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THE PSYCHROPHILIC RESPONSE TO GROWTH AT COLD TEMPERATURES: FUNCTIONAL GENOMICS OF THE PERMAFROST BACTERIUM *PSYCHROBACTER ARCTICUS* 273-4

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PETER WARREN BERGHOLZ

has been accepted towards fulfillment
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Doctoral degree in Microbiology and Molecular Genetics

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THE PSYCHROPHILIC RESPONSE TO GROWTH AT COLD TEMPERATURES: FUNCTIONAL GENOMICS OF THE PERMAFROST BACTERIUM *PSYCHROBACTER ARCTICUS* 273-4

By

Peter Warren Bergholz

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ABSTRACT

THE PSYCHROPHILIC RESPONSE TO GROWTH AT COLD TEMPERATURES: FUNCTIONAL GENOMICS OF THE PERMAFROST BACTERIUM *PSYCHROBACTER ARCTICUS* 273-4

By

Peter Warren Bergholz

Permafrost is an environment that imposes three selective pressures limiting the metabolism of resident microbes: low temperature, low water activity, and low nutrient availability. Furthermore, resident microbes are exposed to low continuous doses of background γ-irradiation which should shear genomic DNA. In spite of these characteristics, microbes have been isolated from Siberian permafrost 2-3 million yr in age. The present work aims to elucidate the cold acclimation responses that permitted maintenance of viability over thousands of years in *Psychrobacter arcticus* 273-4, an isolate from Siberian permafrost. *P. arcticus* could have survived long term storage in the permafrost by inducing a dormant survival stage upon exposure to cold. I demonstrate that this is not the case. Rather survival of lethal stresses decreases with decreasing growth temperature. The transcriptome response to growth at 4°C, a cardinal temperature used in cold acclimation studies was narrow in the scope of genes differentially expressed. However, when *P. arcticus* was challenged with growth at subzero temperatures in a high salt defined medium, global expression decreased for genes involved in energy metabolism, anabolic metabolism, and the basal machinery of transcription and translation. Temperature compensation via gene expression was observed in expression for carbon and nitrogen incorporation, sulfur metabolism, biosynthesis of key amino acids and cell envelope dynamics. Changes in amino acid

metabolism could be correlated to bias in amino acid content of genes highly expressed at subzero temperatures, suggesting that the observed changes have ecological relevance for *P. arcticus* in cryo-environments.

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To Natalie and Warren Bergholz, Rebecca and Jamie Glasgow, Rick Bergholz, Charles Tisdale, the Cunningham family, and my grandparents, who have throughout my life inspired confidence and optimism while encouraging my curiosity for the natural world.

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I would also like to acknowledge the support and friendship of my colleagues in the Center for Genomic and Evolutionary Studies on Microbial Life at Low Temperatures, who contributed in innumerable ways every day to the progress of knowledge of life in the permafrost. None of this work could have been accomplished without the support of the staff scientists in the Research Technology and Support

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Finally, I would like to acknowledge the NASA Astrobiology Institute for funding the work presented in this dissertation. Early in the course of my degree, I have to confess that I did not fully appreciate the value of the NAI research structure which forced geochemists, microbiologists, astronomers, physicists and engineers to interact on a monthly basis in extremely formalized meetings and discussions. However, I'm confident that such an integrative approach to science will ultimately carry biology forward into an age of new global theories of biological systems, and I feel that I've gained the capacity to converse with scientists from extremely diverse fields as a result of my participation on this project.

PREFACE

The research on UV-survival was carried out in the laboratory of Dr. George Sundin at Michigan State University. The slow freeze-thaw experiment was performed by Chia-Kai Chang under my direction. Control *Escherichia coli* strains were provided by the laboratory of Dr. Richard Lenski at Michigan State University.

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CHAPTER 1

MICROBIAL LIFE IN CRYO-ENVIRONMENTS

Cryo-environments and permafrost

Cold environments, those with average temperatures less than or equal to 4°C, represent nearly the entire ocean and approximately 25% of the terrestrial surface area. In spite of the clear impact of cold temperatures on the biosphere, studies of the composition and function of microbial communities in these environments have been limited until recent years (17, 24, 69). Cryo-environments are a subset of cold environments defined by the predominance of frozen water. Polar cryo-environments can be stable on the order of 10^3-10^7 yr. Rates of carbon and nutrient turnover in these environments are slow. Microbes have been detected and isolated from all known cryo-environments including sea ice, glaciers, subglacial lakes, snow, and permafrost (8, 26, 34, 62, 68). The biomass and diversity of these environments are dominated by microbial populations. While no one disputes that the rates of microbially driven geochemical processes are lower in cryo-environments, diverse microbial communities and activities are detected in cryoenvironments. For example, incorporation of acetate was observed by Rivkina and colleagues in Antarctic permafrosts at -20°C (52). Motility of a sea ice microbe *Colwellia psychrerythrea* was observed at -10°C (30). Growth and carbon incorporation by *Psychrobacter* spp. isolated from cryopegs in Siberian permafrosts have been reported (2, 27). Biogenic methane corresponding microbes have been detected in Siberian permafrosts (22, 64). Sulfate reducing bacteria have been detected in Lake Fryxell, Antarctica (32). Rhizobial and cyanobacterial nitrogen fixation have been also been described (19, 75).

Permafrost selects for organisms capable of persisting at subzero temperatures with concomitant reduced rates of biological activity, reduced water activity, and reduced nutrient availability. In spite of these three stressors, microbes have been isolated from permafrost soil cores as old as 3 million years (68). *In situ* radiation levels in permafrost, resulting from soil minerals, dictate that a dormant microbe should only be able to survive on the order of 10^4 yr without the capacity to repair damage to the genome (24, 69). Therefore, organisms that can be resuscitated after residing in permafrost for 10^4-10^6 yr must have been carrying out at least survival level metabolism, defined as metabolism necessary to repair damaged cellular constituents (49). Considering the natural limits on microbial life in cryo-environments, it has been assumed that microbial communities in permafrost are populated by specialized genera of microbes. Examination of permafrost microbial communities with both molecular and culture based techniques have detected members of common soil associated microbial phyla including alpha, beta and gamma Proteobacteria, Cytophaga-Flavobacterium, and High and Low GC Gram Positive Bacteria (58, 68). However, some genera are more easily detected and isolated from the Siberian permafrost suggesting these genera such as *Psychrobacter*, *Exiguobacterium*, and *Arthrobacter* spp may have some specialized traits allowing the maintenance of viability under these conditions. The preceding genera contain members adapted for growth at warmer temperatures with varying degrees loss of psychrotolerance when compared to permafrost species. This introduces the possibility of whole genome comparison as a tool for examination of evolutionary adaptations to cryo-environments (15, 67).

Permafrost is also a major sink of fixed carbon. As much as one-third of the Earth's soil carbon is contained in Arctic permafrosts according to the National Snow and Ice Data Center's "State of the Cryosphere" report on permafrosts (http://www.nsidc.org/sotc/permafrost.html). Such a high level of stored carbon makes permafrost communities important from the perspective of climate change. A small increase in temperature of only a few degrees Celsius could have major implications for microbial carbon turnover in permafrost. The prolonged frozen state and observed persistence of microbes over intervals of millions of years makes permafrost communities particularly interesting as models for hypothetical microbial communities in extraterrestrial habitats such as underneath the polar caps on Mars (51). How do permafrost microbes maintain viability for thousands of yr at conditions near the limits of known life? Are these organisms surviving in the dormant state in the permafrost, or are resident microbes specially adapted to function at rates that could support growth in this extreme environment? Understanding the adaptive traits of microbial species from permafrost will yield insights into the function of microbes in cold environments and also potentially the responses of microbes and communities in temperate soils to transient freezing during winter.

Challenges in cryo-environments

Cold temperatures impact biology on the molecular level by imposing constraints on the rates and magnitude of both chemical and physical processes. Processes as diverse as diffusion, elasticity, turbulent flow, microbial growth rate, biogeochemical processes, and chemical reaction rates all decrease with temperature (18, 49). Central

to our understanding of the behavior of microbes and biochemistry over temperature is the Arrhenius equation (Eq. 1).

$$k = A_0 e^{-E_a/RT} \qquad \text{(Equation 1)}$$

Here, k is the rate constant of a reaction, A_0 is some basal rate constant specific to the process, E_a is the activation energy of a reaction, R is the gas constant and T is the temperature. Thus, rates of chemical processes decrease exponentially with temperature.

There are special exceptions at the extremes of functional temperature for microbial cultures which result in observations of inflections in growth rate near the temperature limits of microbial growth. Ratkowsky proposed a thermodynamic model that takes into account the probability of "denaturation" as a function of distance from the temperature optimum of growth. This equation models the behavior of microbial growth of temperature better than previous Bêlehrádek or square-root models (50, 54). These thermodynamic models still fail to model growth rate behavior accurately over temperature when growth temperatures are near the limits for an organism. The weakness of the thermodynamic model for microbial growth at the thermal limits is probably due to its inherent assumption that function of a single enzyme is limiting growth rate across the entire temperature range of growth. Rather, microbial cells growing near the temperature limit of growth modify their gene expression and physiology to cope with the low temperature condition.

The effects of reduced temperatures can be greatly exasperated below the freezing point of water. During freezing, water molecules are incorporated into ice

crystals, rendering them biologically inaccessible. Solutes and microbes concentrate into the remaining volume. Freezing of extracellular solutions reduces water activity and extracellular osmotic pressure and increases the heterogeneity of solutions by generating physical barriers to already slow diffusion of solutes (65). The effect of sudden osmotic shock on microbial cells due to freezing can be lethal due to loss of water from the hydrated shell of proteins (33). Nucleation of intracellular ice crystals can lead to cell death through physical disruption of the cell membrane (40, 63). However, water in microbial cells is confined due to their small volume, so the probability of intracellular ice nucleation is very small in microbial cells (42). The combined effects of life in cryo-environments force microbes in permafrost to adapt to the interacting stresses of low temperature, low water activity and low nutrient availability. The nature of these adaptations on a genome-wide scale in a model organism is the focus of this dissertation.

Psychrophilic adaptations to low temperature

Physiological acclimation or adaptation to different thermal ranges is a major factor in defining the biogeography of species. Originally, cold growing microbes were divided into broad thermal categories based on cutoffs in optimal temperatures as psychrophiles ($T_{opt} \sim 15°C$, $T_{max} \sim 20°C$, $T_{min} \leq 0°C$) and psychrotrophs ($T_{opt} > 20°C$, $T_{min} < 4°C$). The psychrophile classification persists today, though increased knowledge of the diversity of cold-growing microbes has led to new definitions based on both the biogeography and the growth temperature limits. Microbes which are rarely isolated from cold environments, but exhibit growth at 4°C are considered

broadly as psychrotolerant. Microbes which are commonly isolated from cold environments are now referred to as psychrophiles, and can be subdivided into steno-psychrophiles with a narrow physiological temperature range and eury-psychrophiles with a broad physiological temperature range (20).

Organisms can adapt for function at a temperature through evolution of alleles specific for function at physiological temperatures or evolution of mechanisms for regulation of genes that permit acclimation to different temperature ranges. Evolutionary pressure for allele specialization in the cold should be stronger in steno- rather than eury-psychrophiles. In contrast, eury-psychrophiles should be under pressure to maintain thermostability and function of those alleles at low temperature (23). Eury-psychrophiles would also be under selective pressure to maintain regulatory mechanisms that allow for acclimation for growth to both psychrophilic and mesophilic temperature ranges. Our discussion here will examine both allelic adaptations for low temperature function and gene expression responses to the cold.

Allelic adaptation for low temperature function. The critical challenge to enzyme function at low temperatures is loss of flexibility in the peptide chain. Recent years have witnessed an explosion in research on thermal adaptation of psychrophilic enzymes. Increased flexibility results in decreased enthalpy, $\Delta H^{\#}$, of the enzyme activation energy thus increasing the reaction rates at low temperatures (23). One of the first methods of understanding adaptations for increased flexibility was to examine amino acid contents of psychrophilic enzymes (55). Genome-wide amino acid bias analyses in psychrophilic bacteria and archaea have demonstrated that psychrophiles have increased proportions of polar amino acids in both surface

exposed and buried amino acid positions when compared to mesophilic relatives. Surface exposed residues in these genomes exhibit decreased charged amino acid content. Buried residues are biased away from hydrophobic amino acid content coupled with slight increases in charged amino acid content (41, 56). Other adaptations for increased flexibility of enzymes at low temperature can include glycine clusters particularly near the boundaries of secondary structure elements, and loss of proline residues in coils (35, 39). However, global trends in amino acid content are far less important than the position of amino acid substitutions. Glycine to proline substitutions were found to be most important in the context of the local structure in a psychrophilic chitinase where one such substitution reduced k_{cat} at low temperature and another improved it (38). Similarly, a recent modeling of molecular dynamics of cold adapted enzymes showed higher flexibility localized to coil regions, and that insertions in psychrophilic enzymes were commonly highly disordered (59).

