaccharide biosynthesis protein, predicted pyruvyl transferase	epsO	3.15	COG5039	K19431
2529299791	Sugar transferase involved in LPS biosynthesis (colanic, teichoic acid)		3.02	COG2148	
2529302388	Acyl dehydratase		2.30	COG2030	
2529302869	Putative flippase GtrA	gtrA	2.21	COG2246	
Iron transport and acquisition					
2529300144	NADPH-dependent ferric siderophore reductase, contains FAD-bin		8.13	COG2375	
2529300140	Mycobactin salicyl-AMP ligase	mbtA	6.33	COG1021	K04787
2529301663	ABC-type cobalamin/Fe3+-siderophores transport system, ATPase	ABC.FEV.A	6.17	COG1120	K02013
2529300145	Salicylate synthetase	mbtI	6.02	COG0147	K04781
2529302627	ABC-type Fe3+-hydroxamate transport system, periplasmic component	ABC.FEV.S	5.70	COG0614	K02016
2529301661	ABC-type Fe3+-siderophore transport system, permease component	ABC.FEV.P	5.35	COG0609	K02015
2529300143	ABC-type Fe3+-hydroxamate transport system, periplasmic component	ABC.FEV.S	5.21	COG0614	K02016
2529303132	ABC-type Fe3+-hydroxamate transport system, periplasmic component	ABC.FEV.S	4.71	COG0614	K02016
2529300593	Fe2+ uptake regulator, Fur family transcriptional regulator	fur	4.49	COG0735	K03711
2529300147	L-ornithine N5-oxygenase	pvdA	4.46	COG3486	K10531
2529300139	Mycobactin phenyloxazoline synthetase	mbtB	3.51	COG1020	K04788
2529301174	NADPH-dependent ferric siderophore reductase, contains FAD-bin		2.95	COG2375	
2529299468	ABC-type Fe3+-hydroxamate transport system, periplasmic component	ABC.FEV.S	2.32	COG0614	K02016
2529303362	ABC-type Fe3+ transport system, periplasmic component	afuA, fbpA	2.26	COG1840	K02012
2529300022	Fe2+ uptake regulator, Fur family transcriptional regulator	fur	-4.40	COG0735	K03711
Transporters					

2529299875	ABC-type multidrug transport system, ATPase component	ABC-2.A	7.56	COG1131	K01990
2529302312	Na+/H+-dicarboxylate symporter		7.21	COG1301	
2529302083	ABC-type sulfate transport system, permease component	cysW	5.93	COG4208	K02047
2529302491	ABC-type branched-chain amino acid transport system, permease	livM	5.69	COG4177	K01998
2529300854	ABC-type sugar transport system, periplasmic component	ABC.SS.S	5.18	COG1879	K02058
2529302471	ABC-type dipeptide/oligopeptide/nickel transport system, permease	ABC.PE.P	5.16	COG0601	K02033
2529301448	ABC-type peptide/nickel transport system, periplasmic component	ABC.PE.S	3.54	COG0747	K02035
2529300505	Aerobic C4-dicarboxylate transport protein	dctA	3.09	COG1301	K11103
2529301861	MFS transporter, DHA2 family, multidrug resistance protein	smvA, qacA, lfrA	3.01		K08167
2529301796	ABC-type branched-chain amino acid transport system, periplasmic comp	livK	2.35	COG0683	K01999
2529300180	ABC-type peptide/nickel transport system, periplasmic component	ABC.PE.S	2.29	COG0747	K02035
2529301795	ABC-type branched-chain amino acid transport system, ATPase	livF	1.66	COG0410	K01996
2529301792	Branched-chain amino acid ABC-type transport system, permease	livH	1.59	COG0559	K01997
2529299546	ABC-type dipeptide/oligopeptide/nickel transport system, permease	ABC.PE.P	-4.57	COG0601	K02033
Carbon, Energy, and co-enzyme metabolism					
2529299871	Protoporphyrinogen IX oxidase, menaquinone-dependent (flavodoxin)	hemG	7.62	COG4635	K00230
2529299865	Protoporphyrinogen IX oxidase, menaquinone-dependent (flavodoxin)	hemG	6.73	COG4635	K00230
2529300710	Ethanolamine ammonia-lyase, large subunit	eutB	6.49	COG4303	K03735
2529299868	Alcohol dehydrogenase, propanol-preferring	adhP	7.21	COG1064	K13953
2529300697	Glycolate oxidase/L-lactate dehydrogenase (cytochrome)	glcD, lldD	7.01	COG1304	K00104
2529300499	Nitric oxide dioxygenase	hmp, YHB1	5.96	COG1017	K05916
2529300276	Pimeloyl-ACP methyl ester carboxylesterase		4.77	COG0596	
2529303159	NAD+-dependent secondary alcohol dehydrogenase	adh1	5.50	COG1064	K18382
2529298821	2-(1,2-epoxy-1,2-dihydrophenyl) acetyl-CoA isomerase	paaG	-6.73	COG1024	K15866
2529298822	ring 1,2-phenylacetyl-CoA epoxidase, catalytic subunit PaaA	paaA	-5.37	COG3396	K02609
2529300992	NAD(P)H-nitrite reductase, large subunit	nirB	-4.57	COG1251	K00362
2529298827	Phenylacetate-coenzyme A ligase, adenylate-forming domain	paaK	-4.36	COG1541	K01912
2529298820	3-hydroxybutyryl-CoA dehydrogenase	paaH, hbd, fadB	-3.89	COG1250	K00074
2529298826	ring-1,2-phenylacetyl-CoA epoxidase subunit PaaE	paaE	-3.70	COG1018	K02613
2529298823	ring 1,2-phenylacetyl-CoA epoxidase, PaaB subunit	paaB	-3.47	COG3460	K02610
2529298825	ring-1,2-phenylacetyl-CoA epoxidase subunit PaaD	paaD	-3.22	COG2151	K02612

2529298824	1,2-phenylacetyl-CoA epoxidase, catalytic subunit	paaC	-2.38	COG3396	K02611
2529298814	Acyl-coenzyme A thioesterase PaaI, contains HGG motif	paaI	-2.15	COG2050	K02614
Stress and oxidative responses					
2529299876	Nucleotide-binding universal stress protein, UspA family		6.53	COG0589	
2529299882	Nucleotide-binding universal stress protein, UspA family		6.26	COG0589	
2529299879	Nucleotide-binding universal stress protein, UspA family		4.44	COG0589	
2529299880	Nucleotide-binding universal stress protein, UspA family		3.55	COG0589	
2529300594	Catalase (peroxidase I)	katG	3.17	COG0376	K03782
2529299042	Superoxide dismutase	SOD2	1.53	COG0605	K04564
2529300023	Catalase (peroxidase I)	katG	-5.95	COG0376	K03782
2529300031	Mn-containing catalase (includes spore coat protein CotJC)		-5.10	COG3546	K07217
2529299729	Catalase	katE, CAT, catB	-3.64	COG0753	K03781
Translation, ribosomes, helicases, posttranslational modifications					
2529302299	ADP-ribosylglycohydrolase		5.16	COG1397	
2529300328	Superfamily II DNA and RNA helicase		3.75	COG0513	
2529298761	Queuine/archaeosine tRNA-ribosyltransferase	tgt	3.45	COG0343	K00773
2529301229	Ribosome-binding factor A	rbfA	3.45	COG0858	K02834
2529301134	Ribosomal protein L28		3.18	COG0227	K02902
2529302298	O-acetyl-ADP-ribose deacetylase (regulator of RNase III)		3.12	COG2110	
2529299072	Regulator of RNase E activity RraA	rraA, menG	2.79	COG0684	K02553
2529301894	16S rRNA C1402 N4-methylase RsmH	mraW, rsmH	2.77	COG0275	K03438
2529300757	Ribosomal protein S5	RP-S5, rpsE	2.70	COG0098	K02988
2529300756	Ribosomal protein L18	RP-L18, rplR	2.25	COG0256	K02881
2529301143	Ribosomal protein L32	RP-L32, rpmF	2.07	COG0333	K02911
2529302902	Ribosomal protein L7/L12	RP-L7, rplL	2.04	COG0222	K02935
2529300742	Ribosomal protein L29	RP-L29, rpmC	2.00	COG0255	K02904
2529302067	GTPase Era, involved in 16S rRNA processing	era	2.33	COG1159	K03595
2529300958	Ribosome-associated translation inhibitor RaiA	raiA	-2.18	COG1544	
2529301311	mRNA degradation ribonuclease J1/J2	rnj	-1.61	COG0595	K12574
DNA repair/recombination					
2529299297	Single-strand DNA-binding protein	ssb	2.44	COG0629	K03111

2529301443	Holliday junction DNA helicase RuvA	ruvA	2.01	COG0632	K03550
2529302293	Exodeoxyribonuclease V alpha subunit	recD	1.99	COG0507	K03581
Osmoregulation					
2529301958	ABC-type proline/glycine betaine transport system, ATPase component	opuA	5.49	COG1125	K05847
2529301959	ABC-type proline/glycine betaine transport system, permease component	opuBD	5.20	COG1174	K05846
2529302730	Uncharacterized OsmC-related protein		5.10	COG1765	
2529302675	Periplasmic glycine betaine/choline-binding (lipo)protein of a ABC-type glycine betaine transport system	opuC	3.37	COG1732	K05845
2529303167	Choline-glycine betaine transporter	betT, betS	3.21	COG1292	K02168
2529298829	Choline dehydrogenase	betA, CHDH	-8.14	COG2303	K00108
2529298830	Betaine-aldehyde dehydrogenase	betB, gbsA	-6.46	COG1012	K00130
Transcription					
2529301008	WhiB family transcriptional regulator, redox-sensing regulator	whiB7	5.91		K18958
2529299065	DNA-binding transcriptional regulator, MarR family		5.73	COG1846	
2529301848	DNA-binding transcriptional regulator YtrA, GntR family	ytrA	5.57	COG1725	K07979
2529302494	DNA-binding transcriptional regulator, IclR family		5.60	COG1414	
2529303412	RNA polymerase sigma-70 factor, ECF subfamily	SIG3.2, rpoE	5.51	COG1595	K03088
2529300498	DNA-binding transcriptional regulator, NsrR	nsrR	5.34	COG1959	K13771
2529301247	DNA-binding transcriptional regulator, AcrR family		5.21	COG1309	
2529300684	DNA-binding response regulator, NarL/FixJ family, contains REC		5.04	COG2197	
2529299715	DNA-binding transcriptional regulator, AcrR family		3.24	COG1309	

Table 4.3. Select genes differentially expressed at 0°C compared to 20°C in *Polaromonas* sp. Eur3 1.2.1. logFC is log fold change at 0°C as compared to 20°C. Includes COG and KEGG Orthology (KO) assignments.

Gene	Gene Description	Shortname	logFC	COG	KO
Cell wall/membrane and EPS biosynthesis					
2619647124	OmpA-OmpF porin, OOP family	TC.OOP, ompA	5.62	COG2885	K03286
2619646989	Outer membrane lipoprotein SlyB		2.88	COG3133	
2619645993	Undecaprenyl diphosphate synthase	uppS	2.31	COG0020	K00806
2619647584	Uncharacterized protein involved in exopolysaccharide biosynthesis		2.14	COG3206	
2619644456	Putative colanic acid biosynthesis UDP-glucose lipid carrier transferase	wcaJ	1.98	COG2148	K03606
2619647025	Membrane-bound lytic murein transglycosylase B	mltB	1.91	COG2951	K08305
2619645090	UDP-N-acetylmuramyl tripeptide synthase	murE	1.74	COG0769	K01928
2619645525	Glycosyltransferase, catalytic subunit of cellulose synthase and poly-beta-1,6-N-acetylglucosamine synthase		1.68	COG1215	
Transporters					
2619647128	Tripartite-type tricarboxylate transporter, receptor component TctC	tctC	5.16	COG3181	K07795
2619645035	Magnesium/Calcium-transporting ATPase (P-type)		4.49	COG0474	K01537
2619646220	Predicted arabinose efflux permease, MFS family		3.59	COG2814	
2619646852	TRAP-type C4-dicarboxylate transport system, periplasmic component		3.32	COG1638	
2619643636	ABC-type sulfate transport system, periplasmic component	cysP, sbp	2.89	COG1613	K02048
2619645167	Tripartite-type tricarboxylate transporter, receptor component TctC		2.40	COG3181	
2619646850	TRAP-type C4-dicarboxylate transport system, small permease component		2.32	COG3090	
2619643741	Polar amino acid transport system ATP-binding protein	ABC.PA.A	2.11		K02028
2619645443	ABC-type polar amino acid transport periplasmic component	ABC.PA.S	2.10	COG0834	K02030
Carbon, energy, and co-enzyme metabolism					
2619646396	Tetrathionate reductase subunit A	ttrA	5.69		K08357
2619646394	Tetrathionate reductase subunit B	ttrB	4.60	COG0437	K08358
2619647875	glucose-6-phosphate 1-epimerase		3.51	COG0676	K01792
2619646395	Tetrathionate reductase subunit C	ttrC	2.88		K08359
2619645020	Cytochrome c553		2.56	COG2863	

2619645992	Mannose-6-phosphate isomerase, cupin superfamily		2.41	COG0662	
2619644322	Malate/lactate dehydrogenase	mdh	2.39	COG0039	K00024
2619645995	Mannose-6-phosphate isomerase, cupin superfamily		2.33	COG0662	
2619646941	NADH:ubiquinone oxidoreductase subunit 11 or 4L (chain K)	nuoK	2.07	COG0713	K00340
2619647564	Fe-S cluster assembly iron-binding protein IscA	iscA	1.95	COG0316	K13628
2619646526	Fe-S cluster biogenesis protein NfuA, 4Fe-4S-binding domain		1.66	COG0694	
2619647563	NifU homolog involved in Fe-S cluster formation	iscU, nifU	1.57	COG0822	K04488
2619646928	Triosephosphate isomerase	tpiA	1.97	COG0149	K01803
2619646935	NADH:ubiquinone oxidoreductase 24 kD subunit E	nuoE	1.93	COG1905	K00334
2619644324	Succinate dehydrogenase/fumarate reductase, cytochrome b subunit	sdhC, frdC	1.78	COG2009	K00241
2619646939	Formate hydrogenlyase subunit 6/NADH:ubiquinone oxidoreductase subunit I	nuoI	1.75	COG1143	K00338
2619646934	NADH:ubiquinone oxidoreductase subunit D	nuoD	1.72	COG0649	K00333
2619645466	FoF1-type ATP synthase, membrane subunit c/Archaeal/vacuolar-type H+-ATPase, subunit K	ATPF0C, atpE	1.69	COG0636	K02110
2619646938	NADH:ubiquinone oxidoreductase subunit 1 (H)	nuoH	1.67	COG1005	K00337
2619646943	NADH:ubiquinone oxidoreductase subunit 4 (M)	nuoM	1.67	COG1008	K00342
2619645461	FoF1-type ATP synthase, beta subunit	ATPF1B, atpD	1.66	COG0055	K02112
2619645462	FoF1-type ATP synthase, gamma subunit	ATPF1G, atpG	1.64	COG0224	K02115
2619646411	Thiosulfate dehydrogenase	tsdA	1.56	COG3258	K19713
2619646937	NADH dehydrogenase/NADH:ubiquinone oxidoreductase subunite G	nuoG	1.54	COG1034	K00336
2619646933	NADH:ubiquinone oxidoreductase subunit C	nuoC	1.53	COG0852	K00332
2619643796	Glucose-6-phosphate isomerase	GPI, pgi	1.53	COG0166	K01810
Signal transduction and motility					
2619646417	Two-component response regulator, FixJ family, REC and HTH domains		3.45	COG4566	
2619646293	cAMP-binding domain of CRP or a regulatory subunit of cAMP-dependent protein kinases		2.95	COG0664	
2619645446	CBS domain		2.86	COG0517	
2619646418	Two-component system, LuxR family, sensor histidine kinase TtrS	ttrS	2.74		K13040
2619645212	Two-component system, response regulator	rssB, hnr	2.57		K02485
2619647120	cAMP-binding domain of CRP or a regulatory subunit of cAMP-dependent		2.23	COG0664	

	protein kinases				
Translation, ribosomes, helicases, chaperones					
2619646416	Thiol:disulfide interchange protein DsbG	dsbG	5.85	COG1651	K03805
2619646415	Thiol-disulfide isomerase or thioredoxin		4.07	COG0526	
2619646079	Ribosomal protein S7	RP-S7, rpsG	2.51	COG0049	K02992
2619645369	Ribosomal protein S14	RP-S14, rpsN	2.44	COG0199	K02954
2619646414	Thiol:disulfide interchange protein DsbD	dsbD	2.74	COG4232	K04084
2619645361	Translation initiation factor IF-1	infA	2.10	COG0361	K02518
2619646692	Superfamily II DNA and RNA helicase		2.04	COG0513	
Osmoregulation, stress, and oxidative responses					
2619646962	Osmotically inducible lipoprotein	osmB	3.83		K04062
2619647233	Nucleotide-binding universal stress protein, UspA family	uspA	3.42	COG0589	
2619647880	Catalase	katE, CAT, catB	1.95	COG0753	
2619646406	Catalase	katE, CAT, catB	1.84	COG0753	
2619646404	Cytochrome c peroxidase		1.70	COG1858	
Transcription					
2619647902	DNA-binding transcriptional regulator, Nac, LysR family	lysR, nac	5.19	COG0583	K19338
2619644323	DNA-binding transcriptional regulator, GntR family	GntR	3.80	COG2188	K03710
2619646876	DNA-binding transcriptional regulator, IclR family		2.59	COG1414	
2619645166	DNA-binding transcriptional regulator, LysR family		2.15	COG0583	
2619646581	DNA-binding response regulator, NarL/FixJ family, contains REC and HTH domains		1.55	COG2197	
Transposons					
2619646664	Transposase and inactivated derivatives, IS5 family		1.86	COG3039	K07481
2619647232	Transposase and inactivated derivatives, IS5 family		1.80	COG3039	K07481
2619644443	Plasmid stabilization system protein ParE		1.74	COG3668	
2619647514	Transposase and inactivated derivatives, IS5 family		1.70	COG3039	K07481
2619647785	Transposase and inactivated derivatives, IS5 family		1.63	COG3039	K07481
2619645329	Transposase and inactivated derivatives, IS5 family		1.60	COG3039	K07481

Table 4.4. Genomically-redundant differentially-expressed genes in *Rhodococcus* sp. JG-3 and *Polaromonas* sp. Eur3 1.2.1. logFC = log fold change as compared to the higher temperature culture. Fold changes (right column; logFC) for differentially expressed genes under each function description are separated by a forward slash and correspond to the given gene ID in the same order (left column).