Sensing low temperatures. Virtually every cellular system is impacted by low temperatures, so sensing of low temperatures need not rely on membrane bound histidine kinases (Fig. 1.1) (57). Nevertheless, such histidine kinases have been identified in *Synechocystis* and *Bacillus subtilis* where it is thought that they respond to cytoplasmic membrane fluidity (29, 61). RNA thermometers also play a significant role in cold shock and acclimation. Cold temperatures can induce the formation of secondary structures. Secondary structures impact the stability of mRNAs and their translational efficiency. This strategy s involved in the regulation of cold inducible proteins such as *cspA, pnp*, *rnr*, and the *bkd* operon (10, 37, 45, 70, 73). One open question in cold sensing involves the evolutionary modification of thresholds for

temperature responses. Experiments in psychrophilic microbes suggest that the heat shock response is induced in psychrophiles at lower temperatures than in mesophiles (16, 71).

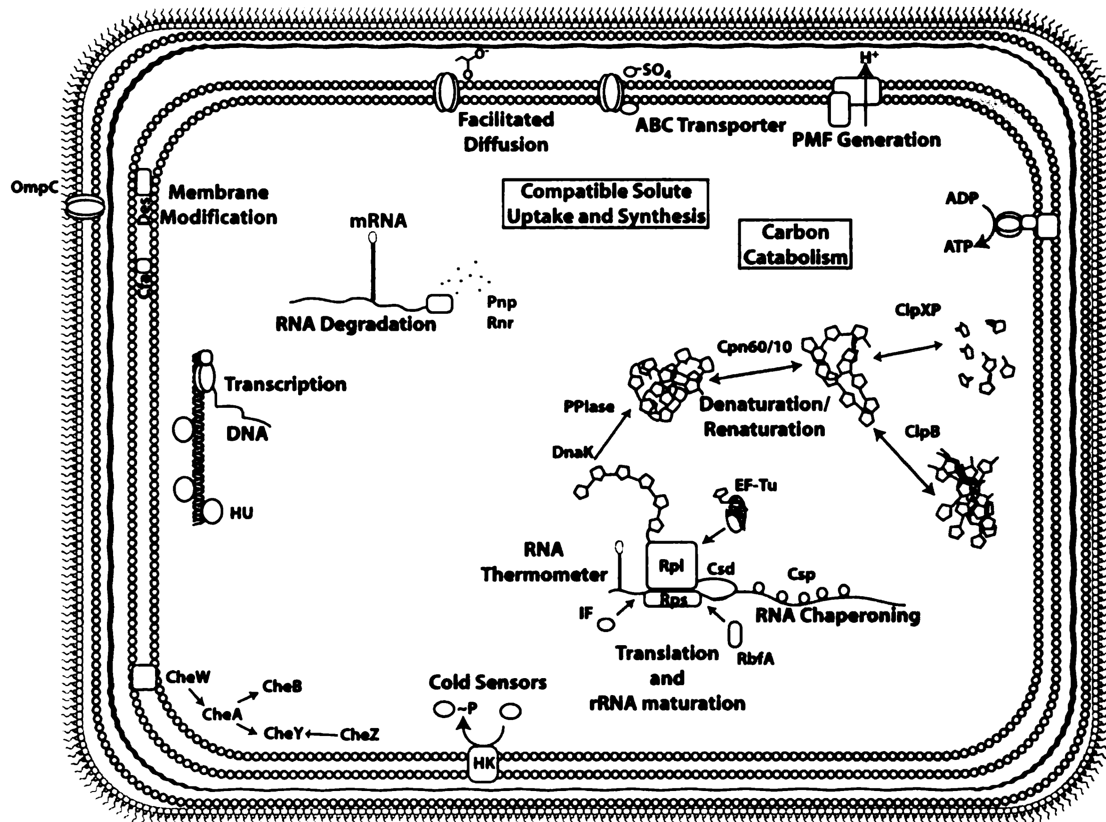

Figure 1.1. Mechanisms of cold acclimation in Gram negative bacteria. Cold temperatures reduce the inherent disorder of molecular systems, decreasing enzymatic reaction rates and membrane fluidity and stabilizing hydrogen bonds in nucleic acids. Depicted genes are up-regulated in diverse mesophilic and psychrophilic microbes including *E. coli*, *B. subtilis*, *Synechococcus* sp. PCC7942, *M. burtonii*, and *Psychrobacter cryohalolentis*. See text for references.

Translation and protein folding responses in the cold. A high proportion of genes up-regulated during cold acclimation or shock have to do with translation functions (Fig. 1.1). RNA chaperones, e.g. the major cold shock proteins, are among the most highly conserved examples. RNA chaperones are up-regulated constitutively in some psychrophiles during growth at low temperatures (1, 4). DEAD-box RNA helicases play a complementary role in unwinding secondary

structures in RNA. Together these genes ensure that RNAs are free from secondary structure, so that translation can proceed. Changes in ribosomal protein complement have previously been detected in organisms growing in the cold, including structural ribosomal proteins, elongation factors, initiation factors and ribosome-binding factor (1, 9, 28, 74). Cold shock induced changes in *Listeria monocytogenes* ribosomes led to decreased thermostability and presumably improved ribosome function in the cold (3).

Mesophiles commonly up-regulate heat shock chaperones during cold shock and express transcripts and proteins thereof at elevated levels during growth at sub-optimal temperatures (9, 36, 46). The chaperone ClpB is strongly induced during low temperature growth presumably due to its role in protein disaggregation and repair (46, 48). While psychrophiles do not increase expression of heat shock chaperones in the cold, increased expression of peptidyl-prolyl *cis-trans* isomerases has been observed in both psychrophiles and mesophiles during growth at suboptimal temperatures (9, 28, 60). Thus, psychrophiles may not experience protein denaturation at low temperatures, but do exhibit increased response for the proline dependent folding of peptides.

Maintenance of membrane fluidity. The fluidity of biological membranes is considered a key factor in dictating metabolic activity levels (Fig. 1.1). Transport proteins and the key proteins of respiratory metabolism are associated with the cell membrane. The activity of these proteins is thus controlled strongly by the viscosity of the cell membrane (44). Maintenance of membrane fluidity is one of the dominant factors in the response to cold shock and for growth in the cold (14). Expression of

fatty acid desaturases and reduction in the activity of long-chain acyl-CoA ligase at low temperatures are common mechanisms of maintaining membrane fluidity in mesophiles (29). Cyclopropane fatty acid synthases are also up-regulated during low temperature growth in mesophiles and can stabilize double-bonds in membrane lipids against oxidative damage. Increased synthesis of branched chain fatty acids has been supposed to result from increased production of isobutyryl-CoA in *B. subtilis*, though this induction appears to occur only during cold shock rather than cold acclimated growth (9, 45). In theory, changes in phospholipids head-group chemistry could also introduce steric hindrance to lipid packing though this has been only rarely reported (14).

Growing up cold and hungry? In cryo-environments, solutes are concentrated into a limited volume of remaining water; in Siberian permafrost, 3-8% of water is unfrozen (25). While substrates are concentrated in briny veins and pockets of water, the rates at which they can be taken up and metabolized is limited at low temperatures (44, 57). Indeed, enzymes in frozen and otherwise heterogeneous solutions exhibit oscillations in activity due to local depletion of substrate due to decreased diffusion rates (65). From the microorganismal perspective, induction of a starvation response, with its consequent reduction in overall metabolic activity is an important strategy to ensure that resources are not exhausted. Such a response can take the form of the stringent response, which controls gene expression during a variety of substrate limited growth conditions (12, 13). Alternatively, permafrost microbes could activate a general stress response such as that controlled by RpoS or SigB. Such responses lead to growth arrest, but also activate a variety of damage

resistance and repair mechanisms, essentially hardening the cell against environmental sources of damage (9, 43, 66). However, proteome studies in a psychrophilic archaeon and a psychrophilic bacterium have detected increased expression of genes involved in carbon catabolism and energy metabolism during growth at sub-optimal temperatures (1, 28). These results suggest that substrate uptake is not limiting for psychrophiles, rather energy conversion must be compensated to maximize growth rate. Directing energy toward damage resistance or growth metabolism could both enable the survival of organisms in permafrost, but the extent to which psychrophilic organisms have adopted either strategy is poorly understood.

Psychrobacter arcticus 273-4: *A model organism for subzero metabolism*

Psychrobacter arcticus 273-4 was isolated from a depth of 12.5 m of a bore hole in the Kolyma lowland (68). This depth places *P. arcticus* firmly in the Edoma suite, a syngenetically frozen layer resulting from alluvial flooding 20,000 to 40,000 years ago in this location. It is a gamma Proteobacterium with an optimal growth temperature of 17°C to 22°C dependent on medium conditions and can be cultured at temperatures ranging from 28°C to -10°C. *P. arcticus* is also capable of metabolism in high salt concentrations up to 2.8 osmolal (47). It has also been found to increase the proportion of unsaturated fatty acids in its cytoplasmic membrane during growth at 4°C and on increased salt and exhibits slight ice nucleation activity (47).

Members of the genus *Psychrobacter* are Gram negative, heterotrophic, aerobic gamma Proteobacteria that are defined by their ability to grow at 4°C and the

ability of a competent strain to take up exogenous DNA from candidate *Psychrobacter* strains (31). *Psychrobacter* species have been detected in clone libraries from Antarctic sea ice (6). In addition to Siberian permafrost, isolates of *Psychrobacter* species have been obtained from a variety of polar environments (7, 11, 21), sea water and marine worms (53), and salted meats (5, 72). The combined tolerance in this genus for low temperatures and reduced water activity make them excellent candidates for model cryo-adapted bacteria.

The genome sequence of *P. arcticus* 273-4 was obtained by the DOE Joint Genome Institute (JGI) in 2002 (http://genome.ornl.gov/microbial/psyc). This completed sequence was manually annotated by a team of scientists from Michigan State University and JGI. Interesting metabolic features of the genome include the lack of a glycolysis pathway and the presence of a rare glyoxylate metabolism pathway. The *P. arcticus* genome is also very repetitive with a single 139 bp dispersed repeat that accounts for 1.92% of the genome sequence and is highly dominant among repeated sequences in the genome (evenness of all repeated sequences = 0.11). Among the potential cryo-adaptive features of the genome are three cold shock proteins, two DEAD box RNA helicases, glycine-betaine biosynthesis pathways, fatty acid desaturase, anti-freeze domain proteins, and a full complement of proteases and chaperones.

Goals of the present research

The central question of this dissertation is:

How does *P. arcticus* acclimate to life near the lower temperature limit?

I seek to answer this question through a combination of survival studies, microarray gene expression experiments, genome analysis, and gene knockout experiments. In Chapter 2, I test the effect of low temperature incubation on survival of lethal stresses. In Chapters 3 and 4, I present analysis of transcriptomes during low temperature incubation in both rich and mineral media developed based on the *P. arcticus* genome. In Chapter 5, I examine changes in amino acid metabolism as a result of low temperature incubation and present evidence for the ecological relevance of the observed changes.

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CHAPTER 2

GROWTH AT LOW TEMPERATURES DOES NOT IMPROVE SURVIVAL OF LETHAL STRESSES BY *PSYCHROBACTER ARCTICUS* 273-4

Abstract

Permafrost microbes experience continuous low temperatures, dessication and low levels of radiation *in situ*. In spite of these conditions, *Psychrobacter arcticus* 273-4 was isolated following 10,000-40,000 yr inhabitation of the Kolyma lowland permafrosts. We examined low temperature effects on survival of UVC and freeze-thaw stress. As growth temperature decreased, *P. arcticus* exhibited decreased survival after both UVC. *P. arcticus* survived freeze-thaw at higher rates than *E. coli* regardless of growth temperature, but *E. coli* survived UVC at higher rates than *P. arcticus* under all conditions. In contrast to repeated observations of increased freeze-thaw survival following cold acclimation in mesophiles and Gram-positive permafrost bacteria, no increase in stress resistance was observed in *P. arcticus* during low temperature growth. Therefore, *P. arcticus* may not respond to low temperature by increasing resistance to lethal stress.

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Introduction

The permafrost soils of the Kolyma lowland, Siberia, Russia have been frozen for periods between 10,000 and 3.5 million yr, depending on the geologic stratum sampled (28). Owing to their frozen state, these soils represent a set of stable environmental stresses to the resident microbes including constant low temperatures (-10°C to -12°C), continuously low water activity and low doses of background radiation from soil minerals at a rate of 1-6 kGy yr^{-1} (7, 8). Recent advances in our understanding of the biogeochemical flux in these environments have shed light on the potential for microbial survival, maintenance and even growth in permafrosts and other cryoenvironments, i.e. environments which undergo long-term freezing on the order of years (4, 21).

While background levels of γ-radiation are low in the permafrost of the Kolyma lowland, exposure to even low levels of background radiation over 10^4-10^6 yr would significantly damage genomic DNA (7, 31). Exposure to ultraviolet radiation can lead to direct DNA damage in the form of pyrimidine dimers and DNA strand breaks, and can also lead to generalized damage to the cell through the formation of reactive oxygen species (13). If radiation-induced damage is an important selective factor in permafrost, one would expect to isolate organisms that are adapted either for resistance to damage from radiation, or improved capacity to repair damage due to radiation. Price and Sowers proposed an alternative hypothesis that psychrophiles might be resilient against DNA damage by adopting a survival level metabolism, defined as the metabolic rate necessary to repair damage to the genome, in nutrient-limited and cold environments (20). Both such strategies could yield organisms with long survival times in permafrost.

Microbes must also undergo freezing upon entry into the permafrost. While survival in a frozen soil matrix over 10^3-10^4 yr cannot be directly assayed, freeze-thaw cycling can be used to assess the cryotolerance of microorganisms. Freeze-thaw stress is thought to impact cells through two principle damage mechanisms: the formation of intracellular ice and the formation of extracellular ice with concomitant increase in solute concentrations due to loss of liquid water (15). Formation of intracellular ice is unlikely in the case of bacteria, because water in the bacterial cell is confined in a very small volume and interacts with proteins and the cytoplasmic membrane (17). However, osmotic stress can be particularly damaging as it leads to the denaturation of cytoplasmic proteins and the damage of cell membranes (12, 25). Diverse permafrost strains survived a single freeze-thaw cycle for 1 yr with less viable cell loss following growth at 4°C versus growth at 25°C (19). *Exiguobacterium* species isolated from the Kolyma lowland were recently demonstrated to increase survival after repeated freeze-thaw cycles when cultured at low temperatures (29). The prevalence of increased resistance to freeze-thaw stress in permafrost isolates after growth at low temperatures remains unknown.

We examined the Gram-negative permafrost isolate, *Psychrobacter arcticus* 273-4 to determine if incubation at low temperatures improved survival of freeze-thaw cycling or UVC irradiation. *P. arcticus* was isolated from 10,000-40,000 yr Kolyma lowland permafrost. It is capable of growth at temperatures ranging from -10°C to 28°C and salinities ranging from 10 mM to 1.3 M NaCl (2, 19). These combined capacities suggest that *P. arcticus* is adapted to inhabit cryoenvironments. If *P. arcticus* exhibits increased resistance to lethal stresses following growth at low temperatures, then this resistance response may explain its capacity for survival over 10^3-10^4 yr in this

environment. Alternatively, *P. arcticus* may not respond to low temperatures by increasing resistance to radiation or freeze-thaw. The lack of such a response would indicate that active metabolism at some low rate was required for survival in the permafrost.

Materials and Methods

UVC Survival Assay. Cultures of *Psychrobacter arcticus* 273-4 and *Escherichia coli* B606 were grown to late-log phase (OD_{600} = 0.6 to 0.9 under all conditions) in 50 ml of ½ Tryptic Soy Broth (TSB, Difco). *P. arcticus* was cultured at either 25°C or 4°C. *E. coli* was cultured at 37°C. *E. coli* was grown to late log phase (OD_{600} = 0.9) in ½ TSB at 25°C. Fifteen ml of each culture was mixed with an equal volume 0.85% NaCl and centrifuged at 6000 x g for 30 min at their cultured temperature. The culture supernatant was discarded, and cell pellets were resuspended in 15 ml 0.85% NaCl. Cells were stored on ice until experiments were completed (never more than 1.5 hours).

Cells were pipetted into a sterile glass Petri plate, and exposed to a UVC fluorescent lamp (equivalent dose 1.5 J m^{-2} s^{-1}) with constant mixing. Cell suspensions were cumulatively exposed to 0, 25, 50, and 100 J m^{-2}. At each exposure level, a 100 μl cell suspension was collected. After all exposures were completed, cell suspensions from each exposure level were decimally diluted to 10^{-8} of their original concentration in 0.85% NaCl. Triplicate 50 μl aliquots of cell suspension at each dilution level were spot plated onto a ½ TSA plate. Plates were incubated in the dark at culture temperature. All steps from UVC exposure through incubation were carried out in the dark. All solutions were kept at the same temperature at which cells were cultured. Colony forming units

were counted on the spot plates after 48 hours of incubation at 25°C or two weeks incubation at 4°C respectively.

Slow freeze thaw survival. *P. arcticus* 273-4 and *E. coli* B606 were grown from -80°C freezer stocks on ½ TSA at 25°C and 37°C respectively. Starter cultures were inoculated by transfer of single colonies from agar medium to 5 ml ½ Tryptic Soy Broth (1/2 TSB, Difco). Two starter cultures were grown to stationary phase ($OD_{600} \cong 0.8$ for *P. arcticus* and $OD_{600} \cong 2$ for *E. coli*) at 25°C or 4°C for *P. arcticus* and 37°C for *E. coli.* Two 25 ml ½ TSB sample cultures were inoculated with 250 µl of starter culture and these cultures were allowed to grow at the same temperature as the starter cultures until exponential phase ($OD_{600} \cong 0.2$). Each sample culture was split into 17 x 1 ml aliquots in 1 ml Corning cryovials (Corning, Corning, NY). These aliquots were transferred to an -15°C incubator and allowed to freeze at that temperature. Samples were incubated at -15°C for 24 hours each freeze-thaw cycle. Each day, aliquots were thawed at room temperature for approximately 10 minutes. After each freeze-thaw cycle, one aliquot of each sample was decimally diluted and plate counts were performed by spreading 100 µl each dilution from 10^{-1} to 10^{-6} on ½ TSB. The remaining aliquots were returned to the -15°C incubator for refreezing. Plated cells were allowed to grow at the original culture temperature of the sample. After 4 days at 25°C or 15 days at 4°C for *P. arcticus* plates or 1 day at 37°C for *E. coli*, colony forming units (CFU) were enumerated on each plate.

Fast freeze thaw survival. *P. arcticus* 273-4 was inoculated from -80°C freezer stock onto a marine agar (MA) plates and grown at 4°C. Marine agar consisted of 5 g l^{-1} tryptone (Difco), 1 g l^{-1} yeast extract (Difco) and 30 g l^{-1} sea salts (Sigma-Aldrich). Starter cultures were inoculated by transferring a single colony for each of six replicate

samples to 5 ml marine broth (MB, Difco) at 25°C, 4°C, and -6°C. Starter cultures were grown to stationary phase ($OD_{600} \cong 0.8$). Freeze-thaw samples were grown at each temperature by inoculating 5 ml MB in a 18 mm culture tube with 50 µl starter culture. Freeze-thaw sample cultures were allowed to reach exponential phase ($OD_{600} \cong 0.2$). Samples were split into 8 x 0.5 ml aliquots in 1.5 ml Eppendorf tubes. Freeze-thaw cycles were carried out by immersing the vials in a dry ice and ethanol bath for 1 minute. Aliquots were then thawed at 25°C in a water bath for 3 minutes. This freeze-thaw process was repeated for 15 cycles. At 0, 1, 3, 5, 10, and 15 freeze-thaw cycles, one aliquot was retained, decimally diluted, and spread plated at 10^{-3} to 10^{-6} dilutions on MA. MA plates were incubated at 4°C. CFU were enumerated after 15 days incubation at 4°C.

Log Survival Calculations and Analysis. For all survival experiments, CFU ml^{-1} of culture were estimated to be plated $volume^{-1}$ · CFU per plate · dilution factor. Log survival was calculated as $Log_{10}(CFU\ ml^{-1}{}_{final}) - Log_{10}(CFU\ ml^{-1}{}_{initial})$. T-test comparisons were carried out in the UVC and fast freeze-thaw survival studies comparing log survival of *E. coli* and *P. arcticus* grown at 25°C. *P. arcticus* cultures grown at 25°C versus cultures at lower temperatures were also tested. A P-value < 0.05 was considered statistically significant.

Results

UVC survival. Cumulative damage due to long term exposure to low levels of background radiation could be an important selective pressure on microbial permafrost communities. We tested *P. arcticus* to determine if it was more resistant to UVC

radiation than a related mesophilic bacterium, *E. coli* B606. We also tested whether there was an increase in survival of UVC irradiation as growth temperature decreased.

Since *P. arcticus* cultured in ½ TSA exhibits a growth rate inflection at 6°C indicative of cold acclimated growth, *P. arcticus* at two growth temperatures (25°C and 4°C) and *E. coli* B606 at 37°C were exposed to cumulative UVC doses equivalent to 0, 25, 50, and 100 J m^{-2}. Average log survival at each of the UVC dosage levels was used to compare *P. arcticus* grown at 25°C to both of the other two samples using a one-tailed two sample t-test assuming unequal variances. The log survival of the 25°C sample of *P. arcticus* was significantly greater than that of the 4°C at 50 and 100 J m^{-2} dosage levels ($P < 0.05$) (Fig. 2.1). The *E. coli* B606 sample was found to exhibit greater survival ($P < 0.05$) than *P. arcticus* grown at 25°C at the 100 J m^{-2} dose.

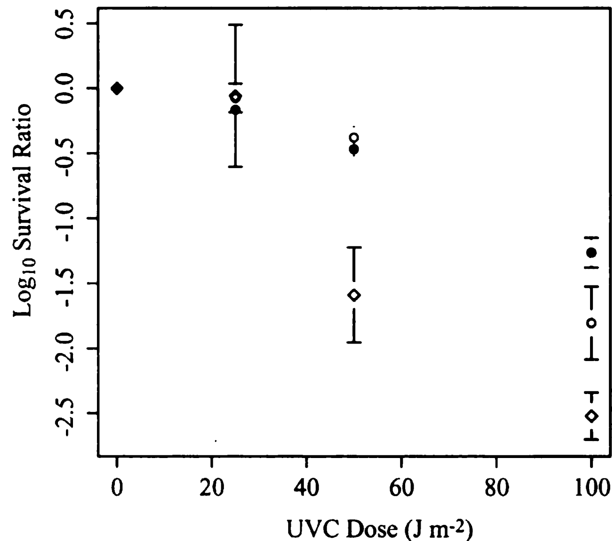

Figure 2.1. Log survival difference over increasing UVC dose. Counts of *E. coli* B606 (●) and *P. arcticus* grown at 25°C (○) and 4°C (◇) after UVC up to 100 J m^{-2}. CFU ml^{-1} was estimated from cultures grown on ½ TSA in the dark. Error bars represent one standard deviation for each sample ($n = 4$).

Slow Freeze-thaw survival. *P. arcticus* cultures were exposed to slow freeze-thaw stress, by freezing 1 ml aliquots of culture at -15°C and thawing at room temperature. Freezing of cell aliquots was verified after 2 h incubation at -15°C. Cooling rates were measured in to be -0.1°C min^{-1} in proxy aliquots when averaged over the freezing period. The freezing point of ½ TSB under our experimental conditions was -7.5°C. Thawing rates at room temperature were also not strictly controlled in the slow freeze-thaw regime. Thawing required approximately 10 minutes. Therefore, warming rates were approximately 2°C min^{-1} averaged over the thawing period.

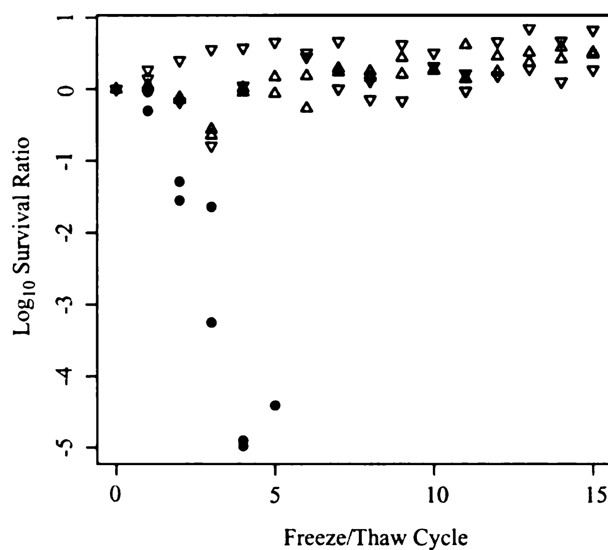

Figure 2.2. Log survival ratios obtained during slow freeze-thaw experiment. Counts of two replicates each of *P. arcticus* grown at 25°C (open downward triangle, ∇) and 4°C (open upward triangle Δ) in ½ TSB medium. Survival of the psychrophile *P. arcticus* is compared with the mesophile *E. coli* B606 (closed circle ●). All assays were carried out with plating on ½ TSA agar.

E. coli B606 cultures were grown at 37°C. *P. arcticus* cultures were grown at 25°C and 4°C to evaluate the effect of low temperature growth below the critical

temperature ($T_{crit} \sim 6$ degC) on cryotolerance. CFU ml^{-1} was estimated for each sample for two replicates after each freeze-thaw cycle by decimal dilution and plating on ½ TSA. *P. arcticus* cultures were found to be cryotolerant, with no detectable decline in viable cells over multiple freeze-thaw cycles in this slow freeze regime (Fig. 2.2). Similar survival and slight growth in *P. arcticus* populations was observed regardless of the growth temperature. In contrast, *E. coli* B606 was much more sensitive to freeze-thaw stress. *E. coli* CFU were undetectable after 5 freeze-thaw cycles. The detection limit of our assay was approximately 10^2 CFU ml^{-1}. The average log survival rate of *E. coli* in this experiment was -0.9 CFU freeze-thaw $cycle^{-1}$.