***Rhodococcus* sp. JG-3**	
Description, COG/KO, Gene ID	**logFC**
Acyl dehydratase, COG2030	
2529298888/2529302388	-2.39/2.30
Catalase-peroxidase katG, COG0376/K03782	
2529300023/2529300594	-5.95/3.17
ABC di-, oligo-peptide/nickel transport, permease, COG0601/K02033	
2529299546/2529302471	-4.57/5.16
Fe2+ or Zn2+ uptake regulation protein, fur, COG0735/K03711	
2529300022/2529300593	-4.40/4.49
DNA-binding transcriptional regulator, AcrR family, COG1309	
2529299557/2529298884/2529299633	-5.36/-2.75/2.01
2529302950/2529299074/2529299715/2529301247	2.27/2.51/3.24/5.21
RNA polymerase sigma-70 factor, ECF subfamily, COG1595/K03088	
2529302703/2529303412	-1.91/5.51
DNA-binding transcriptional regulator, MarR family, COG1846	
2529302179/2529298833/2529299065	-2.27/2.96/5.73
ADP-ribosylglycohydrolase, COG1397	
2529302519/2529302299	-5.19/5.16
Flavin-dependent oxidoreductase, luciferase family, COG2141	
2529299256/2529298656	-4.56/-3.05
2529299395/2529300234/2529302665	2.55/2.76/4.73
***Polaromonas* sp. Eur3 1.2.1**	
Description, COG/KO	**logFC**
2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway), COG0179	
2619646842/2619645902/2619646695	1.81/-2.42/-3.12
Tripartite-type tricarboxylate transporter, receptor, TctC, COG3181	
2619647128/2619645167	5.16/2.40
2619646693/2619647684	-2.30/-4.11
Thiol-disulfide isomerase or thioredoxin, COG0526	
2619646415/2619646606	4.07/-2.54
Nucleotide-binding universal stress protein, UspA family, COG0589	
2619647233/2619644545/2619647313	3.42/-2.38/-5.54
CRP cAMP-binding domain or regulatory unit cAMP kinases, COG0664	
2619646293/2619647120/2619644878	2.95/2.23/-1.81

ABC-type polar A.A. transport, periplasmic component, COG0834	
2619645443/2619646238	2.10/-3.76
TRAP-type C4-dicarboxylate transport, small permease, COG3090	
2619646850/2619647206	2.32/-2.08

4.7 Supplementary Materials

Supplementary Figure 1

Supplementary Tables 1-3

Figure S4.1. Volcano plot showing differentially expressed genes (log2 fold change) at low temperatures that are statistically significant ($p < 0.05$) in: A) *Rhodococcus* sp. JG3 and B) *Polaromonas* sp. Eur3 1.2.1. Negative log 2-fold change are downregulated at low temperatures, while positive points represent upregulated genes, as compared to the higher temperatures. Red dots represent expressed genes with log2 fold changes > 5 for *Rhodococcus* sp. JG3 and > 4 for *Polaromonas* sp. Eur3 1.2.1 at the cold temperatues; orange dots represent gene transcripts at cold temperatures with highly significant fold changes (-log10(pvalue) > 15 for JG3 and > 10 for Eur3), and green dots represent genes that correspond to both categories.

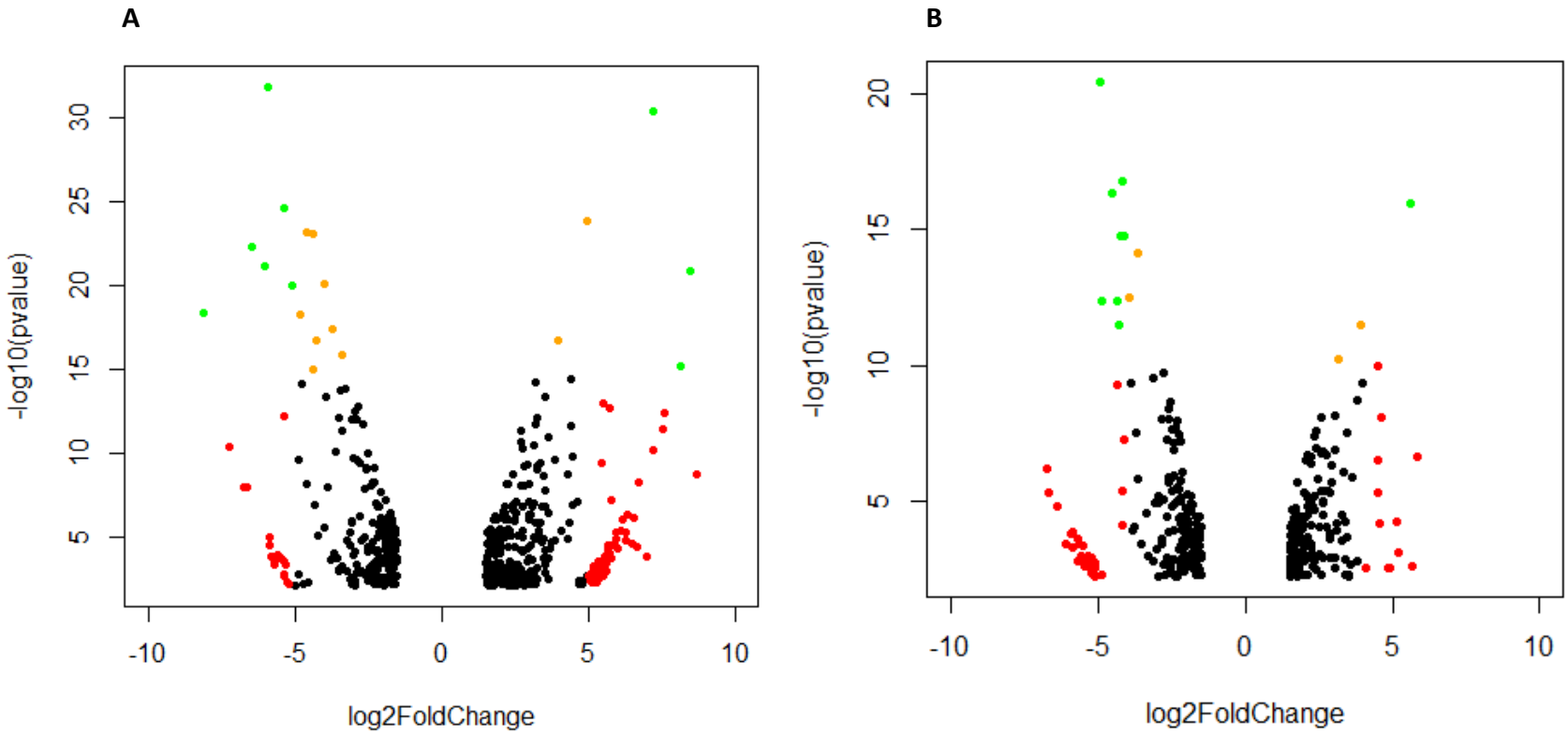

A
-log10(pvalue)
5
10
15
20
25
30
-10
-5
0
5
10
log2FoldChange
B
-log10(pvalue)
5
10
15
20
-10
-5
0
5
10
log2FoldChange

Table S4.1. Sequencing summary for all samples used in this study. Letters in the sample name (A, B, C, and D) represent a specific growth condition, each sampled in triplicate. A = *Polaromonas* sp Eur3 1.2.1 at 20°C; B = *Polaromonas* sp Eur3 1.2.1 at 0°C; C = *Rhodococcus* sp. JG3 at 25°C; D = *Rhodococcus* sp. JG3 at -5°C

Name	Sample Name	Raw Fragments	Surviving Fragments	Surviving Fragments%	Surviving Single	Total Reads QCed	mapped	mapped%	Properly Paired	Properly Paired%
Pola	A1_S1_L001	558,396	540,271	96%	119	1,077,884	441,110	40%	427,206	39%
Pola	A2_S2_L001	333,878	330,776	99%	163	659,318	279,583	42%	266,218	40%
Pola	A3_S3_L001	1,570,954	1,553,501	98%	275	3,071,830	1,274,738	41%	1,219,650	39%
Pola	B4_S4_L001	316,915	315,531	99%	46	628,998	313,978	49%	297,224	47%
Pola	B5_S5_L001	273,550	270,447	98%	56	538,694	281,568	52%	262,892	48%
Pola	B7_S6_L001	630,322	625,343	99%	55	1,241,862	540,485	43%	508,496	40%
Rhodo	C1_S7_L001	157,968	129,930	82%	26	255,956	173,352	67%	170,482	66%
Rhodo	C2_S8_L001	292,608	272,733	93%	65	541,856	407,084	75%	402,236	74%
Rhodo	C3_S9_L001	195,126	193,145	98%	16	384,022	291,804	75%	286,452	74%
Rhodo	D1_S10_L001	267,891	259,603	96%	78	512,126	345,896	67%	340,234	66%
Rhodo	D2_S11_L001	128,679	126,765	98%	15	251,742	164,110	65%	159,418	63%
Rhodo	D3_S12_L001	126,551	125,573	99%	14	249,964	179,168	71%	171,820	68%
		4,852,838	4,743,618		928	9,414,252	4,692,876		4,512,328	
raw bp	727.93									

Table S4.2. Additional genes differentially regulated at -5°C compared to 25°C in *Rhodococcus* sp. JG3.

Gene	Gene Description	Shortname	logFC	COG	KO
Amino acid metabolism					
2529301108	Acetolactate synthase, small subunit	E2.2.1.6S, ilvH, ilvN	2.75	0440	K01653
2529299490	Dihydroxyacid dehydratase/phosphogluconate dehydratase	ilvD	2.44	0129	K01687
2529301808	Glutamate synthase domain 2	gltB	2.40	0069	K00265
2529301109	Ketol-acid reductoisomerase	ilvC	2.39	0059	K00053
2529298629	ABC-type dipeptide/oligopeptide/nickel transport system, perme	oppC	2.22	1173	K15582
2529300014	1-aminocyclopropane-1-carboxylate deaminase/D-cysteine desulf	E3.5.99.7	2.16	2515	K01505
2529298751	Tryptophan 2,3-dioxygenase	E1.13.11.11, TDO2, kynA	2.14	3483	K00453
2529299782	Serine acetyltransferase	cysE	1.99	1045	K00640
2529302280	D-serine deaminase, pyridoxal phosphate-dependent		1.72	3616	
2529299577	Threonine dehydrogenase or related Zn-dependent dehydrogenase	SORD, gutB	1.68	1063	K00008
2529298750	Kynureninase	KYNU, kynU	1.63	3844	K01556
2529299933	Alanine dehydrogenase	ald	-4.74	0686	K00259
2529300105	1-pyrroline-5-carboxylate dehydrogenase		-4.57	1012	K00294
2529300636	Glutamate/leucine/valine dehydrogenase	vdh	-4.34	0334	K00271
2529298885	3-methylcrotonyl-CoA carboxylase beta subunit		-4.25	4799	K01969
2529303137	Prolyl oligopeptidase PreP, S9A serine peptidase family		-2.29	1505	K01322
2529299129	4-aminobutyrate aminotransferase or related aminotransferase		-2.24	0160	K03918

2529299938	Dipeptidyl aminopeptidase/acylaminoacyl peptidase		-2.10	1506	
2529300603	Threonine dehydrogenase or related Zn-dependent dehydrogenase		-1.87	1063	K00100
2529302122	4-aminobutyrate aminotransferase or related aminotransferase		-1.75	0160	K00836
Carbohydrate transport and metabolism					
2529299881	Phosphoketolase	xfp, xpk	4.42	3957	K01621
2529302279	2-dehydro-3-deoxygluconokinase	kdgK	3.72	0524	K00874
2529302262	Glucan/starch phosphorylase	glgP, PYG	3.67	0058	K00688
2529300073	Beta-phosphoglucomutase or related phosphatase, HAD superfamil		3.28	0637	
2529302373	Sugar phosphate isomerase/epimerase		3.19	1082	
2529302770	Glycosyltransferase, GT2 family		2.92	1216	
2529301505	Glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate	GAPDH, gapA	1.70	0057	K00134
2529300360	Glucose-6-phosphate isomerase	GPI, pgi	1.56	0166	K01810
2529299576	Mannitol-1-phosphate/altronate dehydrogenases	E1.1.1.67, mtlK	1.57	0246	K00045
2529298889	Citrate lyase beta subunit	citE	-5.69	2301	K01644
2529301342	1-phosphofructokinase	fruK, PfkB	-3.50	1105	K00882
2529299505	N-acetylglucosaminyl deacetylase, LmbE family		-3.39	2120	
2529303035	Glucose/arabinose dehydrogenase, beta-propeller fold		-3.13	2133	
2529303109	D-arabinose 1-dehydrogenase, Zn-dependent alcohol dehydrogenas		-2.37	1064	K13979
2529301213	Predicted arabinose efflux permease, MFS family		-1.79	2814	
2529298736	Predicted arabinose efflux permease, MFS family		-1.73	2814	
Cell cycle control, cell division, chromosome partitioning					

2529301194	Cell division protein FtsI/penicillin-binding protein 2		-5.60	0768	
2529302119	Fluoride ion exporter CrcB/FEX, affects chromosome condensatio		-2.88	0239	K06199
Cell wall/membrane/envelope biogenesis					
2529300085	2,4-dienoyl-CoA reductase or related NADH-dependent reductase,		4.97	1902	
2529302896	Phospholipid/cholesterol/gamma-HCH transport system substrate-binding protein	mlaD	3.00	1463	K02067
2529302895	Phospholipid/cholesterol/gamma-HCH transport system substrate-binding protein		2.60	1463	
2529302893	Phospholipid/cholesterol/gamma-HCH transport system substrate-binding protein	mlaD, linM	2.45	1463	K02067
2529302897	Phospholipid/cholesterol/gamma-HCH transport system substrate-binding protein	mlaD, linM	1.61	1463	K02067
2529303392	Apolipoprotein N-acyltransferase	Int	-5.68	0815	K03820
2529302696	Membrane-bound lytic murein transglycosylase B		-3.08	2951	
2529301886	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	murG	-3.08	0707	K02563
2529302604	Anionic cell wall polymer biosynthesis enzyme, LytR-Cps2A-Psr		-2.92	1316	
2529303387	Murein DD-endopeptidase MepM and murein hydrolase activator Nl		-2.72	0739	
2529301592	AAA+-type ATPase, SpoVK/Ycf46/Vps4 family		-1.94	0464	K13527
2529300019	Glycosyltransferase involved in cell wall bisynthesis		-1.53	0438	
Coenzyme transport and metabolism					
2529300276	Pimeloyl-ACP methyl ester carboxylesterase		4.77	0596	
2529302665	Flavin-dependent oxidoreductase, luciferase family (includes a		4.73	2141	
2529300146	Aspartate 1-decarboxylase	panD	3.11	0853	K01579
2529301838	Aspartate oxidase	nadB	3.06	0029	K00278

2529299832	Nicotinamidase-related amidase		3.04	1335	
2529300234	Flavin-dependent oxidoreductase, luciferase family (includes a		2.76	2141	
2529299395	Flavin-dependent oxidoreductase, luciferase family (includes a		2.55	2141	
2529301839	Quinolinate synthase	nadA	1.81	0379	K03517
2529302958	Pimeloyl-ACP methyl ester carboxylesterase		1.74	0596	
2529300910	Glutathione synthase/RimK-type ligase, ATP-grasp superfamily		1.57	0189	
2529300026	NH3-dependent NAD+ synthetase	nadE	-6.05	0171	K01916
2529299256	Flavin-dependent oxidoreductase, luciferase family (includes alkanesulfonate monooxygenase SsuD and methylene tetrahydromethanopterin reductase)		-4.56	2141	
2529301905	Geranylgeranyl pyrophosphate synthase	idsA	-4.56	0142	K13787
2529298656	Flavin-dependent oxidoreductase, luciferase family (includes alkanesulfonate monooxygenase SsuD and methylene tetrahydromethanopterin reductase)		-3.05	2141	
2529300025	Nicotinamide mononucleotide (NMN) deamidase PncC		-3.00	1546	K03743
2529302712	Dihydropteroate synthase		-2.33	0294	K00796
2529302107	Phosphoglycerate dehydrogenase or related dehydrogenase		-1.54	0111	
Defense mechanisms					
2529301356	Alkyl hydroperoxide reductase subunit AhpC (peroxiredoxin)	E1.11.1.15, PRDX, ahpC	1.67	0450	K03386
2529298994	S-formylglutathione hydrolase FrmB	fbp	1.67	0627	K18851
2529302178	ABC-type multidrug transport system, ATPase and permease compo		-2.31	1132	K06147
2529298479	Organic hydroperoxide reductase OsmC/OhrA		-1.82	1764	K04063
Energy production and conversion					
2529300679	Alcohol dehydrogenase, class IV		5.78	1454	

2529300685	Anaerobic selenocysteine-containing dehydrogenase		5.77	0243	
2529300706	Acyl-CoA reductase or other NAD-dependent aldehyde dehydrogena	aldB	2.83	1012	K00138
2529302461	Acyl-CoA reductase or other NAD-dependent aldehyde dehydrogena	gabD	2.71	1012	K00135
2529301126	Glycerol-3-phosphate dehydrogenase	gpsA	2.53	0240	K00057
2529299108	NADP-dependent 3-hydroxy acid dehydrogenase YdfG		2.37	4221	
2529303160	Quinol monooxygenase YgiN		2.28	1359	
2529300269	Citrate synthase	CS, gltA	1.69	0372	K01647
2529298839	Glycerol-3-phosphate dehydrogenase	glpA, glpD	1.63	0578	K00111
2529302587	NAD/NADP transhydrogenase alpha subunit	pntA	-5.87	3288	K00324
2529299416	Ferredoxin-NADP reductase		-5.60	1018	
2529298812	Glycolate oxidase	glcD	-3.50	0277	K00104
2529299710	Pyruvate dehydrogenase complex, dehydrogenase (E1) component	aceE	-3.39	2609	K00163
2529303393	Cytochrome c biogenesis protein ResB	resB, ccs1	-3.11	1333	K07399
2529300639	Pyruvate/2-oxoglutarate/acetoin dehydrogenase complex, dehydro		-2.96	0022	K00162
2529303388	Cytochrome c oxidase assembly factor CtaG		-2.86	3336	K07245
2529300192	ABC-type transport system involved in cytochrome bd biosynthes		-2.79	4987	K16012
2529302653	FAD/FMN-containing dehydrogenase		-2.72	0277	K00103
2529302644	CO or xanthine dehydrogenase, Mo-binding subunit		-2.43	1529	K11177
2529300638	TPP-dependent pyruvate or acetoin dehydrogenase subunit alpha		-2.30	1071	K00161
2529299426	NADPH:quinone reductase or related Zn-dependent oxidoreductase		-2.26	0604	K00344
2529303224	Isocitrate/isopropylmalate dehydrogenase		-1.97	0473	K07246

2529302187	Ferredoxin-NADP reductase		-1.94	1018	
2529298805	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamid		-1.73	0508	K00627
2529303384	Heme/copper-type cytochrome/quinol oxidase, subunit 1		-1.72	0843	K02274
2529300640	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamid		-1.67	0508	K00627
2529299617	Phosphoenolpyruvate carboxykinase, GTP-dependent		-1.52	1274	K01596
Inorganic ion transport and metabolism					
2529298548	Arsenite efflux pump ArsB, ACR3 family		4.73	0798	
2529301234	ABC-type molybdate transport system, periplasmic component	modA	4.73	0725	K02020
2529300197	3-mercaptopyruvate sulfurtransferase SseA, contains two rhodan	sseA, TST, MPST	3.23	2897	K01011
2529300708	Divalent metal cation (Fe/Co/Zn/Cd) transporter		3.04	0053	
2529302085	ABC-type sulfate transport system, periplasmic component	cysP	3.11	1613	K02048
2529300395	Mg2+ and Co2+ transporter CorA	corA	2.01	0598	K03284
2529300990	Cyanate permease	MFS.CP	1.54	2807	K03449
2529298976	Membrane protein TerC, possibly involved in tellurium resistan		-2.92	0861	K05794
2529299035	Ferritin		-2.08	1528	
Intracellular trafficking, secretion, and vesicular transport					
2529301447	Preprotein translocase subunit SecF	secF	1.54	0341	K03074
Lipid transport and metabolism					
2529299636	Acetyl-CoA acetyltransferase	atoB	4.90	0183	K00626
2529299781	Lysophospholipase L1 or related esterase		2.06	2755	
2529301248	1-acyl-sn-glycerol-3-phosphate acyltransferase	plsC	1.77	0204	K00655

2529301920	Long-chain acyl-CoA synthetase (AMP-forming)	ACSL, fadD	1.66	1022	K01897
2529298861	Formyl-CoA transferase	frc	-4.57	1804	K07749
2529298818	Acetyl-CoA acetyltransferase		-4.02	0183	K00680
2529298819	Enoyl-CoA hydratase/carnithine racemase		-3.95	1024	
2529298886	Acetyl/propionyl-CoA carboxylase, alpha subunit	bccA	-3.76	4770	K11263
2529298887	Acyl-CoA dehydrogenase related to the alkylation response prot		-3.13	1960	K00257
2529300808	Choline dehydrogenase or related flavoprotein		-2.60	2303	K03333
2529298888	Acyl dehydratase		-2.39	2030	
2529301317	Phosphatidylglycerophosphate synthase		-2.18	0558	K00995
2529299272	Myo-inositol-1-phosphate synthase		-1.92	1260	K01858
2529301182	CDP-diglyceride synthetase		-1.92	0575	K00981
2529302186	Fatty acid desaturase		-1.86	3239	K00508
2529298657	NAD(P)-dependent dehydrogenase, short-chain alcohol dehydrogen		-1.72	1028	
2529298703	3-hydroxyacyl-CoA dehydrogenase		-1.59	1250	K01782
2529302220	NAD(P)-dependent dehydrogenase, short-chain alcohol dehydrogen		-1.58	1028	K00540
2529298540	Acyl-CoA dehydrogenase related to the alkylation response prot		-1.58	1960	K00249
Nucleotide transport and metabolism					
2529302300	ADP-ribose pyrophosphatase YjhB, NUDIX family		3.15	1051	
2529300862	Cytidine deaminase	cdd, CDA	2.78	0295	K01489
2529301730	Cytidylate kinase	cmk	1.90	0283	K00945
2529302406	ppGpp synthetase catalytic domain (RelA/SpoT-type nucleotidylt		1.57	2357	

2529301063	Ribonucleotide reductase beta subunit, ferritin-like domain		-2.12	0208	K00526
2529301060	Protein involved in ribonucleotide reduction		-1.97	1780	K03647
2529301061	Ribonucleotide reductase alpha subunit		-1.68	0209	K00525
Osmoregulation					
2529301957	ABC-type proline/glycine betaine transport system, permease co	opuBD	2.40	1174	K05846
Posttranslational modification, protein turnover, chaperones					
2529300820	Protein-S-isoprenylcysteine O-methyltransferase Ste14		3.43	2020	
2529303158	Metal-sulfur cluster biosynthetic enzyme		3.28	2151	
2529300799	Co-chaperonin GroES (HSP10)	groES	3.25	0234	K04078
2529303135	Zn-dependent protease with chaperone function		2.53	0501	
2529300800	Chaperonin GroEL (HSP60 family)	groEL, HSPD1	2.16	0459	K04077
2529303164	Chaperonin GroEL (HSP60 family)	groEL, HSPD1	1.84	0459	K04077
2529302154	ATP-dependent protease ClpP, protease subunit	clpP, CLPP	1.80	0740	K01358
2529301523	Fe-S cluster assembly scaffold protein SufB	sufB	1.71	0719	K09014
2529299191	Peptidyl-prolyl cis-trans isomerase (rotamase) - cyclophilin f	PPIA	1.70	0652	K03767
2529303394	ABC-type transport system involved in cytochrome c biogenesis,		-5.83	0755	
2529300021	Gamma-glutamyl:cysteine ligase YbdK, ATP-grasp superfamily	ybdK	-5.36	2170	K06048
2529302519	ADP-ribosylglycohydrolase		-5.19	1397	
2529301852	NAD-dependent protein deacetylase, SIR2 family		-2.98	0846	K12410
2529303174	Thioredoxin reductase		-2.57	0492	K00384
2529299466	ATP-dependent Lon protease, bacterial type		-2.53	0466	K01338

2529302124	NAD-dependent protein deacetylase, SIR2 family		-1.58	0846	
Replication, recombination and repair					
2529302415	Predicted ATP-dependent endonuclease of the OLD family, contai		1.89	3593	
2529302709	3-methyladenine DNA glycosylase Tag		-2.21	2818	K01246
Secondary metabolites biosynthesis, transport and catabolism					
2529299331	Catechol 2,3-dioxygenase or other lactoylglutathione lyase fam		5.62	0346	
2529298397	Taurine dioxygenase, alpha-ketoglutarate-dependent	tauD	5.53	2175	K03119
2529300596	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid		4.35	0179	
2529300141	Non-ribosomal peptide synthetase component F		3.84	1020	
2529299792	Isopenicillin N synthase and related dioxygenases		2.84	3491	
2529299150	Phenolic acid decarboxylase	pdc	2.75	3479	K13727
2529300142	Non-ribosomal peptide synthetase component F		2.14	1020	
2529298797	Homogentisate 1,2-dioxygenase	HGD, hmgA	1.88	3508	K00451
2529301617	Catechol 2,3-dioxygenase or other lactoylglutathione lyase fam		1.75	0346	
2529298824	1,2-phenylacetyl-CoA epoxidase, catalytic subunit		-2.38	3396	K02611
2529298814	Acyl-coenzyme A thioesterase PaaI, contains HGG motif		-2.15	2050	K02614
2529299513	Cytochrome P450		-2.10	2124	K00517
Signal transduction mechanisms					
2529300535	Signal transduction histidine kinase		4.80	0642	
2529299858	EAL domain, c-di-GMP-specific phosphodiesterase class I (or it		2.74	2200	
2529299877	DNA-binding response regulator, NarL/FixJ family, contains REC	devR	2.32	2197	K07695

2529301683	Forkhead associated (FHA) domain, binds pSer, pThr, pTyr		1.82	1716	
2529301153	Nitrogen regulatory protein PII	glnB	1.75	0347	K04751
2529299483	Adenylate cyclase, class 3	E4.6.1.1	1.69	2114	K01768
2529300020	Anti-anti-sigma regulatory factor (antagonist of anti-sigma fa		-4.24	1366	
2529300028	Anti-anti-sigma regulatory factor (antagonist of anti-sigma fa		-1.86	1366	
2529299913	Anti-anti-sigma regulatory factor (antagonist of anti-sigma fa		-1.62	1366	
2529300101	Anti-anti-sigma regulatory factor (antagonist of anti-sigma fa		-1.61	1366	
Transcription					
2529303125	DNA-binding transcriptional regulator, XRE-family HTH domain		5.02	1476	K07729
2529298833	DNA-binding transcriptional regulator, MarR family		2.96	1846	
2529302323	DNA-binding transcriptional regulator, ArsR family	arsR	2.75	0640	K03892
2529300496	DNA-binding transcriptional regulator, LysR family		2.66	0583	
2529299074	DNA-binding transcriptional regulator, AcrR family		2.51	1309	
2529302950	DNA-binding transcriptional regulator, AcrR family		2.27	1309	
2529298898	DNA-binding transcriptional regulator, LysR family		2.18	0583	
2529299633	DNA-binding transcriptional regulator, AcrR family		2.01	1309	
2529300506	DNA-binding transcriptional regulator, LysR family		1.94	0583	
2529302580	DNA-binding transcriptional regulator, GntR family	K03710	1.88	2188	K03710
2529298519	DNA-binding transcriptional regulator, GntR family		1.68	1802	
2529299557	DNA-binding transcriptional regulator, AcrR family		-5.36	1309	
2529298828	AraC-type DNA-binding domain and AraC-containing proteins		-4.58	2207	

2529298884	DNA-binding transcriptional regulator, AcrR family		-2.75	1309	
2529299273	DNA-binding transcriptional regulator, PadR family		-2.40	1695	
2529302179	DNA-binding transcriptional regulator, MarR family		-2.27	1846	
2529301343	DNA-binding transcriptional regulator of sugar metabolism, Deo		-2.19	1349	K03436
2529298615	Cold shock protein, CspA family		-2.18	1278	K03704
2529302703	DNA-directed RNA polymerase specialized sigma subunit, sigma24		-1.91	1595	K03088
2529302008	Cold shock protein, CspA family		-1.64	1278	
2529299115	DNA-binding transcriptional regulator, ArsR family		-1.63	0640	
Translation, ribosomes, helicases					
2529301179	Translation elongation factor EF-Ts	tsf, TSFM	1.87	0264	K02357
2529300770	Ribosomal protein L17	RP-L17, MRPL17, rplQ	1.84	0203	K02879
2529299296	Ribosomal protein S6	RP-S6, MRPS6, rpsF	1.77	0360	K02990
2529302903	Ribosomal protein L10	RP-L10, MRPL10, rplJ	1.71	0244	K02864
2529301875	Isoleucyl-tRNA synthetase	IARS, ileS	1.66	0060	K01870
2529300767	Ribosomal protein S11	RP-S11, MRPS11, rpsK	1.65	0100	K02948
2529300383	Ribosomal protein S18	RP-S18, MRPS18, rpsR	1.63	0238	K02963
2529299299	Ribosomal protein L9	RP-L9, MRPL9, rplI	1.57	0359	K02939
2529300754	Ribosomal protein S8	RP-S8, rpsH	1.56	0096	K02994
2529301767	Ribosomal protein L20	RP-L20, MRPL20, rplT	1.55	0292	K02887

2529300738	Ribosomal protein S19	RP-S19, rpsS	1.53	0185	K02965
2529301332	tRNA A37 methylthiotransferase MiaB		-1.53	0621	K06168

Table S4.3. Additional genes differentially regulated at 0°C compared to 20°C in *Polaromonas* sp. Eur3 1.2.1.

Gene	Gene Description	Shortname	logFC	COG	KO
Amino acid transport and metabolism					
2619646948	Glutamate dehydrogenase/leucine dehydrogenase	gdhA	1.79	0334	K00261
2619646216	Kynurenine formamidase		1.68	1878	
2619647247	Archaeal aspartate aminotransferase or a related aminotransferase, includes purine catabolism protein PucG		1.56	0075	
2619647679	oxalyl-CoA decarboxylase	oxc	-3.96	0028	K01577
2619643647	ATP phosphoribosyltransferase regulatory subunit HisZ	hisZ	-2.58	3705	K02502
2619645801	Xaa-Pro aminopeptidase	pepP	-2.32	0006	K01262
2619643644	Cystathionine beta-lyase/cystathionine gamma-synthase	metC	-2.12	0626	K01760
2619645859	3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase	aroF, aroG, aroH	-2.01	0722	K01626
2619647674	NADPH-dependent glutamate synthase beta chain or related oxidoreductase	fdoG, fdfH	-1.96	0493	K00123
2619645815	Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase or related deacylase		-1.92	0624	
2619646235	ABC-type amino acid transport system, permease component	ABC.PA.P	-1.78	0765	K02029
2619644380	Argininosuccinate lyase	argH	-1.54	0165	K01755
Carbohydrate transport and metabolism					
2619644232	Phosphomannomutase	manB	-1.61	1109	K01840
2619645656	Pyruvate kinase	pyk	-1.56	0469	K00873
Cell cycle control, cell division, chromosome partitioning					
2619644948	GTP-binding protein EngB required for normal cell division	engB	-2.19	0218	K03978
Cell motility					
2619643841	Flp pilus assembly protein, secretin CpaC	cpaC, rcpA	-6.10	4964	K02280
2619643846	Flp pilus assembly protein, pilin Flp	flp, pilA	-4.20	3847	K02651

2619643845	Flp pilus assembly protein, pilin Flp	flp, pilA	-4.09	3847	K02651
Cell wall/membrane/envelope biogenesis					
2619644849	D-alanine-D-alanine ligase and related ATP-grasp enzymes	cphA	1.75	1181	K03802
2619646061	ABC-type transporter Mla maintaining outer membrane lipid asymmetry, periplasmic component MlaD	mlaD, linM	1.54	1463	K02067
2619644269	Outer membrane protein OmpA and related peptidoglycan-associated (lipo)proteins		1.50	2885	
2619646018	Alanine racemase	alr	-5.24	0787	K01775
2619645404	Putative effector of murein hydrolase		-5.12	1346	
2619643705	Lipid A disaccharide synthetase	lpxB	-4.34	0763	K00748
2619645251	dTDP-4-dehydrorhamnose 3,5-epimerase	rfbC	-2.81	1898	K01790
2619645698	O-antigen ligase		-2.41	3307	
2619646670	Outer membrane translocation and assembly module TamA	tamA	-2.24	0729	K07278
2619643706	Acyl-[acyl carrier protein]--UDP-N-acetylglucosamine O-acyltransferase	lpxA	-2.23	1043	K00677
2619644424	Glycosyltransferase involved in cell wall bisynthesis		-1.69	0438	
2619646077	D-alanyl-D-alanine carboxypeptidase	dacC, dacA, dacD	-1.67	1686	K07258
Coenzyme transport and metabolism					
2619646403	Non-heme chloroperoxidase	cpo	2.03	0596	K00433
2619647644	Molybdopterin synthase sulfur carrier subunit	moaD	-3.63	1977	K03636
2619646734	Riboflavin kinase / FMN adenylyltransferase	ribF	-2.29	0196	K11753
2619646633	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases		-2.21	0654	
2619646694	Pimeloyl-ACP methyl ester carboxylesterase		-2.14	0596	
2619647643	Molybdopterin molybdotransferase	moeA	-2.03	0303	K03750
2619645550	Glutathione synthase/RimK-type ligase, ATP-grasp superfamily	gshB	-1.73	0189	K01920
2619644467	NAD(P)H-flavin reductase	ascD, ddhD, rfbI	-1.55	0543	K00523

Defense mechanisms					
2619643978	type I restriction enzyme, S subunit	hsdS	2.23		K01154
2619643980	Type I site-specific restriction endonuclease, part of a restriction-modification system	hsdR	1.72	4096	K01153
2619645699	mRNA interferase MazF	mazF	-2.50	2337	K07171
2619643826	DNA-damage-inducible protein J	dinJ	-2.21	3077	K07473
2619645700	Antitoxin MazE of the MazEF toxin-antitoxin module	mazE, chpAI	-2.42	2336	K07172
2619645910	Plasmid maintenance system killer protein	higA	-1.79	3549	K07334
2619646234	Enamine deaminase RidA, house cleaning of reactive enamine intermediates, YjgF/YER057c/UK114 family		-1.64	0251	
Energy production and conversion					
2619644108	Cytochrome c551/c552	CYC	1.94	4654	K08738
2619646935	NADH:ubiquinone oxidoreductase 24 kD subunit (chain E)	nuoE	1.93	1905	K00334
2619644324	Succinate dehydrogenase/fumarate reductase, cytochrome b subunit	sdhC, frdC	1.78	2009	K00241
2619646939	Formate hydrogenlyase subunit 6/NADH:ubiquinone oxidoreductase 23 kD subunit (chain I)	nuoI	1.75	1143	K00338
2619646934	NADH:ubiquinone oxidoreductase 49 kD subunit (chain D)	nuoD	1.72	0649	K00333
2619645466	FoF1-type ATP synthase, membrane subunit c/Archaeal/vacuolar-type H+-ATPase, subunit K	ATPF0C, atpE	1.69	0636	K02110
2619646938	NADH:ubiquinone oxidoreductase subunit 1 (chain H)	nuoH	1.67	1005	K00337
2619646943	NADH:ubiquinone oxidoreductase subunit 4 (chain M)	nuoM	1.67	1008	K00342
2619645461	FoF1-type ATP synthase, beta subunit	ATPF1B, atpD	1.66	0055	K02112
2619645462	FoF1-type ATP synthase, gamma subunit	ATPF1G, atpG	1.64	0224	K02115
2619646411	Cytochrome c	tsdA	1.56	3258	K19713
2619646937	NADH dehydrogenase/NADH:ubiquinone	nuoG	1.54	1034	K00336

	oxidoreductase 75 kD subunit (chain G)				
2619646933	NADH:ubiquinone oxidoreductase 27 kD subunit (chain C)	nuoC	1.53	0852	K00332
2619645803	Rubredoxin		-5.84	1773	
2619645601	(Methyl)Malonate-semialdehyde dehydrogenase (acetylating)	mmsA, iolA	-5.59	1012	K00140
2619644823	Phosphoglycolate phosphatase, HAD superfamily	gph	-5.41	0546	K01091
2619647336	FAD/FMN-containing dehydrogenase		-4.37	0277	
2619647684	Tripartite-type tricarboxylate transporter, receptor component TctC		-4.11	3181	
2619646490	Formate dehydrogenase iron-sulfur subunit	fdoH	-3.35	0437	K00124
2619647673	Formate dehydrogenase alpha subunit	fdhA1	-2.84	3383	K05299
2619646693	Tripartite-type tricarboxylate transporter, receptor component TctC		-2.30	3181	
2619645505	Malate synthase	aceB, glcB	-2.22	2225	K01638
2619646097	Cytochrome b		-2.09	3658	
2619645905	FAD/FMN-containing dehydrogenase	dld	-1.95	0277	K00102
2619646075	(2Fe-2S) ferredoxin		-1.66	3411	
2619646450	Anaerobic selenocysteine-containing dehydrogenase		-1.59	0243	
2619643817	Uncharacterized conserved protein		-1.51	3019	
2619644971	Uncharacterized conserved protein YbjT, contains NAD(P)-binding and DUF2867 domains		-1.94	0702	K00356
2619645655	Membrane-associated enzyme, PAP2 (acid phosphatase) superfamily		-1.84	3907	
2619646324	Predicted dinucleotide-utilizing enzyme	nadX	-1.68	1712	K06989
Inorganic ion transport and metabolism					
2619645596	Mg2+ and Co2+ transporter CorA	corA	1.61	0598	K03284
2619645019	Magnesium-transporting ATPase (P-type)	E3.6.3.2, mgtA, mgtB	1.60	0474	K01531
2619645583	Cu2+-exporting ATPase	copB	-6.71	2217	K01533
2619647685	MFS transporter, OFA family, oxalate/formate antiporter	oxlT	-3.90	2223	K08177
2619645690	Membrane protein TerC, possibly involved in tellurium resistance		-3.07	0861	
2619645724	Membrane protein TerC, possibly involved in tellurium resistance		-2.48	0861	

2619645204	Carbonic anhydrase	cynT, can	-2.19	0288	K01673
2619645159	ABC-type phosphate/phosphonate transport system, periplasmic component	phnD	-1.84	3221	K02044
Intracellular trafficking, secretion, and vesicular transport					
2619647110	Type II secretory pathway component GspD/PulD (secretin)	gspD	1.62	1450	K02453
2619644574	Signal recognition particle GTPase	ftsY	-1.65	0552	K03110
Lipid transport and metabolism					
2619647004	3-hydroxyacyl-CoA dehydrogenase	paaH, hbd, fadB, mmgB	1.51	1250	K00074
2619647678	Formyl-CoA transferase	frc	-4.95	1804	K07749
2619647774	Acetyl-CoA acetyltransferase	atoB	-2.83	0183	K00626
2619647676	Formyl-CoA transferase	frc	-2.77	1804	K07749
2619646556	Glycerophosphoryl diester phosphodiesterase	glpQ, ugpQ	-2.42	0584	K01126
2619647138	Phytoene/squalene synthetase		-2.21	1562	
2619647618	Acyl carrier protein		-1.52	0236	
2619647425	Acyl-CoA thioesterase FadM	ybgC	-1.50	0824	K07107
Mobilome: prophages, transposons					
2619644208	comEA; competence protein ComEA	comEA	1.65		K02237
2619646169	Transposase		-2.66	3436	
2619645345	Transposase		-2.26	3436	
2619647498	Transposase		-2.11	3436	
2619647190	Transposase		-2.01	3436	
2619644154	Transposase		-1.99	3436	
2619646282	Transposase (or an inactivated derivative)		-1.87	3316	
2619645851	Transposase		-1.86	3436	
2619645352	Transposase		-1.81	3436	
2619646176	Transposase		-1.76	3436	
2619643799	Transposase		-1.62	3436	
Nucleotide transport and metabolism					
2619646419	Nucleoside phosphorylase	amn	1.98	0775	K01241
2619646615	Ribonucleotide-diphosphate reductase alpha	nrdA, nrdE	-2.14	0209	K00525

	subunit				
Other transporters					
2619646708	ABC-type protease/lipase transport system, ATPase and permease components	hasD, prtD, aprD	-6.70	4618	K12536
2619645428	MFS transporter, DHA1 family, bicyclomycin/chloramphenicol resistance protein	bcr	-2.36	2814	K07552
Posttranslational modification, protein turnover, chaperones					
2619647143	FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor)	tig	1.64	0544	K03545
2619647912	ATP-dependent Lon protease, bacterial type	lon	-5.71	0466	K01338
2619647792	Collagenase-like protease, PrtC family		-3.72	0826	K08303
2619646606	Thiol-disulfide isomerase or thioredoxin		-2.54	0526	
2619644737	Molecular chaperone IbpA, HSP20 family		-2.28	0071	
2619644738	Molecular chaperone IbpA, HSP20 family		-2.18	0071	K13993
2619647767	Membrane protein implicated in regulation of membrane protease activity		-1.93	1585	
2619645681	Glutamine synthetase adenylyltransferase	glnE	-1.91	1391	K00982
2619644385	Periplasmic serine protease, S1-C subfamily, contain C-terminal PDZ domain	degP, htrA	-1.67	0265	K04771
2619646309	ABC-type glutathione transport system ATPase component, contains duplicated ATPase domain		-1.64	1123	K02032
2619646095	Peroxiredoxin		-1.51	0678	
Replication, recombination and repair					
2619646142	rpoB; DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]	rpoB	1.63		K03043
2619645631	DNA polymerase	dpo	-5.44	1573	K02334
2619645299	ATP-dependent DNA helicase Rep	rep	-2.13	0210	K03656
Secondary metabolites biosynthesis, transport and catabolism					
2619646842	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)		1.81	0179	
2619646695	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)		-3.12	0179	
2619645902	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase		-2.42	0179	