Fast freeze-thaw survival. Use of ½ TSA as our plating medium for fast freeze-thaw experiments failed to yield stable CFU ml^{-1} estimates after several attempts. Therefore, MB was used as the growth and plate count medium for this fast freeze-thaw survival experiment. Sample cultures with separate colonies as source inocula were grown to mid-exponential phase under three temperature conditions, 25°C, 4°C and in the subzero temperature range at -6°C. Six replicate cultures inoculated from separate colonies were assayed at each temperature. Under the conditions of this experiment, cooling and heating rates were strictly controlled by the use of dry ice/ethanol baths and water baths heated to 25°C. The cooling rate was estimated to be greater than -30°C min^{-1} in the interval of our freezing regime from 25°C to -7.5°C. This is a conservative estimate as freezing was evaluated visually in these samples.

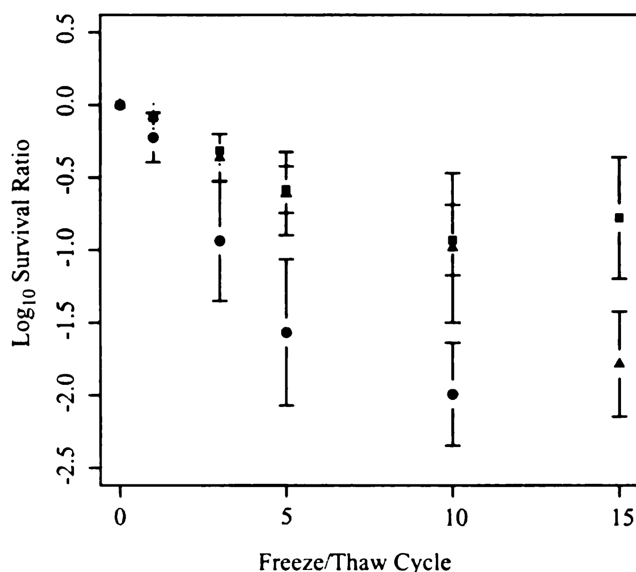

Figure 2.3. Log survival ratio obtained during fast freeze-thaw experiment. CFU ml^{-1} estimates of *P. arcticus* cultures grown at 25°C (closed squares ■), 4°C (closed triangles ▲) and -6°C (closed circles ●). The error bars indicate standard deviations for each sample at each temperature (n = 6). CFU ml^{-1} was estimated using MA as the growth medium.

P. arcticus survived at significantly higher rates at 25°C and 4°C than at -6°C (Fig. 2.3). Cultures grown at 25°C and 4°C were statistically indistinguishable in log survival until the freeze-thaw cycle 15 where 4°C grown cells exhibited statistically lower log survival with $P < 0.05$. Log survival behaved as a log-linear decay in CFU ml^{-1} over the interval of 0-5 freeze-thaw cycles for -6°C samples and 0-10 freeze-thaw cycles for 25°C and 4°C samples. The loss of the expected decay behavior in -6°C samples at 10 freeze-thaw cycles is probably due to CFU ml^{-1} estimates which approach the limit of detection at these time points (detection limit $\cong 10^4$ CFU ml^{-1}). Log survival rates were -0.07 Log_{10} CFU freeze-thaw $cycle^{-1}$, -0.11 Log_{10} CFU freeze-thaw $cycle^{-1}$, and -0.31 Log_{10} CFU freeze-thaw $cycle^{-1}$ at 22°C, 4°C and -6°C respectively.

Discussion

We tested the effect of growth temperature on stress resistance by measuring survival of *P. arcticus* following UVC and freeze-thaw exposure. UVC was chosen because it causes both pyrimidine dimers and occasional single strand DNA breakage, and DNA damage is a relevant stress in the permafrost (7, 13). While estimation of temperature effects on cryotolerance over 10^3-10^4 yr is not possible in a laboratory setting, we used resistance to repeated freeze-thaw as a proxy for cryotolerance. Furthermore, exposure to cold is known to improve cryotolerance in plants and mesophilic bacteria (3, 5, 6, 22, 24).

Freeze-thaw conditions combine the challenges of desiccation and low temperatures. Gene expression responses to low temperatures and desiccation have been linked on a functional genomic level in *Arabidopsis thaliana* (22). Accumulation of compatible solutes is a prevalent response to dessication stress. Compatible solutes protect proteins from denaturation, but may destabilize cell membranes as was observed in model membranes during freeze-thaw assays (10). Nevertheless, some bacteria accumulate compatible solutes to high levels after cold shock. Exposure to 10°Cled to expression of glycine-betaine and carnitine uptake transporters in *Listeria monocytogenes* (1, 16). *E. coli* survival at low temperatures is dependent on the ability to synthesize trehalose (11).

The characteristics of the permafrost soil matrix may also impact survival; thin layers of liquid water will be retained around soil particles far below the freezing point (9). While Gram-positive bacteria appeared to benefit from the presence of a soil matrix in freeze-thaw assays, Gram-negative organisms survived better in liquid environments or high moisture content soils (18, 23). Therefore, we did not assess the effect of

presence vs. absence of a soil matrix on freeze-thaw survival. However, attachment to permafrost soil particles could afford *P. arcticus* protection in ordered liquid water bound to particle surfaces (9).

Our results demonstrate primarily that low temperature growth of *P. arcticus* cells does not yield a benefit to freeze-thaw or UVC survival. Survival at 25°C was greatest in all experiments and survival at low temperatures, 4°C or -6°C, was never greater than at 25°C. While *E. coli* exhibited higher survival in the UVC experiment, *P. arcticus* performed better than *E. coli* in all freeze-thaw conditions. *P. arcticus* may be evolutionarily adapted to survive freeze-thaw stress more successfully than *E. coli*, but this difference in survival is not improved by acclimation of *P. arcticus* for growth at lower temperatures.

The *P. arcticus* genome contains factors for nucleotide excision repair and recombination repair of DNA including but not limited to *recA*, *uvrBCD*, *mut*S and *mutL*. The oxidative stress response gene complement in the *P. arcticus* genome includes *katE*, *soxA*, *ahpC*, and *msrA*. This genome also contains factors for freeze-thaw damage and osmotic shock resistance including *betAB*, betaine/choline/carnitine uptake transporters, small and large conductance mechanosensitive ion channels, and two proteins containing antifreeze domains. *P. arcticus* was also reported to possess significant ice nucleation activity that improved with incubation at 4°C versus 25°C (19). The preceding factors may explain *P. arcticus* inherently higher survival of freeze-thaw challenge versus *E. coli*.

Cold shock improves freeze-thaw survival of lactic acid bacteria and *E. coli* O157:H7 (3, 6). Mesophiles such as *E. coli* commonly harden against lethal stresses

when exposed to growth inhibitory conditions. The stationary phase sigma factor RpoS controls expression of approximately 10% of *E. coli* genes in response to starvation, low pH, cold, heat, osmotic and oxidative stress responses (27, 30). For example, a *Vibrio parahaemolyticus* Δ*rpoS* mutant exhibited substantially decreased survival vs. the wild-type during low temperature storage (26). Similarly, an *E. coli* O157:H7 Δ*rpoS* mutant did not exhibit increased freeze-thaw survival following cold shock (14).

No *rpoS* homologue has been detected in the *P. arcticus* genome, and this may explain the lack of a improved general stress resistance at low temperature. Growth temperature had no influence on freeze-thaw survival in the slow freeze-thaw experiment. In contrast, the fast freeze-thaw experiment resulted in decreased survival as growth temperature decreased. Growth at low temperatures reduces *P. arcticus* ability to cope with exposure to fast freeze-thaw and UVC. Reduced growth temperature may be negatively affecting survival by decreasing the capacity to resist or repair damage.

Exiguobacterium species isolated from the Kolyma lowland permafrosts were recently tested for freeze-thaw tolerance (29). In contrast to our results, low temperature growth improved freeze tolerance of *Exguobacterium* spp. Vishnivetskaya and colleagues observed the formation of highly refractive, spore-like cells at subzero temperatures, and these differentiated cells may have contributed to improved freeze-thaw survival at low temperatures. However, the *P. arcticus* data demonstrate that increased stress resistance after low temperature incubation is not a universal strategy selected for in permafrost microbes.

Exposure to UVC following growth at 25°C and 4°C was used to evaluate the ability of *P. arcticus* 273-4 to survive DNA damage. A decrease in UVC survival at 4°C

relative to 25°C was observed. This indicates that unusual capacity to withstand DNA damage was not necessary for *P. arcticus* to survive and be resuscitated in the lab after 10^4 to 10^6 yr in permafrost. The theoretical time to break a 50% G+C genome into ~ 100 bp fragments is 81,000 yr at -10°C (31). Therefore, one must conclude that some level of repair must have been occurring *in situ*. Price and Sowers compiled data indicating that *in situ* survival metabolism by microbial communities is slightly higher than theoretical rates of DNA depurination over time (20). This suggests that exceptional ability of microbes to resist DNA damage would only be required by organisms that are incapable of survival level metabolism, defined as a state in which cells "can repair macromolecular damage but are probably largely dormant" in permafrost conditions. They reported that survival metabolism would only need to be proportionally 10^{-6} of the rates required for growth metabolism.

We have demonstrated that tolerance of *P. arcticus* to two kinds of lethal stresses does not improve with decreased growth temperature. *P. arcticus* was more resistant to freeze-thaw induced damage than *E.* coli, but this was not the case when UVC damage tolerance was tested. Low temperature does not induce damage resistance phenotypes in *P. arcticus*. *P. arcticus* does grow under subzero temperatures and, so may not sense low temperature growth as stress resistance inducing condition. *P. arcticus* may not possess an *rpoS* gene, and this may also explain its lack of a general stress resistance response. Rates of damage due to the cold, desiccation, and irradiation stresses present in the permafrost must be slow, and the lack of a cold-induced survival response suggests that *P. arcticus* is sufficiently adapted under low temperature conditions to these conditions to at least survive damage *in situ* for periods of ~10^4 yr.

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CHAPTER 3

GROWTH AT 4°C INDUCES A LIMITED TRANSCRIPTIONAL RESPONSE IN THE PSYCHROPHILE *PSYCHROBACTER ARCTICUS* 273-4

Abstract

Eurypsychrophiles are capable of growth at a wide-range of temperatures ranging from < 0°C to optimum growth temperatures near 30°C. Growth at cold temperatures, T ≤ 4°C, impacts functions of all molecules in the cell by reducing rates and amplitude of molecular motion. The transcriptome response to growth at 4°C and 22°C was analyzed in the eurypsychrophilic permafrost bacterium *Psychrobacter arcticus* 273-4. Gene expression for increased production of unsaturated fatty acids, increased metabolism of oxidized substrates, and ribosomal adaptation to the cold were detected during growth at 4°C in rich ½ TSB medium. A defined mineral medium was developed to allow analysis of the biosynthetic demands placed on the cell during growth at low temperatures. During growth in the mineral medium, no statistical differences in gene expression were detected in the comparison between 22°C and 4°C. The results of this study indicate that *P. arcticus* does not differentiate transcriptomes strongly between growth at T_{opt} and at 4°C, a temperature difference of 18°C. This lack of response is probably the result of specialization in *P. arcticus* for life in low temperature environments.

Introduction

Low temperature is a global stress on organisms that leads to reduced enzymatic reaction rates, increased viscosity, liquid-crystalline phase transitions of biological membranes, denaturation of proteins due to weakening of hydrophobic interactions, increased formation of secondary structures in DNA and RNA due to stabilization of hydrogen bonds, increased steady state concentrations of reactive oxygen species, and reduced diffusion rates of solutes (6, 36). Microorganisms subjected to selection at low temperatures have evolved proteins and membrane lipid fatty acid compositions for function in either constantly high or low temperature environments (28, 29). However, microbes adapted to a broader thermal range must respond to these stressors by expressing cold acclimation proteins. Known cold acclimation proteins have roles in unwinding secondary structures, ribosome function at low temperature, protein folding, expression of alternate enzymes that function at low temperature, and membrane lipid modification (3, 4, 14, 21, 24). Transcription of these genes is typically induced at least 2 to 8-fold during cold shock and continues after cold shock at elevated levels typically in the range of 1.5 to 3-fold during cold acclimated growth (15, 22). However, gene expression in a low temperature versus optimal growth state may depend on the evolutionary history of an organism. Psychrophiles likely do not experience cold stress in the same temperature thresholds as mesophiles and are probably differentially adapted to cope with growth at low and moderate temperatures.

Eurypsychrophiles have a great potential for expanding our knowledge of the functional genomics of thermal adaptation because they are capable of metabolism at temperatures ranging from below 0°C up into the mesophilic range (25°C to 40°C).

Psychrobacter arcticus is such an organism with a growth temperature range of -10°C to 28°C (1). *P. arcticus* adjusts its membrane lipid fatty acid composition, capsular carbohydrate composition, and substrate utilization profile during growth at 4°C versus growth at 25°C (23). However, the transcriptional changes contributing to this modified state at 4°C are unclear. Here we present a transcriptome analysis of *P. arcticus* growing under rich medium conditions at 22°C compared to 4°C. A mineral medium was also developed to permit analysis of the biosynthetic challenges of life at low temperature during growth on a single carbon source. This medium enabled us to analyze transcriptome changes for the same temperature comparison in the context of biosynthetic pathways that may not be required in a complex growth medium.