	(catechol pathway)				
Signal transduction mechanisms					
2619647877	Adenylate cyclase, class 3		1.88	2114	
2619647021	CBS domain		1.82	0517	
2619646291	Two-component response regulator, PleD family, consists of two REC domains and a diguanylate cyclase (GGDEF) domain		1.65	3706	
2619646287	RecA-superfamily ATPase, KaiC/GvpD/RAD55 family	kaiC	1.52	0467	K08482
2619647648	DNA-binding transcriptional response regulator, NtrC family, contains REC, AAA-type ATPase, and a Fis-type DNA-binding domains	zraR, hydG	1.51	2204	K07713
2619645472	Putative lipoic acid-binding regulatory protein		-5.88	2921	K09158
2619647655	Periplasmic catabolite regulation protein CreA (function unknown)	creA	-2.96	3045	K05805
2619645734	Two-component system, OmpR family, sensor histidine kinase TctE	tctE	-2.93	0642	K07649
2619646363	Ser/Thr protein kinase RdoA involved in Cpx stress response, MazF antagonist		-1.93	2334	
2619644878	cAMP-binding domain of CRP or a regulatory subunit of cAMP-dependent protein kinases	fnr	-1.81	0664	K01420
Stress and oxidative responses					
2619647313	Nucleotide-binding universal stress protein, UspA family		-5.54	0589	
2619644545	Nucleotide-binding universal stress protein, UspA family		-2.38	0589	
Transcription					
2619645768	Fic family protein		-5.61	3177	
2619643611	DNA-binding transcriptional regulator, LacI/PurR family	lacI, galR	-5.24	1609	K02529
2619645766	DNA-binding transcriptional regulator YiaG, XRE-type HTH domain		-2.61	2944	
2619645955	DNA-binding transcriptional regulator, GntR family		-1.69	1802	
2619644174	DNA-binding transcriptional regulator, GntR family		-1.58	1802	
Translation, ribosomal structure and biogenesis					
2619646087	Ribosomal protein S19	RP-S19, rpsS	1.94	0185	K02965
2619646078	Ribosomal protein S12	RP-S12, MRPS12, rpsL	1.82	0048	K02950

2619647845	Ribonuclease G	rng, cafA	1.76	1530	K08301
2619645368	Ribosomal protein S8	RP-S8, rpsH	1.72	0096	K02994
2619645366	Ribosomal protein L18	RP-L18, MRPL18, rplR	1.66	0256	K02881
2619645367	Ribosomal protein L6P/L9E	RP-L6, MRPL6, rplF	1.59	0097	K02933
2619645218	Glycyl-tRNA synthetase, alpha subunit	glyQ	1.59	0752	K01878
2619646090	Ribosomal protein L16/L10AE	RP-L16, MRPL16, rplP	1.57	0197	K02878
2619644634	Peptide chain release factor RF-3	prfC	-2.75	4108	K02837
2619647275	tRNA-dihydrouridine synthase	dusA	-2.10	0042	K05539
2619644240	Ribonuclease E	rne	-1.99	1530	K08300
2619647870	Peptide deformylase	PDF, def	-1.81	0242	K01462
2619645150	Ribosomal protein L13	RP-L13, MRPL13, rplM	-1.54	0102	K02871

Chapter 5. Discussion and Conclusions

Permafrost, which accounts for 27% of all terrestrial ecosystems, harbors diverse microbial communities. These environments are harsh, often oligotrophic, with low water activity, and ambient *in situ* temperatures of ~-17 °C (Steven et al. 2007b). The results outlined in this thesis describe mechanisms and adaptations that are important for growth and survival of microorganisms inhabiting the extreme subzero environment of permafrost.

5.1 Important proteins and functions during subzero growth in a halophilic eurypsychrophile

Planococcus halocryophilus is a bacterium, isolated from active layer in Eureka, Nunavut, capable of growth from -15 to 37°C (Mykytczuk et al. 2013). In addition, *P. halocryophilus* is commonly detected in the permafrost underlying the activate layer. In its terrestrial environment, *Planococcus halocryophilus* would experience fluctuating temperatures from ~15 °C in summer to -20°C and below in winter. In permafrost and associated frozen environments, ecosystems are theorized to occur within very thin liquid brine veins surrounding soil particles. In the laboratory, we used proteomics to investigate the growth properties of *Planococcus halocryophilus* at subzero temperatures and salty conditions, namely -10°C with 12% NaCl, and compared them to growth at its near-optimal temperature of 23°C. Subzero growth in *P. halocryophilus* involves a series of complex responses and overall significant changes in abundance of proteins involved in many processes and functions, rather than simply an increase or decrease in a specific category. Overall growth at -10°C and in 12% NaCl appeared to be closely tied, with a number of shared changes in protein abundances, and falls within expectations given its natural environment in salty brine veins, where the organisms would have to adapt to both subzero temperatures and high salt.

The most significant change at -10°C, when compared to higher temperatures, was seen in translation and ribosomal processes. We observed strong increases in abundances of numerous ribosomal and translational proteins. The proteomic response of the psychrophile *Colwellia*

psychrerythraea in ice at -10°C was also dominated by an abundance increase in translation processes and protein synthesis (Nunn et al. 2015), as was the response at -5°C in *Psychrobacter* sp. PAMC 21119 (Koh et al. 2017) and *Fragilariopsis cylindrus* at -2°C (Mock et al. 2005). The increased presence of the transcriptional regulator MazF/PemK at -10°C is intriguing and highlights its potentially central role in regulating the subzero response in *P. halocryophilus*, through translational reprogramming and selective translation, which would partly explain the strong changes observed in most translational and ribosomal processes. MazF has been shown to be important during periods of harsh stress in *Escherichia coli*, leading to persistence, through translation reprogramming, activation of 'stress-ribosomes', and selective translation (Sauert et al. 2016). It is worthwhile to ponder whether a similar global regulatory role is at play here and further studies should be carried out to investigate the potential effect of MazF on the cell response and translation processes at subzero temperatures in this organism. In Southern Ocean psychrophilic phytoplankton communities, translation has been postulated to be the rate-limiting step for protein synthesis at low temperatures (Toseland et al. 2013). Taking into account the results of this study would lead us to suggest that induction and modulation of translational processes may be an especially important adaptation to sustain continued growth at subzero temperatures.

Additional pathways and functions observed to change significantly included amino sugar and nucleotide sugar metabolism, transporters, amino acid biosynthesis, and pyrimidine, purine and pyruvate metabolisms. Certain pathways and processes appeared to be specifically important during subzero growth where we observed large increases in abundances of proteins assigned to these categories with few decreased proteins. These included peptidoglycan biosynthesis, DNA repair and recombination, sulfur cycling, mRNA and ribosome biogenesis, nucleotide and RNA turnover, nicotinamide metabolism and fructose/mannose metabolism. The increase in peptidoglycan synthesis at -10°C is consistent with the observed cellular encrustations, composed partly of peptidoglycan, produced by the organism at subzero temperatures and described previously (Mykytczuk et al. 2013, 2015). The exact role of this extracellular structure remains unknown, but has been theorized to be important, at least in part, for protection from the frozen environment. The permafrost bacteria *Exiguobacterium sibiricum* is also believed to

make use of a thicker cell wall at subzero temperatures (Rodrigues et al. 2008), while the sea ice bacterium *Colwellia psychrethrerya* produces a unique polysaccharide capsule (Carillo et al. 2015).

Previous genomic analysis of *Planococcus halocryophilus* revealed evidence for genomic redundancy and possible isozyme exchange (Mykytczuk et al. 2013), and our proteomic results confirmed that *P. halocryophilus* differentially expresses copies of the same gene at different temperatures. This is likely to offer a cold temperature advantage, whereby cold or hot adapted isozymes can catalyze similar reactions at different temperatures (Maki et al. 2006), and may be one mechanism by which *P. halocryophilus* can sustain growth over a broad temperature range.

This study also raised some intriguing questions that warrant further investigation. *P. halocryophilus* can tolerate up to 19% salt and in its terrestrial subzero brine-vein environment, would be exposed to high salt concentrations. However, the mechanism by which *P. halocryophilus* achieves osmoregulation remains unclear. Compatible solutes are utilized by a number of cold-adapted microbes in this capacity, in addition to roles in increasing molecular stability at cold temperatures (Yancey 2005). While *P. halocryophilus* possesses numerous genes involved in compatible solute uptake (Mykytczuk et al. 2013) none of these proteins were found to be significantly more abundant at -10°C, although one transporter component was found to be strongly induced at 23°C and 12% salt. Its presence at 23°C and 12% salt would point to an important role in salt tolerance; however, it was not detected during growth at -10 °C and 12% salt, for reasons unknown. While it is possible that compatible solute transporters were expressed only during the initial cold and salt acclimation period and no longer necessary once appropriate intercellular concentrations of compatible solutes were achieved, this would not explain why it was still detected at 23°C and 12% salt.

Membrane remodeling to overcome decreased membrane fluidity at lower temperatures is common to many psychrophiles, who achieve this by reducing saturated fatty acids (FA) in favor of branched-chain and/or unsaturated FAs. Curiously, *P. halocryophilus* behaves in an opposite manner and increases saturated FAs at subzero temperatures (Mykytczuk et al. 2013).

Nevertheless, strong fold increases observed for proteins involved in fatty acid and phospholipid synthesis and in modulating chain length and membrane fatty acid composition, namely PlsX and KASII, suggests that fatty acid membrane modifications are important during subzero growth. Additional studies will be necessary to better understand the mechanisms utilized by *P. halocryophilus* to modulate membrane fluidity at low temperatures.

One important caveat with regards to this *in vitro* study is that it cannot replicate the harsher permafrost conditions which *P. halocryophilus* would be expected to encounter in its native environment and therefore these results should be weighed in that context. Nevertheless, it remains an ideal model method for observing the cellular and molecular response of this organism in a controlled setting and providing important data that can form a baseline for further research.

5.2 Genomic and amino acid features of cold adaptation in subzero growing permafrost isolates

Using new genomic data from subzero growing permafrost bacteria, we performed in depth comparative genomic and amino acid analysis with mesophilic genomes to investigate and characterize conserved traits of cold adaptation in cryophiles. We found evidence for presence of many cold adaptation genes in the cryophilic genomes, as well as genomic redundancy, theorized to be an important feature of cold adapted organisms, as discussed above. At lower temperatures, psychrophiles must contend with decreased flexibility of proteins, which can have significant impacts on function. Therefore, in addition to changes in cellular mechanisms and processes, cold adaptation in psychrophiles also involves changes in protein amino acid composition, favoring increased flexibility, and increased enzymatic catalytic efficiency at lower temperatures (Bakermans et al. 2012; De Maayer et al. 2014). We compared the amino acid composition of predicted proteins in the cryophiles with the mesophiles focusing on the following indices previously associated with cold adaptation in several studies: arginine to lysine ratio; frequency of acidic, polar uncharged, and charged residues; number of proline, glycine, and serine residues; aromaticity; aliphacity; and grand average of hydropathicity

(GRAVY) (Russell et al. 1998; Aghajari et al. 1998; Georlette et al. 2000; Huston et al. 2004; Collins et al. 2005; Ræder et al. 2008; Metpally and Reddy 2009).

We identified significant cold adaptation, in four of the five cryophiles, for a subset of the amino acid indices we studied, including fewer prolines, more glycine and serine residues, and reduced aromaticity. The reduction in proline may be because being covalently bonded to the nitrogen atom of the peptide group, they impose constraint on rotations of the peptide backbone and create rigid kinks in peptide chains, thus contributing to overall increased stability but reduced flexibility of proteins (Reiersen and Rees 2001). Conversely, due to its small size, glycine provides more conformational freedom, thus increasing flexibility, and serine, as a polar uncharged amino acid, may compensate partly for the general reduction in charged residues in psychrophilic proteins (Saunders et al. 2003). Similar changes in proline, serine, and glycine have been observed in other cold adapted organisms (Huston et al. 2004; Methé et al. 2005; Ræder et al. 2008; Metpally and Reddy 2009; Ayala-del-Río et al. 2010; Mykytczuk et al. 2013; Bakermans et al. 2014; Ronholm et al. 2015; Goordial et al. 2016b). Comparing all cryophiles to all mesophiles, overall, we found trends favoring lower proline and acidic residues, and higher serine content in cryophilic proteins. It is possible that changes in content of these specific amino acids may be especially important to increase protein flexibility during subzero growth. However, additional comparisons between cryophiles, psychrophiles, and mesophiles would be needed to determine if this is indeed the case. Overall, we could not easily detect cold adaptations on a genome-wide scale for many of the amino acid indices we investigated and we found a significant degree of variability between the genera we studied. Therefore, we believe that individual comparative analyses focusing on single proteins and taking into account not only total amino acid numbers, but the location of specific amino acids within the sequence, the overall potential conformations of the protein, and, if possible, functional assays, are more likely to provide an accurate overall representation of cold adaption in psychrophilic proteins.

5.3 Comparison of transcriptional responses during cold growth in a eurypsychrophiles and stenopsychrophile

To expand on the genomic analyses, and look at actively transcribed genes important in cold growth, we selected two subzero growing permafrost isolates, a eurypsychrophile, *Rhodococcus* sp. JG3, and a stenopsychrophile, *Polaromonas* sp. Eur3 1.2.1, for transcriptomic analysis in order to compare and contrast their zero and sub-zero growth strategies. We observed a response that included a number of similarities, but also several striking differences. As with *Planococcus halocryophilus* and other studies on subzero growing organisms (discussed above), we found translation and ribosomal processes to be strongly induced in both organisms in the low temperature conditions, compared to higher temperatures. This highlights the importance of sustaining these processes at low temperatures in organisms capable of subzero growth. Additional common responses observed in both *Rhodococcus* sp. JG3 and *Polaromonas* sp. Eur3 1.2.1 included upregulation of nutrient transport, increased oxidative and osmotic stress responses, induction of EPS synthesis, and accumulation of compatible solutes, though the latter was much more pronounced in *Rhodococcus* sp. JG3. This is not unexpected however, given that *Rhodococcus* sp. JG3 grows in higher salt medium. The presence of the above properties in most psychrophiles (Bakermans et al. 2012; De Maayer et al. 2014), whether eury- or steno-, suggests that these may be conserved adaptive features, necessary for growth at low temperatures in most organisms.

Recombination and genomic redundancy also appeared to be a shared strategy at low temperatures, though the mechanism used to achieve this varied between organisms. *Polaromonas* sp. Eur3 1.2.1 utilizes specific transposases, while *Rhodococcus* sp. JG3 induces recombination proteins. Recombination has not been studied very much in psychrophiles, and evidence for it is not prominent in most transcriptomic and proteomic studies of psychrophiles to date, although transposons and recombination proteins were reported at low temperatures in the psychrophilic archaeon *Methanococcoides burtonii* (Goodchild et al. 2004). Much like *P. halocryophilus*, *Rhodococcus* sp. JG3 and *Polaromonas* sp. Eur3 1.2.1, appear to rely on genomic

redundancy and both differentially express a number of isozymes at different temperatures. Given that we detected isozyme exchange and recombination like processes in *P. halocryophilus*, *Polaromonas* sp. Eur3 1.2.1, and *Rhodococcus* sp. JG3, it is possible that these mechanisms are an important adaptive strategy in cryophiles and confer growth advantages at subzero temperatures, although additional analysis of recombination of cryophiles will be needed to investigate this hypothesis.

There were some important differences in the response of *Rhodococcus* sp. JG3 and *Polaromonas* sp. Eur3 1.2.1 during cold growth. *Rhodococcus* sp. JG3 emphasized increased abundance of transcripts involved in iron transport, amino acid transport and metabolism, modulating fatty acid synthesis and composition, catabolism of alcohols/ethanolamine, and sustaining redox potential, through increased co-factor synthesis. Conversely, *Polaromonas* sp. Eur3 was found to induce energy metabolism relating to the electron transport chain, oxidative phosphorylation, and glycolysis, as well as global signal transduction mechanisms and transport and metabolism of carboxylates. In addition, enzymes vital to peptidoglycan synthesis and modulation were increased. Lastly, *Polaromonas* sp. Eur3 differentially regulated specific subsets of transposases at its colder and warmer temperatures.

Increased activity of primary energy metabolism and the electron transport chain in *Polaromonas* sp. Eur3 1.2.1 and other stenopsychrophiles (Mock and Hoch 2005; Mock et al. 2005; Hwang et al. 2008; Chong et al. 2011), is likely to be one major mechanism by which these organisms are able to sustain optimal growth at low temperatures and, in the case of *Polaromonas* sp. Eur3 1.2.1, consistent growth rates over its narrower temperature range. The more attenuated transcriptomic response in *Polaromonas* sp. Eur3 1.2.1 also suggests that the organism may be, at least partly, constitutively adapted to colder temperatures or for growth over its temperature range and does not need to upregulate its response to the same levels as *Rhodococcus* sp. JG3 at lower temperatures. However, more research will need to be done to determine if this is true and identify those features which are unique to these types of organisms, and differ from eurypsychrophiles. Certainly, it may simply be that stenopsychrophiles are adapted to grow at a narrow range and temperature changes, both

above and below this range, is difficult for these organisms. In the same vein, it is difficult to pinpoint specifically those mechanisms that favor eurypsychrophilic growth at subzero temperatures from this study alone, though in the case of *Rhodococcus* sp. JG3, increased co-factor formation and redox potential, flexibility in use of carbon sources, control of fatty acid composition in the membrane, and iron acquisition, appear to be important factors. These may represent adaptive strategies that allow this organism to grow at subzero temperatures, in addition to sustaining growth over a wide range of temperatures (-5 to 30°C).

5.4 Conclusions and Future Work

Results from both the proteomic and transcriptomic studies suggest that sustaining and modulating translation and ribosomal processes are especially important during subzero growth. Given that this is consistent with subzero responses observed in other cryophiles (Mock et al. 2005; Nunn et al. 2015; Koh et al. 2017), we may theorize that this is a fundamental adaptation required for growth at subzero temperatures. There is evidence for genomic redundancy in all of the organisms studied in this thesis and both the proteomic and transcriptomic studies provide evidence that differential expression of gene copies at different temperatures is an actively used adaptation in subzero growing organisms. Isozyme exchange has been reported almost exclusively in studies of subzero growing isolates (Rodrigues et al. 2008; Bergholz et al. 2009; Mykytczuk et al. 2013; Koh et al. 2017) and, there are very few studies that show evidence for use of this mechanism in organisms with low above zero temperature limits, with the exception of *Shewanella frigidimarina* (García-Descalzo et al. 2014). Control of fatty acid synthesis and membrane composition, seen more specifically during subzero growth in *Planococcus halocryophilus* and *Rhodococcus* sp. JG3, may be especially important in cryophiles and/or organisms that have wide growth ranges and who must modulate membrane flexibility over large temperature ranges.

Some combination of proteins and mechanisms that are usually associated with stress responses, including RNA helicases and cold shock proteins, antioxidants and the ROS response, efflux pumps, glyoxalase proteins, compatible solutes and osmoregulation, carotenoid

synthesis, and repair and recombination proteins, appeared to be of significant importance during subzero growth in all three organisms studied. However, given that exposure to low temperature and high salt is part of their normal environment, it is worth pondering whether this truly represents a stress response in these organisms. It may be that heightened presence of these mechanisms does not actually represent a stress or shock response so much as it is simply part of the normal response or rather normal state of the organisms in these conditions.

In order to elucidate functional roles more appropriately, further studies targeting individual or subsets of genes and proteins that appeared to have an especially important role during subzero growth would be a worthy next step in this research. Cloning, expression, enzymatic assays, knock-out studies, and protein crystallization or 3D modeling, will be needed to more thoroughly explore the specific functions and processes important for subzero growth. Table 5.1 provides a list of the most intriguing candidate genes for further studies based on the results of the proteomic and transcriptomic analyses (Chapters 2 and 4). With regards to modulation of the peptidoglycan layer specifically, the two penicillin binding proteins most increased in abundance in *Planococcus halocryophilus*, along with an amidase, potentially involved in peptidoglycan remodeling, would be interesting targets to better elucidate their role during the subzero formation of the cellular envelope in this organism. *Polaromonas* sp. Eur3 1.2.1 was also observed to potentially increase peptidoglycan production. A murein transglycosylase, with a probable role in recycling of muropeptides during cell elongation/division, was increased in low temperatures and could be selected for deletion to detect its importance in peptidoglycan remodeling and for cell survival during low temperature growth.

Planococcus halocryophilus increases production of long chain and saturated fatty acids at low temperatures (Mykytczuk et al. 2013), an activity that is inconsistent with our understanding of the necessity for heightened membrane flexibility at lower temperatures through increased proportion of unsaturated or branched chain fatty acids in the membrane and is one of the more intriguing aspects of subzero growth in this organism. It is also contradictory to most psychrophile studies which have found that cold-adapted organisms indeed have higher

proportions of branched or unsaturated fatty acids (Rodrigues et al. 2008). Two proteins involved in fatty acid synthesis with potential roles in controlling chain elongation, production of unsaturated fatty acids, and modulating fatty acid synthesis, PlsX and KASII, were strongly increased in abundance at -10 in *P. halocryophilus*. These proteins may be key to the membrane changes we see during subzero growth in this organism and elucidating their functions would provide additional clues as to how the organism modulates membrane flexibility at low temperatures. *Rhodococcus* sp. JG3 increases transcripts at -5°C of an acyl-ACP thioesterase and an acyl dehydrase, with known roles in regulating lipid metabolism and production of double bonds in fatty acids, respectfully. Targeting either enzyme for knockout studies should elucidate their role in modulating membrane composition and increasing membrane flexibility. This should be done in combination with lipid analyses (GC-FID) to determine the phospholipid composition over a range of temperatures from high to subzero and determine the exact composition of the membrane in this organism.

Since production of a polysaccharide capsule has been described at subzero temperatures in the psychrophile *Colwellia psychrerythraea* (Carillo et al. 2015), it would be worthwhile to study whether similar structures are produced by other cold-adapted organisms at subzero temperatures. Both *Rhodococcus* sp. JG3 and *Polaromonas* sp. Eur 3 1.2.1 upregulate transcripts for proteins predicted to be involved in production of exopolysaccharides (Table 5.1) and creating deletion mutants of these, in combination with polysaccharide analyses (GC-MS, NMR, CLSM microscopy) would inform on the potential for polysaccharide production at low temperatures in these organisms, and the role, if any, of these proteins in this process.

Translation and ribosomal processes were strongly increased in the proteomic and transcriptomic datasets at zero and subzero temperatures. As a result, there are many potential candidate genes for further study (see Chapters' 2 and 4). Of these, the most intriguing candidate is a MazF/PemK homologue in *P. halocryophilus*. It has been shown previously to induce broad translational reprogramming under periods of stress in *E. coli* (Sauert et al. 2016). Its role has yet to be elucidated in cold-adapted organisms; it would be important to determine

whether a similar global regulatory function is involved during subzero growth in *P. halocryophilus*.

All three organisms showed evidence for increasing recombination potential through increased transcripts or abundance of proteins involved in DNA recombination and repair. *Polaromonas* sp. Eur3 1.2.1 upregulated a specific subset of transposases at 0°C, belonging to the IS5 family, members of which are suspected of being involved in regulation of bacterial adaptive strategy through insertion in regulatory regions of genomes (Fléchard and Gilot 2014). Searching for insertions of these sequences in the genome of *Polaromonas* sp. Eur3 1.2.1 along with targeted cloning and insertion experiments could provide additional clues as to their function in this organism during low temperature growth.

Increases in a large number of transcripts involved in iron acquisition in *Rhodococcus* sp. JG3 during subzero growth points to the potential importance of iron acquisition for survival at subzero temps in this organism. Interestingly, these increases are not seen in most studies of psychrophiles; in fact, there is evidence for suppression of iron-associated proteins in psychrophiles (Piette et al. 2011a; Ronholm et al. 2015; Tribelli et al. 2015). This leaves the question of why iron acquisition is upregulated to this extent in *Rhodococcus* sp. JG3 and if it is required for growth at low temperatures. As such, selecting a few of these genes for knockout studies (Table 5.1) would be pertinent.

The question of how *P. halocryophilus* achieves osmoregulation remains unanswered. Most cold-adapted bacteria are known to use compatible solutes for osmoregulation. While *P. halocryophilus* is able to tolerate up to 19% salt, and salty subzero conditions in permafrost brine veins would require the ability to tolerate similar salt concentrations (Gilichinsky et al. 2003), the proteomic study described in this thesis, along with previous studies (Mykytczuk et al. 2013; Ronholm et al. 2015), have not found increased abundance of proteins known to be involved in uptake of compatible solutes. Knockouts of these transporters, along with growth studies, would conclusively establish their role and importance in growth under salty subzero conditions. Additional growth studies using minimal media supplemented with a range of salt

concentrations and potential compatible solutes would also be worthwhile to determine which compatible solutes *Planococcus halocryophilus* uses during subzero and high salt growth.

The transcriptomic and proteomic studies in Chapters 2 and 4 found evidence for isozyme exchange and differential expression of gene copies at the different growth temperatures in all three organisms studied (see Tables 2.3 and 4.4), a feature observed almost exclusively in subzero-growing organisms. Knock-out studies targeting these proteins, in order to elucidate function, along with enzyme assays focusing on enzyme kinetics, and amino acid and 3-D structure modeling analyses to determine compositional, structural, and conformational differences in the isozymes would be ideal to compare and contrast their function and better understand the reason for their differential expression patterns over the different growth temperatures.

Finally, 15-20% of the proteins and transcripts differentially expressed in the two omic studies are hypothetical or have only very general function predictions. Some of these were strongly increased in the ≤ 0°C datasets, which would suggest a potentially very important role during subzero growth. A few of the most interesting candidate proteins are listed in Table 5.1. One of these proteins, YloU, an uncharacterized protein with a conserved alkaline shock protein domain, was increased in abundance in both *P. halocryophilus* and *Rhodococcus* sp. JG3 at subzero temperatures. Determining the function of these different hypothetical proteins would be valuable, not only in terms of our understanding of subzero growth, but also for increasing our knowledge of microbial activity and protein function in general.

We saw some evidence of amino acid modifications favoring increased flexibility at subzero temperatures, but these results were incomplete on their own and require additional analyses to conclusively identify conserved amino acid modifications in subzero growing organisms. Overall, our results of the amino acid analyses varied between organisms in the different genera, and it was difficult to identify conserved amino acid modifications that would be indicative of cold adaption own their own, although increased serine and reduced proline and acidic residue content stood out as being potentially important and the best candidates for

further investigation. It may also simply be that changes in protein abundance and in various cellular pathways and mechanisms is more important than specific amino acid modifications for subzero growth. Increasing our database of cryophilic genomes will allow us to do larger scale amino acid and genomic comparisons between cryophiles with mesophiles and even between types of psychrophiles, such as eury- and steno-psychrophiles.

There are two important caveats to mention in this work. First, since post transcriptional regulation can have a significant impact on the number of transcripts that are translated to protein, transcript abundances from transcriptomic data do not necessarily represent final protein abundances in the cell. As a result, some care must be taken when discussing the two. Second, the *in vitro* studies described in this thesis do not replicate the harsher permafrost conditions which the organisms would be expected to encounter in their native environment and therefore the results must be weighed in that context. Nevertheless, these studies allowed us to observe the zero and subzero cellular and molecular responses of each isolate in a controlled setting and form an important baseline for further research.

Overall, the results of the research in this thesis point to the existence of multiple adaptive strategies that can support growth at subzero temperatures. While some appear to be shared, namely, sustaining translation processes, use of genomic redundancy and isozyme exchange, accumulation of compatible solutes, and induction of various stress responses, others are specific to each organism and reflect the potential for multiple approaches towards subzero adaptation and growth in cryophiles.

Table 5.1. Candidate genes in our study strains representing intriguing targets for further functional characterization. For *Planococcus halocryophilus,* GI accession numbers are provided for each gene, while JGI gene IDs are given for *Polaromonas* sp. Eur3 1.2.1 and *Rhodococcus* sp. JG3. FC = fold change.

Protein Name	Function Description	Organism	Gene ID
Peptidoglycan synthesis and modulation			
PBP (Penicillin binding protein)	D-alanyl-D-alanine carboxypeptidase	*Planococcus halocryophilus*	gi|495773085|
PBP	Multimodular transpeptidase-transglycosylase	*Planococcus halocryophilus*	gi|495772622|
Peptidoglycan amidohydrolase	N-acetylmuramoyl-L-alanine amidase	*Planococcus halocryophilus*	gi|495774369|; gi|495772622|
MltB	Membrane-bound lytic murein transglycosylase B	*Polaromonas* sp. Eur3 1.2.1	2619644456
Fatty acid metabolism/Glycerophospholipid metabolism			
PlsX	Phosphate:acyl-ACP acyltransferase	*Planococcus halocryophilus*	gi|495773390|
KASII	3-oxoacyl-[acyl-carrier-protein] synthase	*Planococcus halocryophilus*	gi|495773567|
Thioesterase	Acyl-ACP thioesterase	*Rhodococcus* sp. JG3	2529302855
Dehydratase	Acyl dehydratase	*Rhodococcus* sp. JG3	2529302388
Envelope and exopolysaccharide synthesis			
HbhA	Heparin binding hemagglutinin	*Rhodococcus* sp. JG3	2529303100
EpsO	Exopolysaccharide biosynthesis protein, predicted pyruvyl transferase	*Rhodococcus* sp. JG3	2529302771
Exopolysaccharide protein	Uncharacterized protein involved in exopolysaccharide biosynthesis	*Polaromonas* sp. Eur3 1.2.1	2619647584
WcaJ	Putative colanic acid biosynthesis UDP-glucose lipid carrier transferase	*Polaromonas* sp. Eur3 1.2.1	2619644456
Translation and ribosomal processes			
PemK/MazF	Translational regulator, PemK/MazF family	*Planococcus halocryophilus*	gi|495707836|
YqfR	ATP-dependent RNA helicase YqfR	*Planococcus halocryophilus*	gi|495774437|
CsdL/TcdA	tRNA A37 threonylcarbamoyladenosine dehydratase	*Planococcus halocryophilus*	gi|495774373|
DNA replication, repair, and recombination			
DnaD	DnaD and phage-associated domain-containing protein	*Planococcus halocryophilus*	gi|495773309|
Transposase	Transposase, IS5 family	*Polaromonas* sp. Eur3 1.2.1	2619646664; 2619647232;

			2619647514; 2619647785; 2619645329
Transcription			
RNAP ε	RNA polymerase auxiliary subunit epsilon	*Planococcus halocryophilus*	gi|495705273|
Stress responses (glyoxal metabolism, general stress response, osmoregulation)			
Glyoxalase	Glyoxalase family protein and glyoxalase beta-lactamase superfamily II	*Planococcus halocryophilus*	gi|495774211|; gi|495774457|
UspA	Nucleotide-binding universal stress protein, UspA family	*Rhodococcus* sp. JG3	2529299876; 2529299882; 2529299879; 2529299880
OsmC	Uncharacterized osmoregulation-related protein	*Rhodococcus* sp. JG3	2529302730
OpuABCD	ABC-type proline/glycine betaine transport system	*Rhodococcus* sp. JG3	2529301958; 2529301959; 2529302675
UspA	Nucleotide-binding universal stress protein, UspA family	*Polaromonas* sp. Eur3 1.2.1	2619647233
OsmB	Osmotically inducible lipoprotein	*Polaromonas* sp. Eur3 1.2.1	2619646962
Transporters			
HAE1 exporter	Hydrophobic/amphiphilic exporter-1, HAE1 family	*Planococcus halocryophilus*	gi|495773523|
ABC-2.A	ABC-type multidrug transport system, ATPase component	*Rhodococcus* sp. JG3	2529299875
OmpA	OmpA-OmpF porin, OOP family	*Polaromonas* sp. Eur3 1.2.1	2619647124
Carotenoid synthesis			
PDS	Dehydrosqualene desaturase/phytoene desaturase	*Planococcus halocryophilus*	gi|495773745|
ZDS	Pro-zeta-carotene desaturase, prolycopene producing	*Planococcus halocryophilus*	gi|495774225|
Iron transport and acquisition			
MtbABI	Mycobactin synthesis pathway	*Rhodococcus* sp. JG3	2529300140; 2529300145; 2529300139
Siderophore reductase	NADPH-dependent ferric siderophore reductase, contains FAD-bin	*Rhodococcus* sp. JG3	2529300144
ABC.FEV.A/S/P	Ferric-siderophore transport system	*Rhodococcus* sp. JG3	2529301663; 2529302627; 2529301661; 2529300143; 2529303132;
Hypothetical proteins			
	Hypothetical protein (> 13FC at -10°C vs 23°C)	*Planococcus halocryophilus*	gi|495772261|
YwiB	Uncharacterized beta-barrel protein (> 11FC at -10°C vs 23°C)	*Planococcus halocryophilus*	gi|495772278|
	Uncharacterized gas vesical protein (> 4FC at -10°C vs 23°C)	*Planococcus halocryophilus*	gi|495773715|
	Hypothetical protein (> 3FC at -10°C vs 23°C)	*Planococcus halocryophilus*	gi|495774071|

	Hypothetical protein (> 3FC at -10°C vs 23°C)	*Planococcus halocryophilus*	gi|495773042|
YloU	Uncharacterized conserved protein (> 4FC at -10°C vs 23°C-salt)	*Planococcus halocryophilus*	gi|495706230|
YfkK	Uncharacterized protein (> 3FC at -10°C vs 23°C-salt)	*Planococcus halocryophilus*	gi|495772569|
YphA	Uncharacterized membrane protein (> 4logFC at 0°C vs 20°C)	*Polaromonas* sp. Eur3 1.2.1	2619645833
YcfJ	Uncharacterized conserved protein (> 2.5logFC at 0°C vs 20°C)	*Polaromonas* sp. Eur3 1.2.1	2619645779
YlmC	Uncharacterized sporulation protein (> 2.5logFC at 0°C vs 20°C)	*Polaromonas* sp. Eur3 1.2.1	2619645996
	Uncharacterized conserved protein (> 2logFC at 0°C vs 20°C)	*Polaromonas* sp. Eur3 1.2.1	2619645043
	Predicted sulfurtransferase (> 5logFC at -5°C vs 23°C)	*Rhodococcus* sp. JG3	2529302622
	Predicted amidohydrolase (> 4.5logFC at -5°C vs 23°C)	*Rhodococcus* sp. JG3	2529299481
YloU	Uncharacterized conserved protein (> 3.5logFC at -5°C vs 23°C)	*Rhodococcus* sp. JG3	2529299354
	Predicted metal-dependent hydrolase (> 3.5logFC at -5°C vs 23°C)	*Rhodococcus* sp. JG3	2529303157
	Predicted CoA-binding protein (> 3logFC at -5°C vs 23°C)	*Rhodococcus* sp. JG3	2529302728

References

Adekoya OA, Helland R, Willassen NP, Sylte I (2006) Comparative sequence and structure analysis reveal features of cold adaptation of an enzyme in the thermolysin family. Proteins Struct Funct Genet 62:435–449. doi: 10.1002/prot.20773

Agarwal V, Lin S, Lukk T, et al (2012) Structure of the enzyme-acyl carrier protein (ACP) substrate gatekeeper complex required for biotin synthesis. Proc Natl Acad Sci U S A 109:17406–11. doi: 10.1073/pnas.1207028109

Aghajari N, Feller G, Gerday C, Haser R (1998) Structures of the psychrophilic Alteromonas haloplanctis alpha-amylase give insights into cold adaptation at a molecular level. Structure 6:1503–16.

Aliyu H, De Maayer P, Cowan D (2016) The genome of the Antarctic polyextremophile *Nesterenkonia* sp. AN1 reveals adaptive strategies for survival under multiple stress conditions. FEMS Microbiol Ecol 92:fiw032. doi: 10.1093/femsec/fiw032

Allen MA, Lauro FM, Williams TJ, et al (2009) The genome sequence of the psychrophilic archaeon, Methanococcoides burtonii: the role of genome evolution in cold adaptation. ISME J 3:1012–1035. doi: 10.1038/ismej.2009.45

Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol 215:403–10. doi: 10.1016/S0022-2836(05)80360-2

An M, Mou S, Zhang X, et al (2013) Temperature regulates fatty acid desaturases at a transcriptional level and modulates the fatty acid profile in the Antarctic microalga Chlamydomonas sp. ICE-L. Bioresour Technol 134:151–157. doi: 10.1016/j.biortech.2013.01.142

Arpigny JL, Lamotte J, Gerday C (1997) Molecular adaptation to cold of an Antarctic bacterial lipase. J Mol Catal B Enzym 3 3:29–35. doi: 10.1016/S1381-1177(96)00041-0

Ayala-del-Río HL, Chain PS, Grzymski JJ, et al (2010) The genome sequence of Psychrobacter arcticus 273-4, a psychroactive Siberian permafrost bacterium, reveals mechanisms for adaptation to low-temperature growth. Appl Environ Microbiol 76:2304–12. doi: 10.1128/AEM.02101-09

Bagnoud A, Chourey K, Hettich RL, et al (2016) Reconstructing a hydrogen-driven microbial metabolic network in Opalinus Clay rock. Nat Commun 7:12770. doi: 10.1038/ncomms12770

Bakermans C, Bergholz PW, Rodrigues DF, et al (2012) Genomic and Expression Analyses of Cold-Adapted Microorganisms. In: Miller R V, Whyte LG (eds) Polar Microbiology: Life in a Deep Freeze. ASM Press, Washington D.C., pp 126–155

Bakermans C, Skidmore ML, Douglas S, McKay CP (2014) Molecular characterization of bacteria from permafrost of the Taylor Valley, Antarctica. FEMS Microbiol Ecol 89:331–346. doi: 10.1111/1574-6941.12310

Bakermans C, Tollaksen SL, Giometti CS, et al (2007) Proteomic analysis of Psychrobacter cryohalolentis K5 during growth at subzero temperatures. Extremophiles 11:343–54. doi: 10.1007/s00792-006-0042-1

Baldridge KC, Contreras LM (2013) Functional implications of ribosomal RNA methylation in response to environmental stress. Crit Rev Biochem Mol Biol 9238:1–21. doi: 10.3109/10409238.2013.859229

Bender RA (2010) A NAC for regulating metabolism: The nitrogen assimilation control protein (NAC) from Klebsiella pneumoniae. J Bacteriol 192:4801–4811. doi: 10.1128/JB.00266-10

Bengis-Garber C, Kushner DJ (1982) Role of Membrane-Bound 5'-Nucleotidase in Nucleotide Uptake by the Moderate Halophile Vibrio costicola. J Bacteriol 149:808–815.