Materials and Methods

Culture conditions for ½ TSB temperature comparison. Four biological replicate cultures of *Psychrobacter arcticus* 273-4 were inoculated from ½ tryptic soy agar plate (½ TSA, BD Biosciences, San Jose, CA) into a 5 ml starter culture of ½ tryptic soy broth (½ TSB, BD Biosciences). Experimental cultures consisted of 50 ml ½ TSB and were inoculated with 50 ul stationary phase starter culture. Cultures were grown at either 22°C (T_{opt}) or 4°C with shaking at 150 rpm to mid-exponential phase ($OD_{600} \cong 0.1$) in a 300 ml Nephelo flask (Bellco Biotech., Vineland, NJ) for cell harvest and RNA extraction (below).

Medium development. A basal mineral medium was developed that allowed for growth of *Psychrobacter* in a medium containing 3% sea salts, 20 mM D,L-lactic acid or sodium pyruvate, 5 mM NH_4Cl or 5 mM glutamate, 1 mM K_2HPO_4, 1X Wolfe's Vitamins and

1X Trace Minerals. Wolfe's Vitamins (1000X) and trace minerals (1000X) were produced as described by Kostka and Nealson (18). HEPES, PIPES, MOPSO, MOPS and Phosphate buffer all at pH 7.0 were tested for ability to support growth. Concentrations of 1 mM and 2 mM K_2HPO_4 were tested, but higher concentrations could not be used due to precipitation of phosphate salts particularly in high sea salts medium.

For growth rate determinations, *P. arcticus* was cultured initially in marine broth (MB) containing 5% sea salts, 5 g l^{-1} tryptone, and 1 g l^{-1} yeast extract until it reached stationary phase about 40 h after inoculation. A 1% inoculum was added to the mineral medium containing 5% sea salts and 20 mM lactate, and cells were grown to stationary phase. Cells were then cultured to stationary phase in mineral medium containing the carbon source to be tested. After two successive passages to condition *P. arcticus* to the selected carbon source, growth was measured by OD_{600} in a Spec20D visible light spectrophotometer. Growth rates were estimated from the data in the OD_{600} range from 0.03-0.3. Cultures were considered negative for growth on a substrate if no growth was observed after 5 days at 22°C or 10 days at 4°C.

Culture conditions for acetate medium temperature comparison. Three biological replicate cultures were inoculated from marine agar (MA) plates into a 5 ml starter culture in marine broth (MB) at 22°C. One liter MB contained 5 g tryptone, 1 g yeast extract and 30 g sea salts. *P. arcticus* was acclimated to mineral medium containing 20 mM acetate and 30 g l^{-1} sea salts by growth to stationary phase ($OD_{600} \sim 0.8$) through three passages in 50 ml acetate medium with shaking at 150 rpm. Cultures for analysis by microarrays were grown in 50 ml acetate medium with shaking at 150 rpm in a 300 ml

Nephelo flask at either 22°C or 4°C until the cultures reached mid-exponential phase growth ($OD_{600} \sim 0.1$).

Microarray Characteristics. The *Psychrobacter arcticus* 273-4 microarray consists of 2144 70-mer oligonucleotide probes (Operon, Huntsville, AL). Arrays were printed in duplicate on CMT-UltraGAPS slides (Corning, Corning, NY). Each array consists of 1998 probes for *P. arcticus* genes, 12 random oligonucleotides with no matching sequence to any gene in the probe database, 10 SpotArray (Stratagene, La Jolla, CA) oligonucleotide probes to assay spike-in positive controls, and 10 cloned human sequences with no homology to *P. arcticus* genes. The remaining spots contained only 0.3X SSC printing buffer.

Cell Harvest, RNA Extraction and cDNA synthesis. When cultures reached mid exponential phase, 25 ml of culture was preserved with 2 volumes isothermal RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). Cells in RNAprotect were incubated at room temperature for 5 min then pelleted by centrifugation in a Sorvall RC-5B centrifuge (Dupont) at 4°C for 25 min at 8000 x *g* in an SLA-1500 rotor. Cells were resuspended in 1 ml room temperature RNAprotect and pelleted again in an Eppendorf 5417R centrifuge at 4°C at 8000 x g for 10 min. Supernatant was decanted and the cell pellet was frozen at -80°C.

RNA extractions were performed according to the RNEasy mini kit (Qiagen) with the following modifications: Specificially, lysozyme digestion was carried out for 30 min. Following cell lysis using buffer RLT, 1 µl each of two-fold diluted SpotReport mRNAs (Stratagene) ranging from 1 ng to 1 pg of RNA were added to the lysate as

controls for RNA degradation during sample preparation. Purified RNAs were analyzed by gel electrophoresis in a 1X FA gel containing 0.67% formaldehyde and 1.2% agarose.

Three x 5 μg aliquots of total RNA were denatured with 6 μg random hexamers (Invitrogen, Carlsbad, CA) in a 17.5 μl volume for 10 min at 70°C and snap-cooled 5 min on ice. Denatured total RNA mixtures were reverse transcribed to amino-allyl labeled cDNAs by combining the total RNA mixture with 6 μl 5X First Strand Synthesis Buffer, 3 μl 0.1 M dithiothreotol, 1 μl RNaseOUT (Invitrogen), 1.2 μl 25X dNTPs (3 aa-dUTP:2 dTTP), and 2 μl Superscript II reverse transcriptase (Invitrogen). Amino-allyl dUTP was obtained from Ambion (Austin, TX). Reactions were incubated at 42°C overnight and stopped by addition of 10 μl 0.5 M EDTA. RNA was hydrolyzed by incubation with 10 μl 1 M NaOH at 65°C for 15 min. RNA hydrolysis was neutralized with 10 μl 1 M HCl. Amino-allyl labeled cDNAs were purified according the amino-allyl labeling protocol posted by The Institute for Genomic Research (12).

Microarray Hybridization. Amino-allyl labeled cDNAs were resuspended in 4.5 μl 0.1 M Na_2CO_3, pH 9.0 for 10 min at room temperature and combined with 4.5 μl Cy3 or Cy5 NHS-ester in DMSO (GE Biosciences, Piscataway, NJ). Dye incorporation reactions were incubated at 25°C for 1.5 h. Equipicomolar nucleotide amounts of dye-labeled cDNA samples to be hybridized such that neither Cy3 nor Cy5 exceeded 550 pmoles. cDNAs were dried in a SpeedVac for 1.5 h. Hybridization mix was prepared by resuspending cDNA pellets in 50 μl 5X SSC, 25% formamide, 0.1% SDS, 0.1 mg ml^{-1} Salmon Testes DNA.

Glass slide arrays were prehybridized by incubation in 5X SSC, 0.1% SDS, 0.1 mg ml^{-1} BSA for 60 min at 49°C. Slides were then washed twice in room temperature

0.1X SSC for 5 min. Slides were washed twice for 30 sec in sterile water. Prehybridized slides were then immediately dried by centrifugation for 3 min at 1600 rpm in a Sorvall RT 6000D centrifuge with an H1000B hanging basket rotor. Lifterslips (22 x 60 mm, Erie Scientific, Portsmouth, NH) were washed in sterile water followed by 100% ethanol and dried under filtered forced air. Hybridization mix was preheated to 95°C for 10 min, then pipetted onto the slide. Hybridization cassettes were sealed and immediately placed in a 49°C water bath shaking at 30 rpm for 20 h.

Hybridized slides were first washed in 2X SSC, 0.1% SDS for 5 min at 49°C. A second wash was performed twice in 0.1X SSC, 0.1% SDS at room temperature for 5 min. A third wash was performed four times in 0.1X SSC for 1 min each. The final wash was in 0.01X SSC for 10 sec. Washed slides were dried by centrifugation as described above. Slides were scanned using a Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA). Images were analyzed and quality control analysis was performed using Genepix 6.0 software. Only slides passing the quality control statistical analysis were included in the biological data analysis.

Microarray data analysis. Fluorescence data from microarray hybridizations were analyzed in the LIMMA 2.7.10 package in R (33). Minimum background correction was applied and data were normalized by print-tip lowess normalization (37). Differential expression was estimated using a simple linear model to estimate the effect of 22°C or 4°C incubation on gene expression. Statistical analysis was performed by t-test with false discovery rate control correction for multiple hypothesis tests (25). Genes were considered differentially expressed with $P < 0.05$.

Accession Numbers. The *P. arcticus* genome was deposited under GenBank accession number CP000082 by H.L. Ayala-del-Rio *et al.*

Results

Analysis of differentially expressed genes at 22°C and 4°C in rich medium. Transcriptomes of *P. arcticus* grown at 22°C (T_{opt}) and 4°C in ½ TSB medium were compared using oligonucleotide microarrays. Gene expression changes were analyzed using the LIMMA 2.7.10 with false-discovery rate control for multiple hypothesis tests. We deemed a post correction P-value cutoff of 0.05 to be significant in these experiments across four biological replicates. Only 44 genes were differentially expressed with a $P < 0.05$ (Fig. 3.1).

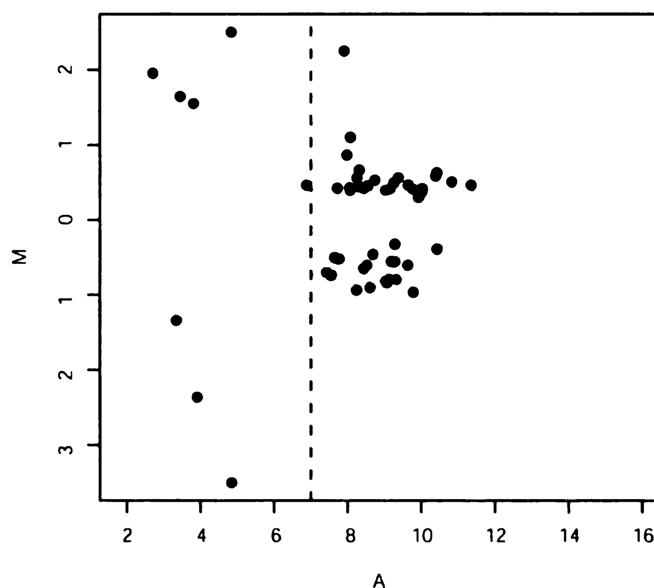

Figure 3.1. M vs. A plot in *P. arcticus* grown at 4°C (positive M-values) and 22°C (negative M-values). M is defined as $\log_2$(4°C/22°C). A is $\log_2$(4°C · 22°C)$^{1/2}$). Only spots with mean intensity (A) greater than 7 were considered relevant for our analysis due to our use of background correction. The dashed line represents the intensity cut-off. Black points are genes that were statistically differentially expressed ($P < 0.05$).

Eighteen genes were down-regulated during growth at 4°C with fold change ranging from 1.3 to 2-fold. These included genes for the isobutyryl-CoA degradation, signal transduction, and fatty acid biosynthesis (Table 3.1). Isobutyryl-CoA may be required in higher concentrations by the cell at 4°C, possibly as a substrate for branched chain fatty acid biosynthesis. Other fatty acid biosynthesis genes which were down-regulated included a long-chain fatty acid acyl-CoA ligase and an enoyl-ACP reductase which might indicate decreased need for long-chain and saturated fatty acids at low temperature. Signal transduction functions in nitrogen metabolism and twitching motility were down-regulated. Dicarboxylate uptake (*Psyc_1965*) and malic enzyme (*Psyc_1367*) were down-regulated indicating that dicarboxylic acids may be the substrate of choice for growth at 22°C but not at 4°C.

In contrast, up-regulated genes at 4°C had roles in transport, translation, transcription and energy metabolism. Up-regulated genes at 4°C exhibited fold changes ranging from 1.2 to 4.8-fold. *Psyc_1653*, a glycolate and lactate uptake symporter was up-regulated, as was the periplasmic binding subunit of a basic amino acid ABC transporter (Table 3.2). The septum ring formation antagonist *minE* was up-regulated at 4°C. Several genes involved in energy metabolism and the metabolism of glycolate or glyoxylate were also up-regulated at 4°C including genes for the β-hydroxyaspartate pathway of glyoxylate metabolism, an alternative to malate synthase driven glyoxylate metabolism. Two out of three acetone carboxylase subunits were strongly up-regulated during growth at 4°C. A peptidyl-prolyl-*cis-trans* isomerase was up-regulated at 4°C possibly to speed peptide folding.

Table 3.1. Genes wth decreased expression during growth at 4°C. Genes are grouped by functional category.