Bennett S (2004) Solexa Ltd. Pharmacogenomics 5:433–438.

Berger F, Morellet N, Menu F, Potier P (1996) Cold shock and cold acclimation proteins in the psychrotrophic bacterium Arthrobacter globiformis SI55. J Bacteriol 178:2999–3007.

Bergholz PW, Bakermans C, Tiedje JM (2009) Psychrobacter arcticus 273-4 Uses Resource Efficiency and Molecular Motion Adaptations for Subzero Temperature Growth. J Bacteriol 191:2340–2352. doi: 10.1128/JB.01377-08

Bertrand EM, McCrow JP, Moustafa A, et al (2015) Phytoplankton-bacterial interactions mediate micronutrient colimitation at the coastal Antarctic sea ice edge. Proc Natl Acad Sci U S A 112:9938–43. doi: 10.1073/pnas.1501615112

Bhattachan A, Wang L, Miller MF, et al (2015) Antarctica's Dry Valleys: A potential source of soluble iron to the Southern Ocean? Geophys Res Lett 42:1912–1918. doi: 10.1002/2015GL063419

Blanco Y, Prieto-Ballesteros O, G??mez MJ, et al (2012) Prokaryotic communities and operating metabolisms in the surface and the permafrost of Deception Island (Antarctica). Environ Microbiol 14:2495–2510. doi: 10.1111/j.1462-2920.2012.02767.x

Bloom LB (2009) Loading Clamps for DNA Replication and Repair. DNA Repair 8:570–578. doi: 10.1016/j.dnarep.2008.12.014.Loading

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. doi: 10.1093/bioinformatics/btu170

Bordo D, Bork P (2002) The rhodanese/Cdc25 phosphatase superfamily. EMBO Rep 3:741–746.

Boyd W (1958) Microbiological Studies of Arctic Soils. Ecology 39:332–336.

Boyd WL, Boyd JW (1962) Viability of thermophiles and coliform bacteria in arctic soils and water. Can J Microbiol 8:189–92.

Boyd WL, Boyd JW (1964) the Presence of Bacteria in Permafrost of the Alaskan Arctic. Can J Microbiol 10:917–9.

Boyd WL, Boyd JW (1971) Studies of Soil Microorganisms Inuvik, Northwest Territories. Arctic 24:162–176.

Brown SD (2006) Molecular Dynamics of the Shewanella oneidensis Response to Chromate Stress. Mol Cell Proteomics 5:1054–1071. doi: 10.1074/mcp.M500394-MCP200

Buelow HN, Kooser AS, Van Horn DJ, et al (2016) Microbial Community Responses to Increased Water and Organic Matter in the Arid Soils of the McMurdo Dry Valleys, Antarctica. Extrem Microbiol 7:1040. doi: 10.3389/fmicb.2016.01040

Burg DW, Lauro FM, Williams TJ, et al (2010) Analyzing the hydrophobic proteome of the Antarctic archaeon Methanococcoides burtonii using differential solubility fractionation. J Proteome Res 9:664–676. doi: 10.1021/pr9007865

Burian J, Ramón-García S, Sweet G, et al (2012) The mycobacterial transcriptional regulator whiB7 gene links redox homeostasis and intrinsic antibiotic resistance. J Biol Chem 287:299–310. doi: 10.1074/jbc.M111.302588

Cacace G, Mazzeo MF, Sorrentino A, et al (2010) Proteomics for the elucidation of cold adaptation mechanisms in Listeria monocytogenes. J Proteomics 73:2021–2030. doi: 10.1016/j.jprot.2010.06.011

Calogero S, Gardan R, Glaser P, et al (1994) RocR, a novel regulatory protein controlling arginine utilization in Bacillus subtilis, belongs to the NtrC/NifA family of transcriptional activators. J Bacteriol 176:1234–1241.

Camacho C, Coulouris G, Avagyan V, et al (2009) BLAST plus: architecture and applications. BMC Bioinformatics 10:1. doi: Artn 421\nDoi 10.1186/1471-2105-10-421

Campanaro S, Williams TJ, Burg DW, et al (2011) Temperature-dependent global gene expression in the Antarctic archaeon Methanococcoides burtonii. Environ Microbiol 13:2018–2038. doi: 10.1111/j.1462-2920.2010.02367.x

Campbell J, Cronan Jr J (2001) Bacterial Fatty Acid Biosynthesis. Annu Rev Microbiol 55:305–32. doi: 10.1146/annurev.biochem.74.082803.133524

Carabetta VJ, Tanner AW, Greco TM, et al (2013) A complex of YlbF, YmcA and YaaT regulates sporulation, competence and biofilm formation by accelerating the phosphorylation of

Spo0A. Mol Microbiol 88:283–300. doi: 10.1111/mmi.12186

Carillo S, Casillo A, Pieretti G, et al (2015) A unique capsular polysaccharide structure from the psychrophilic marine bacterium Colwellia psychrerythraea 34H that mimics antifreeze (glyco)proteins. J Am Chem Soc 137:179–189. doi: 10.1021/ja5075954

Cavicchioli R (2016) On the concept of a psychrophile. ISME J 10:793–795. doi: 10.1038/ismej.2015.160

Chao H, Zhou NY (2013) GenR, an IclR-type regulator, activates and represses the transcription of gen genes involved in 3-hydroxybenzoate and gentisate catabolism in Corynebacterium glutamicum. J Bacteriol 195:1598–1609. doi: 10.1128/JB.02216-12

Chattopadhyay MK, Raghu G, Sharma YVRK, et al (2011) Increase in oxidative stress at low temperature in an antarctic Bacterium. Curr Microbiol 62:544–546. doi: 10.1007/s00284-010-9742-y

Chen Z, Yu H, Li L, et al (2012) The genome and transcriptome of a newly described psychrophilic archaeon, Methanolobus psychrophilus R15, reveal its cold adaptive characteristics. Environ Microbiol Rep 4:633–641. doi: 10.1111/j.1758-2229.2012.00389.x

Chin C-S, Alexander DH, Marks P, et al (2013) Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods 10:563–569. doi: 10.1038/nmeth.2474

Chin JP, Megaw J, Magill CL, et al (2010) Solutes determine the temperature windows for microbial survival and growth. Proc Natl Acad Sci U S A 107:7835–40. doi: 10.1073/pnas.1000557107

Choi KM, Lee MY (2012) Differential protein expression associated with heat stress in Antarctic microalga. Biochip J 6:271–279. doi: 10.1007/s13206-012-6310-5

Chong G-L, Chu W-L, Othman RY, Phang S-M (2011) Differential gene expression of an Antarctic Chlorella in response to temperature stress. Polar Biol 34:637–645. doi: 10.1007/s00300-010-0918-5

Chourey K, Jansson J, Verberkmoes N, et al (2010) Direct Cellular Lysis / Protein Extraction Protocol for Soil Metaproteomics research articles. J Proteome Res 9:6615–6622.

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23. doi: 10.1016/j.femsre.2004.06.005

Coolen MJL, Orsi WD (2015) The transcriptional response of microbial communities in thawing Alaskan permafrost soils. Front Microbiol 6:1–14. doi: 10.3389/fmicb.2015.00197

Crevecoeur S, Vincent WF, Comte J, Lovejoy C (2015) Bacterial community structure across

environmental gradients in permafrost thaw ponds: Methanotroph-rich ecosystems. Front Microbiol 6:1–15. doi: 10.3389/fmicb.2015.00192

Dall'Agnol HPMB, Baraúna R a, de Sá PHCG, et al (2014) Omics profiles used to evaluate the gene expression of Exiguobacterium antarcticum B7 during cold adaptation. BMC Genomics 15:986. doi: 10.1186/1471-2164-15-986

Dammel CS, Noller HF (1995) Suppression of a cold-sensitive mutation in 16s rRNA by overexpression of a novel ribosome-binding factor, RbfA. Genes Dev 9:626–637. doi: 10.1101/gad.9.5.626

Davies PL, Baardsnes J, Kuiper MJ, Walker VK (2002) Structure and function of antifreeze proteins. Philos Trans R Soc Lond B Biol Sci 357:927–935. doi: 10.1098/rstb.2002.1081

De Maayer P, Anderson D, Cary C, Cowan D a (2014) Some like it cold: understanding the survival strategies of psychrophiles. EMBO Rep 15:508–517. doi: 10.1002/embr.201338170

de Mendoza D, Cronan Jr JE (1983) Thermal regulation of membrane lipid fluidity in bacteria. Trends Biochem Sci 8:49–52. doi: 10.1016/0968-0004(83)90388-2

Deana A, Belasco JG (2004) The function of RNase G in Escherichia coli is constrained by its amino and carboxyl termini. Mol Microbiol 51:1205–1217. doi: 10.1046/j.1365-2958.2003.03905.x

Deng W, Li C, Xie J (2013) The underling mechanism of bacterial TetR/AcrR family transcriptional repressors. Cell Signal 25:1608–1613. doi: 10.1016/j.cellsig.2013.04.003

Donegan NP, Thompson ET, Fu Z, Cheung AL (2010) Proteolytic regulation of toxin-antitoxin systems by ClpPc in Staphylococcus aureus. J Bacteriol 192:1416–1422. doi: 10.1128/JB.00233-09

Doyle S, Dieser M, Broemsen E, Christner B (2012) General Characteristics of Cold-Adapted Microorganisms. In: Miller R V, Whyte LG (eds) Polar Microbiology: Life in a Deep Freeze. ASM Press, Washington D.C., pp 103–125

Fedøy A-E, Yang N, Martinez A, et al (2007) Structural and functional properties of isocitrate dehydrogenase from the psychrophilic bacterium Desulfotalea psychrophila reveal a cold-active enzyme with an unusual high thermal stability. J Mol Biol 372:130–49. doi: 10.1016/j.jmb.2007.06.040

Feller G, Lonhienne T, Deroanne C, et al (1992) Purification, Characterization, and Nucleotide Sequence of the Thermolabile alpha Amylase from the Antarctic Psychrotroph Alteromonas haloplanctis A23. J Biol Chem 267:5217–5221.

Fenton HJH (1894) Oxidation of Tartaric Acid in the Presence of Iron. J Chem Soc 65:899–910. doi: doi:10.1039/ct8946500899

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. Science (80-) 281:237–40. doi: 10.1126/science.281.5374.237

Figge RM (2006) Methionine Biosynthesis in Escherichia coli and Corynebacterium glutamicum. In: Wendisch VF (ed) Amino Acid Biosynthesis. Springer-Verlag, Berlin Heidelberg, pp 163–193

Fléchard M, Gilot P (2014) Physiological impact of transposable elements encoding DDE transposases in the environmental adaptation of Streptococcus agalactiae. Microbiology 160:1298–1315. doi: 10.1099/mic.0.077628-0

Galiniers A, Garnerone A, Kahn D, Batutn J (1994) Phosphorylation of the Rhizobium meZiZoti FixJ Protein Induces Its fixK Promoter * Binding to a Compound Regulatory Region at the. Biochemistry 23784–23789.

Gao H, Yang ZK, Wu L, et al (2006) Global transcriptome analysis of the cold shock response of Shewanella oneidensis MR-1 and mutational analysis of its classical cold shock proteins. J Bacteriol 188:4560–4569. doi: 10.1128/JB.01908-05

García-Descalzo L, García-López E, Alcázar A, et al (2014) Proteomic analysis of the adaptation to warming in the Antarctic bacteria Shewanella frigidimarina. Biochim Biophys Acta - Proteins Proteomics 1844:2229–2240. doi: 10.1016/j.bbapap.2014.08.006

Garcia A, Quivey RG, Cross B, Faustoferri R (2016) PlsX deletion impacts fatty acid synthesis and acid adaptation in Streptococcus mutans. Microbiology 162:662–671. doi: 10.1099/mic.0.000252

Gardiol A, Arias A, Cervenansky C, et al (1980) Biochemical characterization of a fructokinase mutant of Rhizobium meliloti. J Bacteriol 144:12–16.

Georlette D, Jónsson ZO, Van Petegem F, et al (2000) A DNA ligase from the psychrophile Pseudoalteromonas haloplanktis gives insights into the adaptation of proteins to low temperatures. Eur J Biochem 267:3502–12.

Gerdes K, Christensen SK, Lobner-Olesen A (2005) Prokaryotic Toxin-Antitoxin Stress Response Loci. Nat Rev Microbiol 3:371–382. doi: 10.1038/nrmicro1147

Gilichinsky DA, Soina VS, Petrova MA (1993) Cryoprotective properties of water in the Earth cryolithosphere and its role in exobiology. Orig Life Evol Biosph 23:65–75.

Gilichinsky DA, Wilson GS, Friedmann EI, et al (2007) Microbial Populations in Antarctic Permafrost: Biodiversity, State, Age, and Implication for Astrobiology. Astrobiology 7:275–311. doi: 10.1089/ast.2006.0012

Gilichinsky D, Rivkina E, Bakermans C, et al (2005) Biodiversity of cryopegs in permafrost. FEMS

Microbiol Ecol 53:117–128. doi: 10.1016/j.femsec.2005.02.003

Gilichinsky D, Rivkina E, Shcherbakova V, et al (2003) Supercooled Water Brines Within Permafrost—An Unknown Ecological Niche for Microorganisms: A Model for Astrobiology. Astrobiology 3:331–341. doi: 10.1089/153110703769016424

Gilichinsky D, Vishnivetskaya T, Petrova M, et al (2008) Bacteria in Permafrost. In: Margesin R, Schinner F, Marx J-C, Gerday C (eds) Psychrophiles: from Biodiversity to Biotechnology. Springer Berlin Heidelberg, pp 83–102

Gleiter S, Bardwell JCA (2008) Disulfide bond isomerization in prokaryotes Stefan. Biochim Biophys Acta 1783:530–534.

Gnerre S, Maccallum I, Przybylski D, et al (2011) High-quality draft assemblies of mammalian genomes from massively parallel sequence data. Proc Natl Acad Sci U S A 108:1513–8. doi: 10.1073/pnas.1017351108

Goodchild A, Raftery M, Saunders NFW, et al (2004) Biology of the cold-adapted archaeon, Methanococcoides burtonii, determined by proteomics using liquid chromatography-tandem mass spectrometry. J Proteome Res 3:1164–1176. doi: 10.1021/pr0498988

Goodchild A, Raftery M, Saunders NFW, et al (2005) Cold Adaptation of the Antarctic Archaeon , Methanococcoides burtonii Assessed by Proteomics Using ICAT. J Proteome Res 4:473–480.

Goordial J, Davila A, Lacelle D, et al (2016a) Nearing the cold-arid limits of microbial life in permafrost of an upper dry valley, Antarctica. ISME J 10:1–12. doi: 10.1038/ismej.2015.239

Goordial J, Lamarche- G, Lay C, Whyte L (2012) Left out in the cold: Life in Cryoenvironments. In: Seckbach J, Oren A, Stan-Lotter H (eds) Polyextremophiles. Springer Netherlands, pp 335–363

Goordial J, Raymond-Bouchard I, Zolotarov Y, et al (2016b) Cold adaptive traits revealed by comparative genomic analysis of the eurypsychrophile Rhodococcus sp. JG3 isolated from high elevation McMurdo Dry Valley permafrost, Antarctica. FEMS Microbiol Ecol 92:1–11. doi: 10.1093/femsec/fiv154

Grove A (2013) MarR family transcription factors. Curr Biol 23:R142–R143. doi: 10.1016/j.cub.2013.01.013

Gunde-Cimerman N, Wagner D, Häggblom M (2012) Polar and alpine microbiology. FEMS Microbiol Ecol 82:215–6. doi: 10.1111/1574-6941.12004

Ha HC, Sirisoma NS, Kuppusamy P, et al (1998) The natural polyamine spermine functions directly as a free radical scavenger. Proc Natl Acad Sci U S A 95:11140–5. doi: 10.1073/pnas.95.19.11140

Hall EK, Neuhauser C, Cotner JB (2008) Toward a mechanistic understanding of how natural bacterial communities respond to changes in temperature in aquatic ecosystems. ISME J 2:471–481.

Hamma T, Ferré-D'Amaré AR (2006) Pseudouridine Synthases. Chem Biol 13:1125–1135. doi: 10.1016/j.chembiol.2006.09.009

Hettich LR, Pan C, Chourey K, Giannone RJ (2013) Metaproteomics: Harnessing the power of high performance mass spectrometry to identify the suite of proteins that control metabolic activities in microbial communities. Anal Chem 85:4203–4214. doi: 10.1021/ac303053e.Metaproteomics

Hinsa-Leasure SM, Bhavaraju L, Rodrigues JLM, et al (2010) Characterization of a bacterial community from a Northeast Siberian seacoast permafrost sample. FEMS Microbiol Ecol 74:103–13. doi: 10.1111/j.1574-6941.2010.00945.x

Hosfield DJ, Guan Y, Haas BJ, et al (1999) Structure of the DNA repair enzyme endonuclease IV and its DNA complex: Double-nucleotide flipping at abasic sites and three-metal-ion catalysis. Cell 98:397–408. doi: 10.1016/S0092-8674(00)81968-6

Hultman J, Waldrop MP, Mackelprang R, et al (2015) Multi-omics of permafrost, active layer and thermokarst bog soil microbiomes. Nature 521:208–212. doi: 10.1038/nature14238

Hunt MC, Alexson SEH (2002) The role Acyl-CoA thioesterases play in mediating intracellular lipid metabolism. Prog Lipid Res 41:99–130. doi: 10.1016/S0163-7827(01)00017-0

Huston AL, Methe B, Deming JW (2004) Purification , Characterization , and Sequencing of an Extracellular Cold-Active Aminopeptidase Produced by Marine Psychrophile Colwellia psychrerythraea Strain 34H. Appl Environ Microbiol 70:3321–3328. doi: 10.1128/AEM.70.6.3321

Hwang Y s., Jung G, Jin E (2008) Transcriptome analysis of acclimatory responses to thermal stress in Antarctic algae. Biochem Biophys Res Commun 367:635–641. doi: 10.1016/j.bbrc.2007.12.176

Hyatt D, Chen G-L, Locascio PF, et al (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119. doi: 10.1186/1471-2105-11-119

Irgens RL, Gosink JJ, Staley JT (1996) Polaromonas vacuolata gen. nov., sp. nov., a Psychrophilic, Marine, Gas Vacuolate Bacterium from Antarctica. Int J Syst Bacteriol 46:822–826.