Genbank Gene Name	Gene Name	Annotation	Function	Fold Change[a]	P-value
Psyc_1604	*Psyc_1604*	putative Pyridoxal phosphate-dependent deaminase family protein	Amino acid metabolism	-1.4	0.019
Psyc_0900	*mmsA*	methylmalonate-semialdehyde dehydrogenase	Branched chain amino acid degradation	-1.5	0.014
Psyc_0901	*Psyc_0901*	putative acyl-CoA dehydrogenase	Branched chain amino acid degradation	-1.8	0.008
Psyc_0903	*Psyc_0903*	enoyl-CoA hydratase/isomerase family protein	Branched chain amino acid degradation	-1.9	0.008
Psyc_0904	*mmsB*	putative 3-hydroxyisobutyrate dehydrogenase	Branched chain amino acid degradation	-1.8	0.007
Psyc_1367	*maeB*	putative NADP dependent malate oxireductase	Central Carbon Metabolism	-1.3	0.040
Psyc_1815	*pilJ*	putative chemotaxis sensory transducer	Chemotaxis/motility	-1.4	0.001
Psyc_1816	*pilI*	putative type IV pili signal transducer (chemotaxis/motility, CheW domain)	Chemotaxis/motility	-1.5	0.005
Psyc_1965	*Psyc_1965*	TRAP-T family transporter, periplasmic substrate binding subunit	Dicarboxylate Uptake	-1.7	0.007
Psyc_1190	*rubA*	putative rubredoxin protein	Energy metabolism	-1.4	0.031
Psyc_0308	*Psyc_0308*	putative long-chain fatty acid acyl-CoA ligase	Fatty acid biosynthesis	-1.9	0.001
Psyc_0309	*Psyc_0309*	putative acyl-CoA carboxylase (beta subunit) protein	Fatty acid biosynthesis/degradation	-1.7	0.005
Psyc_0313	*acd*	putative acyl-CoA dehydrogenase	Fatty acid biosynthesis/degradation	-2.0	0.005
Psyc_1045	*fabI*	enoyl acyl carrier protein reductase	Fatty acid biosynthesis/saturation	-1.6	0.040
Psyc_0395	*glnD*	probable protein-P-II uridylyltransferase	Signal transduction, N-incorporation	-1.5	0.005
Psyc_0384	*Psyc_0384*	hypothetical protein	Unknown	-1.5	0.040
Psyc_1645	*Psyc_1645*	hypothetical protein	Unknown	-1.7	0.030
Psyc_1917	*Psyc_1917*	conserved hypothetical protein	Unknown	-1.3	0.025

[a] Fold change is expressed relative to 4°C transcriptome

Table 3.2. Genes increasing in expression at during growth at 4°C. Genes are grouped by functional category.

Genbank Gene Name	Gene Name	Annotation	Function	Fold Change[a]	P-value
Psyc_0158	*dadA*	probable D-amino acid dehydrogenase subunit	Amino Acid Metabolism	1.3	0.007
Psyc_1810	*minE*	probable septum formation topological specificity factor MinE	Cell Division Septum Formation	1.3	0.013
Psyc_1158	*Psyc_1158*	possible glycosyl transferase	Cell Wall Biogenesis	1.3	0.014
Psyc_1389	*tdcB*	putative threonine dehydratase	D-β-hydroxyaspartate pathway	1.5	0.016
Psyc_1390	*dhaa*	putative D-b-hydroxy-aspartate aldolase	D-β-hydroxyaspartate pathway	1.5	0.000
Psyc_1419	*Psyc_1419*	AAA ATPase	Energy Metabolism	1.3	0.040
Psyc_1440	*Psyc_1440*	putative cytochrome c, class I	Energy Metabolism	1.8	0.016
Psyc_1448	*Psyc_1448*	possible Acetone carboxylase alpha subunit	Energy Metabolism	2.1	0.005
Psyc_1449	*Psyc_1449*	probable Acetone carboxylase beta subunit	Energy Metabolism	4.8	0.001
Psyc_1871	*fabG*	probable 3-oxoacyl-(acyl-carrier-protein) reductase	Fatty Acid Biosynthesis	1.3	0.008
Psyc_0173	*ispE*	possible 4-diphosphocytidyl-2C-methyl-D-erythritol kinase	LPS Biosynthesis	1.6	0.005
Psyc_1523	*asd*	putative aspartate semialdehyde dehydrogenase	Lysine and Threonine Biosynthesis	1.4	0.008
Psyc_0283	*slyD*	putative peptidyl-prolyl cis-trans isomerase, FKBP-type	PPIase; Protein Folding	1.3	0.031
Psyc_0627	*Psyc_0627*	putative PhoH-like protein, predicted ATPase	Signal Transduction; Phosphate Starvation	1.4	0.048
Psyc_1887	*rpoB*	RNA polymerase beta-subunit	Transcription	1.4	0.019
Psyc_0507	*rpmD*	ribosomal protein L30	Translation	1.3	0.048
Psyc_0511	*rpsM*	ribosomal protein S13	Translation	1.5	0.005
Psyc_0512	*rpsK*	ribosomal protein S11	Translation	1.4	0.034
Psyc_1565	*rplM*	ribosomal protein L13	Translation	1.2	0.048
Psyc_1773	*glyS*	glycyl-tRNA synthetase, beta subunit	Translation	1.4	0.003
Psyc_0296	*Psyc_0296*	ABC basic amino acid transporter, periplasmic binding protein	Transport	1.3	0.039
Psyc_1653	*glcA*	glycolate/L-lactate:H+ symporter	Transport	1.3	0.048
Psyc_0406	*Psyc_0406*	transposase, IS4 family related	Transport Pseudogene: Transposase	1.4	0.039
Psyc_1639	*Psyc_1639*	hypothetical protein	Unknown	1.4	0.003
Psyc_1718	*Psyc_1718*	conserved hypothetical protein	Unknown	1.4	0.007
Psyc_1959	*Psyc_1959*	conserved hypothetical protein	Unknown	1.5	0.014

[a] Fold change is expressed relative to 4°C.

Development of a mineral medium. We concluded that the observation of only a small number of differentially expressed genes in the rich medium might be due to the nutritional complexity of ½ TSB. Hence, a mineral medium was developed to analyze biosynthetic challenges resulting from growth at 4°C. This medium formulation was based on hypotheses from a metabolic pathway reconstruction which predicted that *P. arcticus* possesses the necessary gene complement for growth on proline, serine, propionate, malonate, glycolate, acetate, fatty acids, lactate, and dicarboxylic TCA cycle intermediates (N.N. Ivanova, personal communication). Lactate was chosen as the carbon source for development of the medium because *P. arcticus* contains a lactate dehydrogenase cytochrome which was not differentially expressed in the 22°C vs 4°C comparison of growth in ½ TSB. Furthermore, transport of lactate was up-regulated at 4°C, indicating that this substrate might be more important to *P. arcticus* in the cold (Table 3.2). After several trials with various buffers and salt mixtures, a basal medium was developed for growth on 20 mM lactate. The medium contained 5% sea salts (w/v), 50 mM MOPS, pH 7.0, 5 mM NH_4Cl, 1 mM K_2HPO_4, 1X Wolfe's Vitamins, and 1X trace minerals mix. *Psychrobacter* was tested for growth on a variety of carbon sources (Table 3.3). No growth was observed on glucose, isovalerate, isobutyrate, glycerol, malate, succinate, citrate, glycolate, glyoxylate, serine, glycine or propionate at 22°C or 4°C after repeated attempts using inocula pre-cultured on acetate, lactate and marine broth.

Table 3.3. Growth rates of *Psychrobacter arcticus* 273-4 on defined basal medium with various sole carbon sources[a]

Substrate	Growth Rate ($\Delta log_{10}OD_{600}$ hr^{-1}) 22°C	4°C
Acetate	0.069 ± 0.003	0.016 ± 0.001
Acetate + 5 mM Glutamate	0.073 ± 0.002	ND[d]
Malonate	0.067 ± 0.003	ND[d]
Pyruvate	0.003 ± 0.0008	0.005 ± 0.0006
Lactate	0.027 ± 0.002	0.010 ± 0.002
Lactate + 5 mM Glutamate	0.028 ± 0.004	ND[d]
Butanoic Acid	0.041 ± 0.001	0.011 ± 0.0008
Decanoic Acid[c]	0.014 ± 0.004	0.006 ± 0.002
Palmitic Acid[c]	0.007	NG[b]
Glutamate	0.040 ± 0.004	ND[d]
Marine Broth, 5% Sea Salts	0.106 ± 0.006	0.026 ± 0.0008
1/2 TSB + 5% NaCl	0.019 ± 0.0004	0.005 ± 0.0007

[a] Unless otherwise noted, all carbon sources were at 20 mM concentration. No growth was observed when glyoxylate, glycolate, glycine, proline, serine, glycerol, isovaleric acid, isobutyric acid, propionic acid, citrate, succinate, malate or glucose at 22°C or 4°C with

[b] NG = No growth after 10 days at 4°C

[c] No significant growth observed on 0.5% Ethanol introduced to cultures as solvent for decanoic and

[d] ND = No data collected under this condition.

We hypothesized from the microarray data that lactate would support the fastest growth rates. However, cultures in lactate medium grew at only 39% of the rates achieved on acetate. *P. arcticus* had the fastest rate of growth on acetate and malonate media, the two basic units of fatty acid metabolism, and growth rate decreased as fatty acid chain length increased. Acetate enabled growth at 65% of the rate observed in the complex marine broth with 5% sea salts. Supplementation of the medium with 5 mM

glutamate yielded only a slight increase in growth rate over medium containing acetate or lactate alone.

Analysis of differentially expressed genes at 22°C and 4°C in a defined medium. Microarray hybridizations were performed on cultures grown to mid-exponential phase at 22°C and 4°C in the 3% sea salts medium with acetate as the sole source of heterotrophic carbon. We expected that by culturing *P. arcticus* in the acetate medium, temperature specific biosynthetic challenges could be elucidated through transcriptome analysis. However, no differential gene expression was observed between the two temperatures with an FDR corrected P < 0.05 (Fig. 3.2).

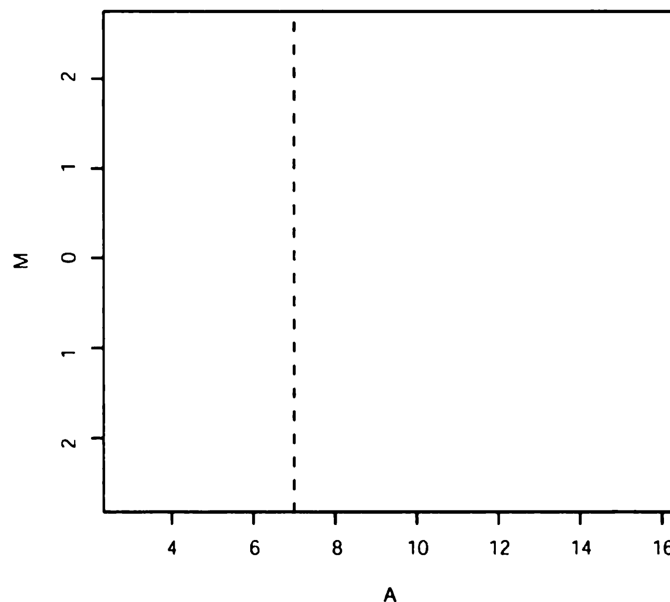

Figure 3.2. M vs. A plot comparing the transcriptome a 4°C (positive M values) to the transcriptome at 22°C (negative M values) in acetate medium. No statistically significant differential expression was observed between the two temperatures this experiment.

Table 3.4. Genes with >1.5-fold change in mean expression between 22°C and 4°C

GenBank	Gene	Annotation	Fold Change[a]	P-Value
Psyc_0397	*Psyc_0397*	hypothetical protein	2.11	1.000
Psyc_0286	*Psyc_0286*	probable RNA pseudouridine synthase	-2.93	1.000
Psyc_1872	*Psyc_1872*	conserved hypothetical protein	-2.65	1.000
Psyc_0243	*Psyc_0243*	conserved hypothetical protein	-1.70	1.000
Psyc_0876	*Psyc_0876*	conserved hypothetical protein	-2.13	1.000
Psyc_1126	*Psyc_1126*	probable exonuclease V, alpha subunit	-1.76	1.000
Psyc_1377	*Psyc_1377*	conserved hypothetical protein	1.73	1.000
Psyc_0721	*Psyc_0721*	hypothetical protein	2.13	1.000

[a] Fold change is expressed relative to 4°C.

In an attempt to learn as much as possible about the temperature response in the nutritionally simple medium, we relaxed our differential expression criteria to include any gene with an average fold change > 1.5 in this experiment. Only eight genes were detected as differentially expressed based on fold change alone (Table 3.4). *Psyc_0286* and *Psyc_1126* had functions in rRNA maturation and recombination respectively. The remaining six genes were hypothetical proteins with no known function.

Discussion

In spite of genomic capacity to acquire and metabolize a variety of amino acids and organic acids, *P. arcticus* demonstrated a clear preference for growth supported by a limited range of oxidized mono-carboxylic acids and fatty acids. When rich and mineral media were used as a growth medium for transcriptome comparisons at 22°C and 4°C, only small magnitude changes in < 50 genes were observed. This suggests that 4°C does not significantly alter growth physiology compared with growth at 22°C (T_{opt}). It seems likely from our transcriptome results that adjustments to metabolism at 4°C might be better explained on the level of posttranscriptional regulation.

P. arcticus may be optimized to conserve energy and carbon by utilizing acetate, a ubiquitous substrate, as the basis for its biosynthesis and energy metabolism. Acetate could originate in permafrost from numerous biological and chemical processes including the degradation and metabolism of reduced molecules. Acetate metabolism has several features that may be desirable at low temperatures. Uptake of acetate can occur directly via the cytoplasmic membrane. *P. arcticus* does not contain a homologue of ActP, the acetate uptake transporter (11). Utilization of the glyoxylate shunt can allow *P. arcticus* to conserve much of the carbon from this substrate. Metabolic activation of acetate requires only one to two enzymes to generate acetyl-CoA from acetate and coenzyme A, so the net energy investment in acetate metabolism is low though this may be offset by increased net cost to synthesize other key cell constituents such as carbohydrates or amino acids.