Jones DS, Flood BE, Bailey J V. (2015) Metatranscriptomic analysis of diminutive Thiomargarita-like bacteria ("Candidatus Thiopilula" spp.) from abyssal cold seeps of the barbados accretionary prism. Appl Environ Microbiol 81:3142–3156. doi: 10.1128/AEM.00039-15

Jones PG, Inouye M (1996) RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. Mol Microbiol 21:1207–1218. doi: 10.1111/j.1365-2958.1996.tb02582.x

Kawamoto J, Kurihara T, Esaki N (2017) Proteomic Insights of Psychrophiles. In: Margesin R (ed) Psychrophiles: From Biodiversity to Biotechnology, 2nd edn. pp 423–435

Kawamoto J, Kurihara T, Kitagawa M, et al (2007) Proteomic studies of an Antarctic cold-adapted bacterium, Shewanella livingstonensis Ac10, for global identification of cold-inducible proteins. Extremophiles 11:819–26. doi: 10.1007/s00792-007-0098-6

Keller AN, Yang X, Wiedermannová J, et al (2014) ε, a new subunit of RNA polymerase found in gram-positive bacteria. J Bacteriol 196:3622–32. doi: 10.1128/JB.02020-14

Kim S, Kim MJ, Jung MG, et al (2013) De novo transcriptome analysis of an Arctic microalga, Chlamydomonas sp. Genes and Genomics 35:215–223. doi: 10.1007/s13258-013-0085-5

Kirby BM, Easton S, Tuffin IM, Cowan DA (2012) Bacterial Diversity in Polar Habitats. In: Miller R V, Whyte LG (eds) Polar Microbiology: Life in a Deep Freeze. ASM Press, Washington D.C., pp 3–31

Koh HY, Park H, Lee JH, et al (2017) Proteomic and transcriptomic investigations on cold-responsive properties of the psychrophilic Antarctic bacterium Psychrobacter sp. PAMC 21119 at subzero temperatures. Environ Microbiol 19:628–644. doi: 10.1111/1462-2920.13578

Kuhn E (2012) Toward understanding life under subzero conditions: the significance of exploring psychrophilic “cold-shock” proteins. Astrobiology 12:1078–86. doi: 10.1089/ast.2012.0858

Kvint K, Nachin L, Diez A, Nyström T (2003) The bacterial universal stress protein: Function and regulation. Curr Opin Microbiol 6:140–145. doi: 10.1016/S1369-5274(03)00025-0

La Terza A, Papa G, Miceli C, Luporini P (2001) Divergence between two Antarctic species of the ciliate Euplotes, E. focardii and E. nobilii, in the expression of heat-shock protein 70 genes. Mol Ecol 10:1061–1067. doi: 10.1046/j.1365-294X.2001.01242.x

Lacelle D, Radtke K, Clark ID, et al (2011) Geomicrobiology and occluded O2–CO2–Ar gas analyses provide evidence of microbial respiration in ancient terrestrial ground ice. Earth Planet Sci Lett 306:46–54. doi: 10.1016/j.epsl.2011.03.023

Lau MC, Stackhouse BT, Layton AC, et al (2015) An active atmospheric methane sink in high Arctic mineral cryosols. ISME J 9:1880–1891. doi: 10.1038/ismej.2015.13

Lauro FM, Allen MA, Wilkins D, et al (2011) Psychrophiles: Genetics, Genomics, Evolution. In: Horikoshi K (ed) Extremophiles Handbook. Springer, pp 1118–1130

Lauro FM, Tran K, Vezzi A, et al (2008) Large-scale transposon mutagenesis of Photobacterium profundum SS9 reveals new genetic loci important for growth at low temperature and high pressure. J Bacteriol 190:1699–1709. doi: 10.1128/JB.01176-07

Lee C, Park C (2017) Bacterial Responses to Glyoxal and Methylglyoxal: Reactive Electrophilic Species. Int J Mol Sci 18:169. doi: 10.3390/ijms18010169

Lee M, Hesek D, Suvorov M, et al (2003) A Mechanism-Based Inhibitor Targeting the DD - Transpeptidase Activity of Bacterial Penicillin-Binding Proteins. J Am Chem Soc 125:16322–16326. doi: 10.1021/ja038445l

Li H, Liu X, Wang Y, et al (2009) Enhanced expression of antifreeze protein genes drives the development of freeze tolerance in an Antarctica isolate of Chlorella vulgaris. Prog Nat Sci 19:1059–1062. doi: 10.1016/j.pnsc.2008.10.012

Li M, Copeland A, Han J (2011) DUK – A Fast and Efficient Kmer Matching Tool. Lawrence Berkeley Natl Lab LBNL Pap LBNL-4516E-Poster p 1–3.

Lide D (2005) Aqueous Solubility of Inorganic Compounds at Various Temperatures. In: CRC Handbook of Chemistry and Physics, 87th edn. CRC Press, pp 1–6

Lin TH, Hu YN, Shaw GC (2014) Two enzymes, TilS and HprT, can form a complex to function as a transcriptional activator for the cell division protease gene ftsH in Bacillus subtilis. J Biochem 155:5–16. doi: 10.1093/jb/mvt081

Linding R, Jensen LJ, Diella F, et al (2003) Protein disorder prediction: Implications for structural proteomics. Structure 11:1453–1459. doi: 10.1016/j.str.2003.10.002

Liu C, Huang X (2015) Transcriptome-wide analysis of DEAD-box RNA helicase gene family in an Antarctic psychrophilic alga Chlamydomonas sp. ICE-L. Extremophiles 19:921–931. doi: 10.1007/s00792-015-0768-8

Liu C, Yu X (2015) ADP-ribosyltransferases and poly ADP-ribosylation. Curr Protein Pept Sci 16:491–501.

Liu X, Wang Y, Gao H, Xu X (2011) Identification and characterization of genes encoding two novel LEA proteins in Antarctic and temperate strains of Chlorella vulgaris. Gene 482:51–58. doi: 10.1016/j.gene.2011.05.006

López-García P, Forterre P (2000) DNA topology and the thermal stress response, a tale from mesophiles and hyperthermophiles. BioEssays 22:738–746. doi: 10.1002/1521-1878(200008)22:8<738::AID-BIES7>3.0.CO;2-5

Lowe TM, Eddy SR (1996) TRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res 25:955–964. doi: 10.1093/nar/25.5.0955

Ludwig B, Akundi A, Kendall K, et al (1995) A Long-Chain Secondary Alcohol Dehydrogenase from Rhodococcus erythropolis ATCC 4277 . These include : A Long-Chain Secondary Alcohol Dehydrogenase from Rhodococcus erythropolis ATCC 4277. 61:3729–3733.

Mader HM, Pettitt ME, Wadham JL, et al (2006) Subsurface ice as a microbial habitat. Geology 34:169. doi: 10.1130/G22096.1

Maki S, Yoneta M, Takada Y (2006) Two isocitrate dehydrogenases from a psychrophilic bacterium, Colwellia psychrerythraea. Extremophiles 10:237–249. doi: 10.1007/s00792-005-0493-9

Margesin R, Spröer C, Zhang DC, Busse HJ (2012) Polaromonas glacialis sp. nov. and Polaromonas cryoconiti sp. nov., isolated from alpine glacier cryoconite. Int J Syst Evol Microbiol 62:2662–2668. doi: 10.1099/ijs.0.037556-0

Markowitz VM, Mavromatis K, Ivanova NN, et al (2009) IMG ER: A system for microbial genome annotation expert review and curation. Bioinformatics 25:2271–2278. doi: 10.1093/bioinformatics/btp393

Math RK, Jin HM, Kim JM, et al (2012) Comparative genomics reveals adaptation by Alteromonas sp. SN2 to marine tidal-flat conditions: Cold tolerance and aromatic hydrocarbon metabolism. PLoS One. doi: 10.1371/journal.pone.0035784

Mazzon RR, Lang EAS, Silva CAPT, Marques M V. (2012) Cold shock genes CspA and CspB from caulobacter crescentus are post transcriptionally regulated and important for cold adaptation. J Bacteriol 194:6507–6517. doi: 10.1128/JB.01422-12

Menozzi FD, Rouse JH, Alavi M, et al (1996) Identification of a heparin-binding hemagglutinin present in mycobacteria. J Exp Med 184:993–1001. doi: 10.1084/jem.184.3.993

Methé BA, Nelson KE, Deming JW, et al (2005) The psychrophilic lifestyle as revealed by the genome sequence of Colwellia psychrerythraea 34H through genomic and proteomic analyses. Proc Natl Acad Sci U S A 102:10913–8. doi: 10.1073/pnas.0504766102

Metpally RPR, Reddy BVB (2009) Comparative proteome analysis of psychrophilic versus mesophilic bacterial species: Insights into the molecular basis of cold adaptation of proteins. BMC Genomics 10:11. doi: 10.1186/1471-2164-10-11

Miller R V, Whyte LG (eds) (2012) Polar Microbiology: Life in a Deep Freeze. ASM Press, Washington D.C.

Minkovsky N, Zarimani A, Chary VK, et al (2002) Bex, the Bacillus subtilis homolog of the essential Escherichia coli GTPase era, is required for normal cell division and spore formation. J Bacteriol 184:6389–6394. doi: 10.1128/JB.184.22.6389-6394.2002

Miyauchi K, Kimura S, Suzuki T (2013) A cyclic form of N6-threonylcarbamoyladenosine as a

widely distributed tRNA hypermodification. Nat Chem Biol 9:105–11. doi: 10.1038/nchembio.1137

Mock T, Hoch N (2005) Long-term temperature acclimation of photosynthesis in steady-state cultures of the polar diatom Fragilariopsis cylindrus. Photosynth Res 85:307–317. doi: 10.1007/s11120-005-5668-9

Mock T, Krell A, Valentin K, et al (2005) Analysis of Expressed Sequence Tags (ESTs) from the Polar Diatom Fragilariopsis cylindrus. J Phycol 42:78–85. doi: 10.1111/j.1529-8817.2005.00164.x

Mulligan C, Fischer M, Thomas GH (2011) Tripartite ATP-independent periplasmic (TRAP) transporters in bacteria and archaea. FEMS Microbiol Rev 35:68–86. doi: 10.1111/j.1574-6976.2010.00236.x

Mykytczuk NC, Foote SJ, Omelon CR, et al (2013) Bacterial growth at -15 °C; molecular insights from the permafrost bacterium Planococcus halocryophilus Or1. ISME J 7:1211–26. doi: 10.1038/ismej.2013.8

Mykytczuk NCS, Lawrence JR, Omelon CR, et al (2015) Microscopic characterization of the bacterial cell envelope of Planococcus halocryophilus Or1 during subzero growth at −15 °C. Polar Biol 39:701–712. doi: 10.1007/s00300-015-1826-5

Mykytczuk NCS, Trevors JT, Foote SJ, et al (2011) Proteomic insights into cold adaptation of psychrotrophic and mesophilic Acidithiobacillus ferrooxidans strains. Antonie Van Leeuwenhoek 100:259–77. doi: 10.1007/s10482-011-9584-z

Mykytczuk NCS, Wilhelm RC, Whyte LG (2012) Planococcus halocryophilus sp. nov., an extreme sub-zero species from high Arctic permafrost. Int J Syst Evol Microbiol 62:1937–44. doi: 10.1099/ijs.0.035782-0

Nakajima T (2015) Roles of Sulfur Metabolism and Rhodanese in Detoxification and Anti-Oxidative Stress Functions in the Liver : Responses to Radiation Exposure. Med Sci Monit 21:1721–1725. doi: 10.12659/MSM.893234

Nawrocki EP, Eddy SR (2013) Infernal 1.1: 100-fold faster RNA homology searches. Bioinformatics 29:2933–2935. doi: 10.1093/bioinformatics/btt509

Nedwell DD. (1999) Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. FEMS Microbiol Ecol 30:101–111. doi: 10.1111/j.1574-6941.1999.tb00639.x

Ng C, DeMaere MZ, Williams TJ, et al (2010) Metaproteogenomic analysis of a dominant green sulfur bacterium from Ace Lake, Antarctica. ISME J 4:1002–1019. doi: 10.1038/ismej.2010.28

North JA, Sriram J, Chourey K, et al (2016) Metabolic Regulation as a Consequence of Anaerobic 5-Methylthioadenosine Recycling in Rhodospirillum rubrum. MBio 7:e00855-16. doi: 10.1128/mBio.00855-16.Editor

Nunn BL, Slattery K V., Cameron KA, et al (2015) Proteomics of Colwellia psychrerythraea at subzero temperatures - a life with limited movement, flexible membranes and vital DNA repair. Environ Microbiol 17:2319–2335. doi: 10.1111/1462-2920.12691

Osinski GR, Léveil-lé R, Berinstain A, et al (2006) Terrestrial Analogues to Mars and the Moon: Canada's Role. Geosci Canada 33:175–188.

Paget MSB, Helmann JD (2003) The sigma70 family of sigma factors. Genome Biol 4:203. doi: 10.1186/gb-2003-4-1-203

Paoletti AC, Parmely TJ, Tomomori-Sato C, et al (2006) Quantitative proteomic analysis of distinct mammalian Mediator complexes using normalized spectral abundance factors. Proc Natl Acad Sci U S A 103:18928–18933. doi: 10.1073/pnas.0606379103

Paoletti L, Lu YJ, Schujman GE, et al (2007) Coupling of fatty acid and phospholipid synthesis in Bacillus subtilis. J Bacteriol 189:5816–5824. doi: 10.1128/JB.00602-07

Park S-K, Jin ES, Lee M-Y (2008) Expression and antioxidant enzymes in Chaetoceros neogracile, an Antarctic alga. Cryo Letters 29:351–61.

Parsons JB, Frank MW, Eleveld MJ, et al (2015) A thioesterase bypasses the requirement for exogenous fatty acids in the plsX deletion of Streptococcus pneumoniae. Mol Microbiol 96:28–41. doi: 10.1111/mmi.12916

Parsons JB, Frank MW, Jackson P, et al (2014) Incorporation of extracellular fatty acids by a fatty acid kinase-dependent pathway in Staphylococcus aureus. Mol Microbiol 92:234–245. doi: 10.1111/mmi.12556

Pati A, Ivanova NN, Mikhailova N, et al (2010) GenePRIMP: a gene prediction improvement pipeline for prokaryotic genomes. Nat Methods 7:455–457. doi: 10.1038/nmeth.1457

Pearson GA, Lago-Leston A, Canovas F, et al (2015) Metatranscriptomes reveal functional variation in diatom communities from the Antarctic Peninsula. Isme J 9:2275–2289. doi: 10.1038/ismej.2015.40

Peng X, Taki H, Komukai S, et al (2006) Characterization of four Rhodococcus alcohol dehydrogenase genes responsible for the oxidation of aromatic alcohols. Appl Microbiol Biotechnol 71:824–832. doi: 10.1007/s00253-005-0204-6

Pereira-Medrano AG, Margesin R, Wright PC (2012) Proteome characterization of the unsequenced psychrophile Pedobacter cryoconitis using 15N metabolic labeling, tandem mass spectrometry, and a new bioinformatic workflow. Proteomics 12:775–789. doi:

10.1002/pmic.201100159

Piette F, D'Amico S, Mazzucchelli G, et al (2011a) Life in the cold: A proteomic study of cold-repressed proteins in the antarctic bacterium Pseudoalteromonas haloplanktis TAC125. Appl Environ Microbiol 77:3881–3883. doi: 10.1128/AEM.02757-10

Piette F, D'Amico S, Struvay C, et al (2010) Proteomics of life at low temperatures: trigger factor is the primary chaperone in the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125. Mol Microbiol 76:120–32. doi: 10.1111/j.1365-2958.2010.07084.x

Piette F, Struvay C, Feller G (2011b) The protein folding challenge in psychrophiles: Facts and current issues. Environ Microbiol 13:1924–1933. doi: 10.1111/j.1462-2920.2011.02436.x

Pollard W, Haltigin T, Whyte L, et al (2009) Overview of analogue science activities at the McGill Arctic Research Station, Axel Heiberg Island, Canadian High Arctic. Planet Space Sci 57:646–659. doi: 10.1016/j.pss.2009.01.008

Pruesse E, Quast C, Knittel K, et al (2007) SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196. doi: 10.1093/nar/gkm864

Pruss GJ, Drlica K (1989) DNA Supercoiling and Prokatyotic Transcription Minireview. Cell 56:521–523.

Qin QL, Xie B Bin, Yu Y, et al (2013) Comparative genomics of the marine bacterial genus Glaciecola reveals the high degree of genomic diversity and genomic characteristic for cold adaptation. Environ Microbiol 16:1642–1653. doi: 10.1111/1462-2920.12318

Qiu Y, Kathariou S, Lubman DM (2006) Proteomic analysis of cold adaptation in a Siberian permafrost bacterium - Exiguobacterium sibiricum 255-15 by two-dimensional liquid separation coupled with mass spectrometry. Proteomics 6:5221–5233. doi: 10.1002/pmic.200600071

Quinlan AR, Hall IM (2010) BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26:841–842. doi: 10.1093/bioinformatics/btq033

Rabin RS, Stewart V (1993) Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in Escherichia coli K-12. J Bacteriol 175:3259–3268.

Rabus R, Ruepp A, Frickey T, et al (2004) The genome of Desulfotalea psychrophila, a sulfate-reducing bacterium from permanently cold Arctic sediments. Environ Microbiol 6:887–902. doi: 10.1111/j.1462-2920.2004.00665.x

R Core Team (2017) R: A language and environment for statistical computing.

Ræder ILU, Leiros I, Willassen NP, et al (2008) Uracil-DNA N-glycosylase (UNG) from the marine, psychrophilic bacterium Vibrio salmonicida shows cold adapted features. Enzyme Microb Technol 42:594–600. doi: 10.1016/j.enzmictec.2008.02.005

Ramachandran V, Chandrakala B, Kumar VP, et al (2006) Screen for Inhibitors of the Coupled Transglycosylase-Transpeptidase of Peptidoglycan Biosynthesis in Escherichia coli Screen for Inhibitors of the Coupled Transglycosylase-Transpeptidase of Peptidoglycan Biosynthesis in Escherichia coli. Antimicrob Agents Chemother 50:1425–1432. doi: 10.1128/AAC.50.4.1425

Raymond-Bouchard I, Whyte LG (2017) From Transcriptomes to Metatranscriptomes: Cold Adaptation and Active Metabolisms of Psychrophiles from Cold Environments. In: Margesin R (ed) Psychrophiles: From Biodiversity to Biotechnology, 2nd edn. Springer International, pp 437–457

Raymond-Bouchard I, Chourey K, Altshuler I, et al (2017) Mechanisms of subzero growth in the cryophile Planococcus halocryophilus determined through proteomic analysis. Environ Microbiol. doi: 10.1111/1462-2920.13893

Reiersen H, Rees AR (2001) The hunchback and its neighbours: Proline as an environmental modulator. Trends Biochem Sci 26:679–684. doi: 10.1016/S0968-0004(01)01957-0

Robinson MD, McCarthy DJ, Smyth GK (2009) edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. doi: 10.1093/bioinformatics/btp616

Rodrigues DF, Ivanova N, He Z, et al (2008) Architecture of thermal adaptation in an Exiguobacterium sibiricum strain isolated from 3 million year old permafrost: a genome and transcriptome approach. BMC Genomics 9:547. doi: 10.1186/1471-2164-9-547

Rodrigues DF, Tiedje JM (2008) Coping with our cold planet. Appl Environ Microbiol 74:1677–1686. doi: 10.1128/AEM.02000-07

Rodríguez-Moya J, Argandoña M, Reina-Bueno M, et al (2010) Involvement of EupR, a response regulator of the NarL/FixJ family, in the control of the uptake of the compatible solutes ectoines by the halophilic bacterium Chromohalobacter salexigens. BMC Microbiol 10:256. doi: 10.1186/1471-2180-10-256

Ronholm J, Raymond-Bouchard I, Creskey M, et al (2015) Characterizing the surface-exposed proteome of Planococcus halocryophilus during cryophilic growth. Extremophiles 19:619–629. doi: 10.1007/s00792-015-0743-4

Roof DM, Roth JR (1988) Ethanolamine utilization in *Salmonella typhimurium*. J Bacteriol 170:3855–63.