The reasons for lack of growth on dicarboxylates and uncharged amino acids are unclear. Transporters are present for the dicarboxylates (TRAP-T dicarboxylate uptake transporter *Psyc_1963-1965*), glycolate (glycolate:H+ symporter *Psyc_1653*), and glycine (BCCT uptake transporter *Psyc_1301*). *P. arcticus* has only a 2.7 Mbp genome, and small genomes possess smaller proportions of regulatory genes than large genomes (16). So, it is possible that culture conditions used here were unable to induce utilization of these substrates for growth, or that unpredicted aspects of the metabolic network of *P. arcticus* prevented growth on these substrates despite the presence of the complete gene complement. Growth on palmitic acid was only observed at 22°C. The reasons for this seeming temperature preference are unclear, but could be due to a combination of

reduced water solubility of the compound at low temperatures and reduced uptake of the compound due to decreased membrane fluidity at 4°C.

A slight inflection of growth rate decline over temperature was observed at 6°C in *P. arcticus* growing in ½ TSB (23). In the same study, *P. arcticus* was reported to decrease fatty acid chain length, increase the proportion of unsaturated fatty acids in membrane lipids and to increase cell length and cell volume during growth at 4°C. The transcriptome in ½ TSB generally supports the hypotheses from the results of the physiological analyses that showed fatty acid metabolism changes. Genes for long chain fatty acid synthesis and saturation of fatty acids during biosynthesis were transcriptionally down-regulated at 4°C. In *Bacillus subtilis*, degradation of valine to isobutyryl-CoA was up-regulated during cold shock (15). An increase in the metabolic pool of isobutyryl-CoA could lead to increased formation of *iso*-branched chain fatty acids. Down-regulation of genes for isobutyryl-CoA degradation may indicate increased need for this branched-chain fatty acid substrate. However, only a small but statistically insignificant increase in branched chain fatty acids was observed in *P. arcticus* at 4°C (M. Ponder, personal communication).

MinE antagonizes the formation of the cell division septum. Location of the division septum formation by FtsZ polymerization is controlled by diffusion of MinCD, and MinE from pole to pole in rod shaped cells (26, 30). Diffusion rates decrease over decreasing temperature but an increase in concentration at the high end of a concentration gradient can increase diffusion rates. Therefore, the increase in cell length observed by Ponder *et al* may be due to overcompensation by increased expression of *minE*.

P. arcticus has a rare mode of glyoxylate metabolism in which glyoxylate, resulting from isocitrate lyase activity, is transformed to glycine then condensed with another glyoxylate to form β-hydroxyaspartate (17). β-hydroxyaspartate aldolase produces oxaloacetate from beta-hydroxyaspartate. Activities associated with this pathway were up-regulated at 4°C. This increase can be interpreted as a demand to process the products of acetate or glyoxylate metabolism faster or to regenerate oxaloacetate as a precursor for gluconeogenesis. Acetone carboxylase was also up-regulated at low temperature which carboxylates acetone or isopropanol to organic acids acids (32). However, the source of acetone in the cell is unclear.

The ribosome responds to cold shock in a variety of organisms. For example, *Listeria monocytogenes* ribosomes become more thermally sensitive after cold shock (2). Translation was also clearly affected in *P. arcticus* by growth at 4°C. Four ribosomal proteins were detected as up-regulated at 4°C. Loss of ribosomal protein L30 is reported to produce an *E. coli* mutant deficient in ribosome assembly at 20°C (9). Ribosomal proteins S13, S11 and L13 have not been reported to be involved in cold acclimated ribosome biogenesis. RNA binding genes associated with translation in the cold, e.g. *cspA, csdA*, were not induced during growth at 4°C, but *P. arcticus* did induce one PPIase at low temperature which may indicate that *P. arcticus* is increasing expression for peptide folding.

When taken together, the transcription response to growth at 4°C in ½ TSB suggests only a fine-tuned compensation for translation and carbon catabolism, coupled with a membrane fluidity response. Importantly, a proteome comparison of *P. arcticus* growth at 22°C and 4°C shows similarities in the functional categories of up-regulated

genes at 4°C including increased expression of PPIases, PhoH, and acetone carboxylase (39).

When *P. arcticus* was placed in a medium with a single source of carbon, no change gene expression due to growth temperature was observed. In the permafrost, responses to low temperatures, low water activity and decreased nutrient availability may be linked (10). Exposure to low temperatures is known to effect survival during osmotic shock and starvation (8, 19, 20). Therefore, carbon source limitation or salt content of the mineral medium may have moderated the effect of temperature on the transcriptional response of *P. arcticus* to growth at 4°C. Furthermore, the Q_{10}, or change in growth rate over 10°C, in ½ TSB is ~2.25 versus ~2.15 in the acetate medium and may not demand adjustments in the cell metabolism to support different growth rates. Growth at temperatures exhibiting a $Q_{10} > 4$ would be expected to yield major physiological changes over temperature and likely exhibit substantial changes to the transcriptome (6).

Effects of temperature on physiology and the differentiation of species is a long researched area in biological science (27, 34). Adaptations of polar species are of particular interest because their physical environment is different in many ways from those in temperate or tropical environments (35). Polar environments are commonly energy and nutrient limited systems with reduced biomass and slower rates of microbial activity (5). Numerous adaptations to these environments have been elucidated including observations of a reduced threshold to induce heat shock responses, reduced hemoglobin content in Antarctic fishes, production of antifreeze and ice nucleation proteins, and adaptation of proteins for high activity rates at low temperatures with concomitant increased thermolability of those proteins (7, 13, 31, 38). Adaptation to the permafrost

environment has likely impacted the *P. arcticus* response to temperature. *P. arcticus* has undergone selection in the cold and may not sense a temperature or growth stress at 4°C relative to growth at 22°C. *P. arcticus* may have regulatory mechanisms that modify the activity of specific enzymes in response to temperatures. Indeed, evidence for post-translational modifications was observed in a *P. arcticus* proteome comparison at 22°C and 4°C (39). Furthermore, reductions in enzyme activity can be attained due to simple temperature effects on enzymes without changes to transcription. Therefore, changes in physiology observed by Ponder *et al* may be regulated more directly on a post-transcriptional level.

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CHAPTER 4

RESOURCE EFFICIENCY AND MOLECULAR DYNAMICS DOMINATE THE TRANSCRIPTOME RESPONSE FOR GROWTH BELOW 0°C IN *PSYCHROBACTER ARCTICUS* 273-4

Abstract

We analyzed the trancriptome of *Psychrobacter arcticus* 273-4 grown at two low temperatures: 0°C and -6°C, and two high temperature: 17°C and 22°C using a mixed effects ANOVA model. Genes involved in transcription, translation, energy production and the majority of biosynthetic pathways were down-regulated at low temperatures. However, specific genes in the transcriptome were up-regulated at 0°C and -6°C presumably to compensate for reduced function of their products. Evidence of enzyme exchange was detected during growth at low vs high temperature, particularly for DD-peptidase, DEAD-box RNA helicase and energy efficient substrate incorporation pathways for ammonium and acetate. Other genes exhibited compensatory up-regulation at low temperature including specific genes for uradine biosynthesis and the biosynthesis of proline, tryptophan, methionine, and histidine. RNases and peptidases were generally up-regulated at low temperatures. In contrast to results observed in other psychrophiles and mesophiles, only *clpB* and *hsp33* were up-regulated at low temperature with no up-regulation of other chaperones and peptidyl-prolyl isomerases. Knockout mutants of *katE*, *msrA*, *csdA*, and *dac2* were all deficient in low temperature growth, but a mutant in *dac1* was deficient in growth at 22°C. Our data suggest that the majority of the basal biological machinery including translation, transcription and energy metabolism are well adapted to function across the -6°C to 22°C growth range of *P. arcticus* and temperature compensation by gene expression was employed to address specific challenges to low temperature life.

Introduction

Growth at low temperatures presents several challenges for life including reduced chemical reaction rates, rates of physical processes and water activity (53). Metabolism at subzero temperatures has been proven for psychrotrophs and psychrophiles isolated from cryoenvironments, i.e. environments which exhibit transient or long-term freezing conditions, including sea ice, cryopegs, and permafrost soils (9, 15, 37). Proteomic studies of psychrophile responses to growth at suboptimal temperatures have identified differentially expressed genes including energy metabolism, translation factors and peptidyl-prolyl isomerases (29, 51). It remains unclear how a cryoadapted microbe must globally modify metabolism for growth at subzero temperatures. Gerday and others have reasoned that eurypsychrophiles could be most energy efficient if their key metabolic proteins were able to function under the full growth temperature range (14, 21, 48). Enzyme adaptations for localized flexibility of the peptide chain surrounding the active site and counteradaptations for thermostability of the peptide chain can allow proteins to function over a wide range of temperatures (21, 50). However, an organism approaching the lower temperature limit of its growth would benefit from differential gene expression as a means of metabolic flexibility versus the alternative of growth inhibition. Organisms can increase expression of genes in order to compensate for reduced biochemical reaction rates or can differentially express isozymes to adapt to temperatures as has been observed in fish and some bacteria (43, 55). Gene expression studies on organisms growing at suboptimal growth temperatures have identified increased expression of genes important to several responses necessary for life in the cold, including DNA and RNA binding

proteins to destabilize secondary structures, maintenance of membrane fluidity, chaperones, uptake of compatible solutes, and energy production (3, 6, 46, 57).

Psychrobacter arcticus 273-4 was isolated from Kolyma lowland permafrost sediments continuously frozen for 10,000 to 40,000 yr (4, 60). Permafrost poses four continuous stressors on resident microbes including continuous cold, dessication, reduced nutrient availability and low continuous levels of radiation resulting from soil minerals (22). Ponder *et al* (2005) showed that *P. arcticus* was capable of growth below 0°C, and that it exhibited modified membrane fatty acid profiles and substrate utilization profiles during growth in the cold (47). *P. arcticus* grows in an experimentally confirmed temperature range of -10°C to 28°C and in a salinity range from 10 mM to 1.3 M NaCl (4). The capacity to endure temperatures and salinities in these ranges suggests that *P. arcticus* is adapted for life in cryoenvironments, which are characterized by low water activity and low temperature. Therefore, we consider *P. arcticus* 273-4 to be a good model for a cryo-adapted eurypsychrophile, i.e. a psychrophile capable of growth at a wide-range of temperatures (12).

Here we assayed the genome-wide modifications in transcription that are necessary for *P. arcticus* to grow in ice-like conditions at -6°C and used these data to learn about the physiology of *P. arcticus* under these conditions. We cultured *P. arcticus* to mid-exponential phase at two warm temperatures: 22°C and 17°C, and two cold temperatures: 0°C, and -6°C in a mineral medium with acetate as the sole source of heterotrophic carbon. We used our transcriptome data to develop a conceptual model of growth at subzero temperatures, in which the first analysis of the transcriptome of a bacterium growing at temperatures below 0°C.

Materials and Methods

Microarray Characteristics and Printing. The *Psychrobacter arcticus* 273-4 microarray consists of 2144 70-mer oligonucleotide probes (Operon, Huntsville, AL). Arrays were printed in duplicate on CMT-UltraGAPS slides (Corning, Corning, NY). Each array consists of 1998 probes for *P. arcticus* genes, 12 random oligonucleotides with no matching sequence to any gene in the probe database, 10 SpotArray (Stratagene, La Jolla, CA) oligonucleotide probes to assay spike-in positive controls, and 10 cloned human sequences with no homology to *P. arcticus* genes. Additional spots contained only 0.3X SSC printing buffer.

Strains, Media and Culture Conditions For all experiments, single colonies of *Psychrobacter arcticus* 273-4 were first grown to $OD_{600} \geq 0.8$ at 4°C in marine broth medium consisting of 3% (w/v) sea salts (Sigma-Aldrich, St. Louis, MO), 5 g/l tryptone (BD, Sparks, MD) and 1 g/l yeast extract (Difco). A 1% (v/v) inoculum of this starter culture was transferred to 7 ml of acetate medium. One liter acetate medium contained 75 g l^{-1} sea salts (Sigma-Aldrich), 50 mM MOPS buffer (Sigma-Aldrich), 20 mM sodium acetate (Baker), 5 mM NH_4Cl (Baker), pH 7.0 with NaOH, 1 mM K_2HPO_4 (Baker), 1X Wolfe's Vitamins, and 1X trace metal mix. Wolfe's Vitamins (1000X) and trace minerals solutions were made according to M1 defined medium from Kostka and Nealson (38).

P. arcticus 273-4 was acclimatized to the acetate medium through two passages to $OD_{600} \geq 0.8$ at 4°C shaken at 150 rpm before inoculation into experimental samples. A 5 ml inoculum from the final starter culture of *P. arcticus* in acetate medium was introduced to 500 ml acetate medium in 300 cm^2 untreated and vent-capped Falcon tissue

culture flask. Cultures were allowed to grow without shaking at -6°C, 0°C, 17°C or 22°C until they reached an OD_{600} between 0.09 and 0.25 (mid-exponential phase at all temperatures).