Roux D, Cywes-Bentley C, Zhang YF, et al (2015) Identification of Poly-N-acetylglucosamine as a major polysaccharide component of the bacillus subtilis biofilm matrix. J Biol Chem 290:19261–19272. doi: 10.1074/jbc.M115.648709

Russell RJ, Gerike U, Danson MJ, et al (1998) Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. Structure 6:351–61.

Saikolappan S, Das K, Sasindran SJ, et al (2011) OsmC proteins of Mycobacterium tuberculosis and Mycobacterium smegmatis protect against organic hydroperoxide stress. Tuberculosis 91:1–22. doi: 10.1016/j.tube.2011.10.021

Sauert M, Wolfinger MT, Vesper O, et al (2016) The MazF-regulon: A toolbox for the post-transcriptional stress response in Escherichia coli. Nucleic Acids Res 44:6660–6675. doi: 10.1093/nar/gkw115

Saunders NFW, Thomas T, Curmi PMG, et al (2003) Mechanisms of thermal adaptation revealed from the genomes of the Antarctic Archaea Methanogenium frigidum and Methanococcoides burtonii. Genome Res 13:1580–8. doi: 10.1101/gr.1180903

Seckbach J, Oren A, Stan-lotter H (eds) (2013) Polyextremophiles. Springer

Seo JB, Kim HS, Jung GY, et al (2004) Psychrophilicity of Bacillus psychrosaccharolyticus: A proteomic study. Proteomics 4:3654–3659. doi: 10.1002/pmic.200401025

Shah P, Swiatlo E (2008) A multifaceted role for polyamines in bacterial pathogens. Mol Microbiol 68:4–16. doi: 10.1111/j.1365-2958.2008.06126.x

Shao F, Bader MW, Jakob U, Bardwell JCA (2000) DsbG, a Protein Disulfide Isomerase with Chaperone Activity. J Biol Chem 275:13349–13352. doi: 10.1074/jbc.275.18.13349

Sharma MR, Koc EC, Datta PP, et al (2003) Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. Cell 115:97–108. doi: 10.1016/S0092-8674(03)00762-1

Sharma N, Goswami UC (2011) Functioning of Lycopene in Mammalian System: A Review. Proc Zool Soc 64:1–7. doi: 10.1007/s12595-011-0005-0

Sharma R, Dill BD, Chourey K, et al (2012) Coupling a detergent lysis/cleanup methodology with intact protein fractionation for enhanced proteome characterization. J Proteome Res 11:6008–6018. doi: 10.1021/pr300709k

Shen L, Yao T, Liu Y, et al (2014) Downward-shifting Temperature Range for the Growth of Snow-bacteria on Glaciers of the Tibetan Plateau. Geomicrobiol J 00–00. doi: 10.1080/01490451.2014.891418

Shi T, Reeves RH, Gilichinsky DA, Friedmann EI (1997) Characterization of Viable Bacteria from

Siberian Permafrost by 16S rDNA Sequencing. Microb Ecol 33:169–179.

Siddiqui KS, Williams TJ, Wilkins D, et al (2013) Psychrophiles. Annu Rev Earth Planet Sci 41:87–115. doi: 10.1146/annurev-earth-040610-133514

Steven B, Briggs G, McKay CP, et al (2007a) Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. FEMS Microbiol Ecol 59:513–23. doi: 10.1111/j.1574-6941.2006.00247.x

Steven B, Léveillé R, Pollard WH, Whyte LG (2006) Microbial ecology and biodiversity in permafrost. Extremophiles 10:259–67. doi: 10.1007/s00792-006-0506-3

Steven B, Niederberger TD, Bottos EM, et al (2007b) Development of a sensitive radiorespiration method for detecting microbial activity at subzero temperatures. J Microbiol Methods 71:275–80. doi: 10.1016/j.mimet.2007.09.009

Steven B, Niederberger TD, Whyte LG (2009) Bacterial and Archaeal Diversity in Permafrost. In: Permafrost Soils. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 59–72

Steven B, Pollard WH, Greer CW, Whyte LG (2008) Microbial diversity and activity through a permafrost/ground ice core profile from the Canadian high Arctic. Environ Microbiol 10:3388–3403. doi: 10.1111/j.1462-2920.2008.01746.x

Stevenson A, Cray JA, Williams JP, et al (2015) Is there a common water-activity limit for the three domains of life? ISME J 9:1333–1351. doi: 10.1038/ismej.2014.219

Sunnarborg A, Klumpp D, Chung T, LaPorte DC (1990) Regulation of the glyoxylate bypass operon: Cloning and characterization of iclR. J Bacteriol 172:2642–2649.

Suvorova IA, Korostelev YD, Gelfand MS (2015) GntR Family of Bacterial Transcription Factors and Their DNA Binding Motifs: Structure, Positioning and Co-Evolution. PLoS One 10:e0132618. doi: 10.1371/journal.pone.0132618

Tabb DL, Fernando CG, Chambers MC (2008) MyriMatch:highly accurate tandem mass spectral peptide identificaiton by multivariate hypergeometric analysis. J Proteome Res 6:654–661. doi: 10.1021/pr0604054.MyriMatch

Tamppari LK, Anderson RM, Archer PD, et al (2012) Effects of extreme cold and aridity on soils and habitability: McMurdo Dry Valleys as an analogue for the Mars Phoenix landing site. Antarct Sci 24:211–228. doi: 10.1017/S0954102011000800

Tamura K, Stecher G, Peterson D, et al (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. doi: 10.1093/molbev/mst197

Thomas T, Cavicchioli R (1998) Archaeal cold-adapted proteins: structural and evolutionary

analysis of the elongation factor 2 proteins from psychrophilic, mesophilic and thermophilic methanogens. FEBS Lett 439:281–6.

Thomas T, Cavicchioli R (2000) Effect of Temperature on Stability and Activity of Elongation Factor 2 Proteins from Antarctic and Thermophilic Methanogens. J Bacteriol 182:1328–1332. doi: 10.1128/JB.182.5.1328-1332.2000.Updated

Thomas T, Kumar N, Cavicchioli R (2001) Effects of Ribosomes and Intracellular Solutes on Activities and Stabilities of Elongation Factor 2 Proteins from Psychrotolerant and Thermophilic Methanogens. J Bacteriol 183:1974–1982. doi: 10.1128/JB.183.6.1974

Thompson MR, Verberkmoes NC, Chourey K, et al (2007) Dosage-Dependent Proteome Response of Shewanella oneidensis MR-1 to Chromate Insult. J Proteome Res 6:1745–1757.

Ting L, Williams TJ, Cowley MJ, et al (2010) Cold adaptation in the marine bacterium, Sphingopyxis alaskensis, assessed using quantitative proteomics. Environ Microbiol 12:2658–2676. doi: 10.1111/j.1462-2920.2010.02235.x

Tiwari P, Arora G, Singh M, et al (2015) MazF ribonucleases promote Mycobacterium tuberculosis drug tolerance and virulence in guinea pigs. Nat Commun 6:6059. doi: 10.1038/ncomms7059

Tomoyasu T, Yuki T, Morimura S, et al (1993) The E. coli FtsH protein is a prokaryotic member of a protein family involved in membrane functions, cell cycle control, and gene expression. J Bacteriol 1344:1344–1351.

Toseland A, Daines SJ, Clark JR, et al (2013) The impact of temperature on marine phytoplankton resource allocation and metabolism. Nat Clim Chang 3:979–984. doi: 10.1038/nclimate1989

Tribelli PM, Venero ECS, Ricardi MM, et al (2015) Novel essential role of ethanol oxidation genes at low temperature revealed by transcriptome analysis in the Antarctic bacterium Pseudomonas extremaustralis. PLoS One 10:e0145353. doi: 10.1371/journal.pone.0145353

Tripathy S, Sen R, Padhi SK, et al (2014) Upregulation of transcripts for metabolism in diverse environments is a shared response associated with survival and adaptation of Klebsiella pneumoniae in response to temperature extremes. Funct Integr Genomics 14:591–601. doi: 10.1007/s10142-014-0382-3

Troxell B, Hassan HM (2013) Transcriptional regulation by Ferric Uptake Regulator (Fur) in pathogenic bacteria. Front Cell Infect Microbiol 3:1–13. doi: 10.3389/fcimb.2013.00059

Tschitschko B, Williams TJ, Allen MA, et al (2016) Ecophysiological distinctions of haloarchaea

from a hypersaline Antarctic lake as determined by metaproteomics. Appl Environ Microbiol 82:3165–3173. doi: 10.1128/AEM.00473-16

Tsoy O, Ravcheev D, Mushegian A (2009) Comparative genomics of ethanolamine utilization. J Bacteriol 191:7157–7164. doi: 10.1128/JB.00838-09

Tveit AT, Urich T, Frenzel P, Svenning MM (2015) Metabolic and trophic interactions modulate methane production by Arctic peat microbiota in response to warming. Proc Natl Acad Sci U S A 112:E2507-16. doi: 10.1073/pnas.1420797112

Tyanova S, Temu T, Sinitcyn P, et al (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13:731–40. doi: 10.1038/nmeth.3901

Verstraeten N, Fauvart M, Versées W, Michiels J (2011) The universally conserved prokaryotic GTPases. Microbiol Mol Biol Rev 75:507–42, second and third pages of table of content. doi: 10.1128/MMBR.00009-11

Vishnivetskaya TA (2009) Viable Cyanobacteria and Green Algae from the Permafrost Darkness. In: Margesin R (ed) Permafrost Soils. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 73–84

Vorobyova E, Soina V, Gorlenko M, et al (1997) The deep cold biosphere: facts and hypothesis. FEMS Microbiol Rev 20:277–290. doi: 10.1111/j.1574-6976.1997.tb00314.x

Voshol GP, Meyer V, van den Hondel CAMJJ (2015) GTP-binding protein Era: a novel gene target for biofuel production. BMC Biotechnol 15:21. doi: 10.1186/s12896-015-0132-1

Wallon G, Lovett ST, Magyar C, et al (1997) Sequence and homology model of 3-isopropylmalate dehydrogenase from the psychrotrophic bacterium Vibrio sp. I5 suggest reasons for thermal instability. Protein Eng 10:665–72.

Wang J, Liu B, Liu G, et al (2015) Draft Genome Sequence of Sporosarcina globispora W 25 T (DSM 4), a Psychrophilic Bacterium Isolated from Soil and River Water. Genome Announc 3:2002–2003. doi: 10.1128/genomeA.01230-15.Copyright

Wang N, Xu B, Liu Y, et al (2016) Polaromonas eurypsychrophila sp. nov., isolated from an ice core. Int J Syst Evol Microbiol 66:2497–2501. doi: 10.1099/ijsem.0.001079

Wang X, Wood TK (2011) Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. Appl Environ Microbiol 77:5577–5583. doi: 10.1128/AEM.05068-11

Watanabe S, Yasutake Y, Tanaka I, Takada Y (2005) Elucidation of stability determinants of cold-adapted monomeric isocitrate dehydrogenase from a psychrophilic bacterium, Colwellia maris, by construction of chimeric enzymes. Microbiology 151:1083–94. doi:

10.1099/mic.0.27667-0

Wilhelm RC, Niederberger TD, Greer C, Whyte LG (2011) Microbial diversity of active layer and permafrost in an acidic wetland from the Canadian High Arctic. Can J Microbiol 57:303–315. doi: 10.1139/W11-004

Wilhelm RC, Radtke KJ, Mykytczuk NCS, et al (2012) Life at the wedge: the activity and diversity of arctic ice wedge microbial communities. Astrobiology 12:347–60. doi: 10.1089/ast.2011.0730

Williams P, Smith M (1989) The Frozen Earth: Fundamentals of Geocryomicrobiology. Cambridge University Press, Cambridge, United Kingdom

Williams TJ, Burg DW, Raftery MJ, et al (2010) Global Proteomic Analysis of the Insoluble, Soluble, and Supernatant Fractions of the Psychrophilic Archaeon Methanococcoides burtonii Part I : The Effect of Growth Temperature. J Proteome Res 9:640–652. doi: 10.1021/pr9005102

Williams TJ, Long E, Evans F, et al (2012) A metaproteomic assessment of winter and summer bacterioplankton from Antarctic Peninsula coastal surface waters. ISME J 6:1883–1900. doi: 10.1038/ismej.2012.28

Xiong W, Giannone RJ, Morowitz MJ, et al (2015) Development of an enhanced metaproteomic approach for deepening the microbiome characterization of the human infant gut. J Proteome Res 14:133–141. doi: 10.1021/pr500936p

Yan W, Xiao X, Zhang Y (2016) Complete genome sequence of the Sporosarcina psychrophila DSM 6497 , a psychrophilic Bacillus strain that mediates the calcium carbonate precipitation. J Biotechnol 226:14–15. doi: 10.1016/j.jbiotec.2016.03.036

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 208:2819–30. doi: 10.1242/jeb.01730

Yang L-L, Tang S-K, Huang Y, Zhi X-Y (2015) Low Temperature Adaptation Is Not the Opposite Process of High Temperature Adaptation in Terms of Changes in Amino Acid Composition. Genome Biol Evol 7:3426–3433. doi: 10.1093/gbe/evv232

Yergeau E, Arbour M, Brousseau R, et al (2009) Microarray and real-time PCR analyses of the responses of high-arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. Appl Environ Microbiol 75:6258–67. doi: 10.1128/AEM.01029-09

Yergeau E, Hogues H, Whyte LG, Greer CW (2010) The functional potential of high Arctic permafrost revealed by metagenomic sequencing, qPCR and microarray analyses. ISME J 4:1206–14. doi: 10.1038/ismej.2010.41

Yergeau E, Kang S, He Z, et al (2007) Functional microarray analysis of nitrogen and carbon cycling genes across an Antarctic latitudinal transect. ISME J 1:163–79. doi: 10.1038/ismej.2007.24

Yu Y, Xin YH, Liu HC, et al (2008) Sporosarcina antarctica sp. nov., a psychrophilic bacterium isolated from the Antarctic. Int J Syst Evol Microbiol 58:2114–2117. doi: 10.1099/ijs.0.65838-0

Yukl E, Elbaz M, Nakano M, Moënne-Loccoz P (2008) Transcription Factor NsrR from Bacillus subtilis Senses Nitric Oxide with a 4Fe− 4S Cluster†. Biochemistry 47:13084–13092. doi: 10.1021/bi801342x.Transcription

Zhang Z, Saier MH (2012) Transposon-mediated adaptive and directed mutations and their potential evolutionary benefits. J Mol Microbiol Biotechnol 21:59–70. doi: 10.1159/000333108

Zhao JS, Deng Y, Manno D, Hawari J (2010) Shewanella spp. genomic evolution for a cold marine lifestyle and in-situ explosive biodegradation. PLoS One. doi: 10.1371/journal.pone.0009109

Zheng S, Ponder MA, Shih JY, et al (2007) A proteomic analysis of Psychrobacter articus 273-4 adaptation to low temperature and salinity using a 2-D liquid mapping approach. Electrophoresis 28:467–488. doi: 10.1002/elps.200600173

Appendix 1.

The work outlined below is published in:

J. Ronholm, **I. Raymond-Bouchard**, M. Creskey, T. Cyr, E.A. Cloutis, and L.G. Whyte. 2015. Characterizing the surface-exposed proteome of *Planococcus halocryophilus* during cryophilic growth. *Extremophiles.*19:619-629.

In addition to my work on whole cell proteomics of *Planococcus halocryophilus*, I contributed to another study which aimed to identify the surface-exposed proteins of *Planococcus halocryophilus* at -5 and -10°C with salt when compared to higher temperature growth (24°C) with and without salt. This study was conducted to investigate cell surface proteomic changes in *P. halocryophilus* over several temperatures and their potential role in the nodular encrustations seen on the organism's surface at subzero temperatures (refer to Chapter 2). My contributions to this study included helping with culturing of the organism, performing amino acid analyses with proteins increased in abundance in the subzero conditions, writing the corresponding section of the manuscript, and providing critical feedback and editing.

Significant differences were seen at -5 and -10°C in proteins involved in peptidoglycan synthesis, iron uptake, and transport, with notable increases in the cell division protein FtsZ and cysteine and manganese transport. *P. halocryophilus* appears to switch mechanisms of iron acquisition at lower temperatures from siderophore-mediated to ferric uptake. My main contribution involved investigating potential amino acid changes in proteins preferentially expressed at -5 and -10°C, which would favor increased flexibility, postulated to be an important strategy for subzero growth (refer to Chapter 3 for additional details). These results are appended in Table 1. Overall few amino acid differences were observed between the conditions. However, hydrophobicity was found to be significantly lower in proteins expressed at -5°C, while significantly fewer aromatic residues could be seen at -10°C. Lower amounts of proline and acidic residues were also observed in proteins at -10°C. These changes are consistent with modifications observed in other psychrophiles (see Chapter 3).

Table 1. Cold-Adaptive Amino Acid Traits Found in *P. halocryophilus* Surfaceome.

	24°C in TSB Media	**24°C in LSM Media**	**24°C in MSM Media**	**-5°C in LSM Media**	**-10°C in MSM Media**
Proline residues	12.4107	**8.9839***	10.4021	11.4436	9.2581*
Arg/Lys ratio	0.6443	**0.8582***	**0.8358***	**0.7817***	**0.8182***
Acidic residues	49.1786	**37.0161***	43.8866	47.5038	38.5161*
Hydrophobicity	-0.2845	**-0.3913***	**-0.3850***	**-0.3707***	-0.3406
Aromaticity	0.0655	**0.0568***	0.0583*	0.0621	**0.0553***
Aliphatic index	87.3956	86.8459	88.0474	87.7566	89.5192

Values indicate the average number of each trait present in the proteins at the particular condition. Traits were first measured independently for each protein expressed in each of the five conditions and then an average calculated for the total set of proteins present in that condition. Bold starred values represent traits that were found to be significantly different at $P < 0.05$ from the 24°C in TSB (optimal growth condition) value, while starred values were found to be marginally different at $P < 0.1$, as determined by an unpaired *t* test.

Appendix 2.

The work outlined below is published in:

J. Goordial, **I. Raymond-Bouchard**, Y. Zolotorov, L. de Bethencourt, J. Ronholm, N. Shapiro, T. Woyke, M. Stromvik, C. Greer, L. Whyte, C. Bakermans. 2016. Cold adaptive traits revealed by comparative genomic analysis of the eurypsychrophile Rhodococcus sp. JG3 isolated from high elevation McMurdo Dry Valley permafrost, Antarctica. *FEMS Microbiology Ecology*. 92(2):1-11

Genomic and amino acid traits of cold adaptation were also investigated in the genome of the Antarctic permafrost cryophile *Rhodococcus* sp. JG3. My role involved helping to design and develop the amino acid bioinformatic analysis pipeline (also used in Chapter 3), helping to compare the *Rhodococcus* sp. JG3 genome to mesophilic relatives, and providing critical feedback and editing.

Overall, the *Rhodococcus* sp. JG3 genome was found to have a higher copy number, when compared to genomes of mesophilic relatives, for several genes with known roles in cold adaptation and growth including the general stress response, UV protection, and osmotic and oxidative stress. Results of the analysis for potential amino acid modifications in the JG3 genome, identified through comparative analyses with the mesophilic genomes, are provided in Figure 1. While the majority of proteins in JG3 did not show significant evidence of cold adaptation, some amino acid modifications, including fewer proline and acidic residues, as well as a lower arginine to lysine ratio, were detected to be significantly different in subsets of proteins. Cell division, transcription, lipid metabolism, and energy production and conversion had the highest number of cold adapted proteins.

Figure 1. Genome wide molecular adaptations to cold in JG3 compared to mesophilic *Rhodococci.* Cold adaptation ratio of cold to hot genes is shown on the right. Number of hot, cold and neutral proteins is shown on the left for each index. Significant indices are indicated with * (Bonferonni corrected P< 0.00001305483).