Cell Harvest, RNA Extraction and cDNA synthesis. When cultures reached mid-exponential phase, 25 ml of culture was preserved with 2 volumes of isothermal RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). Cells in RNAprotect were incubated at room temperature for 5 min then pelleted by centrifugation in a Sorvall RC-5B centrifuge at 4°C. Cells were resuspended in 1 ml room temperature RNAprotect and pelleted again in an Eppendorf 5417R centrifuge at 4°C. Supernatant was decanted and the cell pellet was frozen at -80°C.

RNA extractions were performed according to the RNEasy mini kit (Qiagen) with some modifications. Specificially, lysozyme digestion was carried out for 30 min. Following cell lysis using buffer RLT (Qiagen), 1 µl each of two-fold diluted SpotReport mRNAs (Stratagene) ranging from 1 ng to 1 pg of RNA were added to the lysate as controls for RNA degradation during sample preparation. Purified RNAs were analysed by gel electrophoresis in a 1X FA gel containing 0.67% formaldehyde and 1.2% agarose.

Three 5 µg aliquots of total RNA were denatured with 6 µg random hexamers (Invitrogen, Carlsbad, CA) in a 17.5 µl volume for 10 min at 70°C and snap-cooled for 5 min on ice. Denatured total RNA mixtures were reverse transcribed to amino-allyl labeled cDNAs by combining the total RNA mixture with 6 µl 5X First Strand Synthesis Buffer, 3 µl 0.1 M dithiothreitol, 1 µl RNaseOUT (Invitrogen), 1.2 µl 25X dNTPs (3 aa-dUTP:2 dTTP), and 2 µl Superscript II reverse transcriptase (Invitrogen). Amino-allyl dUTP was obtained from Ambion (Austin, TX). Reactions were incubated at 42°C

overnight and stopped by addition of 10 µl 0.5 M EDTA. RNA was hydrolyzed by incubation with 10 µl 1 M NaOH at 65°C for 15 min. RNA hydrolysis was neutralized with 10 µl 1 M HCl. Amino-allyl labeled cDNAs were purified according to the amino-allyl labeling protocol posted by The Institute for Genomic Research (TIGR) (33).

Microarray Hybridization. Amino-allyl labeled cDNAs were resuspended in 4.5 µl 0.1 M Na_2CO_3, pH 9.0 for 10 min at room temperature and combined with 4.5 µl Cy3 or Cy5 NHS-ester in DMSO (GE Biosciences, Piscataway, NJ). Dye incorporation reactions were incubated at 25°C for 1.5 h. Equipicomolar nucleotide amounts of dye-labeled cDNA samples were hybridized such that neither Cy3 nor Cy5 exceeded 550 pmoles. cDNAs were dried in a SpeedVac for 1.5 h. Hybridization mix was prepared by resuspending cDNA pellets in 50 µl 5X SSC, 25% formamide, 0.1% SDS and 0.1 mg ml^{-1} Salmon Testes DNA (Sigma-Aldrich).

Glass slide arrays were prehybridized by incubation in 5X SSC, 0.1% SDS, 0.1 mg ml^{-1} BSA for 60 min at 49°C. Slides were then washed twice in room temperature 0.1X SSC for 5 min. Slides were washed twice for 30 sec in sterile water. Prehybridized slides were then immediately dried by centrifugation for 3 min at 1600 rpm in a Sorvall RT 6000D centrifuge with an H1000B hanging basket rotor. Lifterslips (22 x 60 mm, Erie Scientific, Portsmouth, NH) were washed in sterile water followed by 100% ethanol and dried under filtered forced air. Hybridization mix was preheated to 95°C for 10 min, then pipeted onto the slide. Hybridization cassettes were sealed and immediately placed in a 49°C water bath shaking at 30 rpm for 20 h.

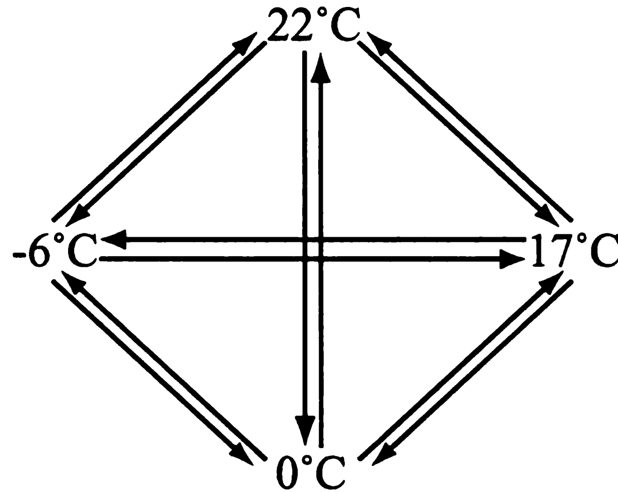

Figure 4.1. Experimental design of temperature comparisons using microarray hybridizations. The experiment consisted of five biological replicates at each temperature. Three cDNAs were synthesized from each RNA extract resulting from a biological replicate and hybridizations were carried out for each biological replicate in direct comparison to samples from all temperatures.

Hybridizations were performed for all growth temperature comparisons with five biological replicates at each temperature (Fig. 4.1). Hybridized slides were first washed in 2X SSC, 0.1% SDS for 5 min at 49°C. A second wash was performed twice in 0.1X SSC, 0.1% SDS at room temperature for 5 min. A third wash was performed four times in 0.1X SSC for 1 min each. The final wash was in 0.01X SSC for 10 sec. Washed slides were dried by centrifugation as described above. Slides were scanned using a Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA). Images were analyzed using Genepix 6.0 software and results were analyzed for quality using the marray and arrayQuality packages in R 2.3.0 (http://www.r-project.org/). Only slides passing the quality control statistical analysis were included in the biological data analysis.

Fluorescence data were analyzed using MAANOVA 1.0 to determine statistical significance and LIMMA 2.7.10 to calculate pairwise comparisons of gene expression. In both cases data were normalized using regional (print-tip) lowess normalization (16,

62). Normalized data were fit to a mixed-effects ANOVA model with gene, dye and temperature as fixed terms. Array, i.e. slide, and sample, i.e. biological replicate, were random (17). F tests were carried out using the Fs statistic in R/MAANOVA to determine the statistical significance of differential expression (18). Genes were considered differentially expressed if $P < 0.01$ after False Discovery Rate Control (FDR) adjustment for multiple hypothesis testing (17). All pairwise temperature comparison coefficients were estimated using LIMMA (54).

Genetics. Knockout mutants *katE* (*Psyc_570*), *dacC1* (*Psyc_0687*), *dacC2* (*Psyc_0704*), *msrA* (*Psyc_1043*), and *csdA* (*Psyc_1082*) were generated using essentially the strategy described in Denef *et al* (2006). Primers, plasmids and strains for this study are described in Table 4.1. Flanking regions of each gene were PCR amplified with primers containing restriction sites for insertion into pJK100. Upstream flanking regions were amplified with primers containing *Bgl*II and *Not*I restriction sites on the forward and reverse primers respectively. Downstream flanking regions were amplified with *Sac*II and *Sac*I site containing primers on the forward and reverse primers respectively.

P. arcticus 273-4 cultures for conjugation were grown in Luria Bertani (LB) broth at 22°C for 36 h. *E. coli* WM3064 containing the suicide vector for knockout was grown at 37°C in LB supplemented with 25 μg ml^{-1} kanamycin (Kan), 20 μg ml^{-1} tetracyclin (Tet) and 100 μg ml^{-1} diaminopimellic acid (DAP) overnight.

Table 4.1: Strains, primers and plasmids used in this study.

Strain, Primer or Plasmid	Description[a]	Reference or Source
Strains		
Psychrobacter arcticus		
273-4	Wild type	Bakermans *et al* (2006a)
PB0570	Δ*katE* ::*kan*	This study
PB0687	Δ*dacC1* ::*kan*	This study
PB0704	Δ*dacC2* ::*kan*	This study
PB1043	Δ*msrA* ::*kan*	This study
Escherichia coli WM3064	*thrB1* 004 *pro thi rpsL hsdS lacZ* Δ*M15* RP4-1360 Δ(*araBAD)567* Δ*dapA* 1341::[*erm pir*]	Denef *et al* (2006)
Primers		
del570UF	AGCTAGATCTgcgtatttcaaaaccgatcc	This study
del570UR	AGCTGCGGCCGCccattgccacgttccatatt	This study
del570DF	AGCTGAATTCaaggttcgtgaccttgccta	This study
del570DR	AGCTCATATGacatcacatcgacaccctga	This study
del687UF	AGCTAGATCTgccctttattgatacgctgttt	This study
del687UR	AGCTGCGGCCGCcacaatcgttggcagactgt	This study
del687DF	AGCTGAATTCgctgggcacaaaacttatt	This study
del687DR	AGCTCATATGaagtgggatggtctgactgc	This study
del704UF	AGCTAGATCTgcgagcatacggctaatacc	This study
del704UR	AGCTGCGGCCGCcactgcccaaactcaatgaa	This study
del704DF	AGCTGAATTCggtgccaatagctcagcaac	This study
del704DR	AGCTCATATGtagatgcacgccaataccaa	This study
del1043UF	AGCTAGATCTgaaaggtatgccggctcata	This study
del1043UR	AGCTGCGGCCGCgtggcaaatccgtaaacgtc	This study
del1043DF	AGCTGAATTCatggaagcagcaggttatgg	This study
del1043UR	AGCTCATATGgtgttgcgcacattggttac	This study
del1082UF	AGCTAGATCTctgcgattttgggtctagga	This study
del1082UR	AGCTGCGGCCGCgtttggggtgtcagtgcttt	This study
del1082DF	AGCTGAATTCtccaagtggtagccaaggtc	This study
del1082DR	AGCTCATATGccataaatctgcgagccaat	This study
kanF	GAAGATCTctcaaaatctctgatgttacattgc	This study
kanR	GAAGATCTtggttgatgagagctttgttgtaggt	This study
Plasmids		
pJK100	Allelic Exchange Vector	Denef *et al* (2006)
p570D	pJK100 with *Psyc_0570* downstream flanking sequence	This study
p570UD	pJK100 with both *Psyc_0570* flanking sequences	This study
p687D	pJK100 with *Psyc_0687* downstream flanking sequence	This study
p687UD	pJK100 with both *Psyc_0687* flanking sequences	This study
p704D	pJK100 with *Psyc_0704* downstream flanking sequence	This study
p704UD	pJK100 with both *Psyc_0704* flanking sequences	This study
p1043D	pJK100 with *Psyc_1043* downstream flanking sequence	This study
p1043UD	pJK100 with both *Psyc_1043* flanking sequences	This study
p1082D	pJK100 with *Psyc_1082* downstream flanking sequence	This study
p1082UD	pJK100 with both *Psyc_1082* flanking sequences	This study

[a] Capital letters include a 4 nucleotide extension on the 5' end of primers and a restriction site for insertion into pJK100.

Cells were combined in a ratio of 200 μl *E. coli* donor and either 200 μl or 800 μl *P. arcticus* 273-4 recipient. Conjugants were allowed to grow for 72 h at 22°C. Putative knockouts were isolated on LB plates with 25 μg ml^{-1} Kan and screened for a Kan^r Tet^s

phenotype. PCR screening of knockouts revealed amplicons of the expected product size and no wild-type amplicons were observed in mutant strains used for growth experiments.

Mutant Growth Experiments. Representative strains of the knockout mutants were grown in MB supplemented with 25 μg ml^{-1} Kan for 48 h at 22°C. Two subsequent rounds of culturing were carried out in acetate medium with 25 μg ml^{-1} Kan for 72 h.

Mutant growth rates were assayed using 96 well plates. Eight replicates of each mutant were inoculated into 200 μl acetate medium in each well. Sixteen replicates of wild-type *P. arcticus* 273-4 were present on each plate along with 12 blank wells. Plates were incubated at 22°C, 17°C, 4°C, 0°C, and -2.5°C. Blank subtracted OD_{600} data were collected using a SpectraMax M2 spectrophotometer (Molecular Devices). Data were compiled using a Perl script to parse data by growth temperature, mutant strain and time point. T-tests and plotting of data were carried out using R 2.4.0. The script calculated average growth rates for each replicate of each mutant for optical density increases between OD_{600} values 0.03 and 0.4. Two-tailed t-tests were performed to determine if growth rates were statistically different between wild-type *P. arcticus* and PB0343 within a temperature. Strains were considered statistically different if $P < 0.05$ after Bonferoni multiple-testing correction over the five hypothesis tests within a temperature.

Results

Growth physiology changes with temperature. Growth rate measurements were performed at -6°C, 0°C, 4°C, 17°C and 22°C in acetate medium (Fig. 4.2). Comparison of growth rates revealed that the Q10 of growth rate, defined as the fold-decline in growth rate over 10°C, exhibited the expected relationship of $Q_{10} \sim 2$ between growth

rates at 17°C and 4°C but not at 22°C or at temperatures below 4°C. The inflection in growth rate behavior between 17°C and 22°C suggested that growth at 22°C was being impacted by heat-stress on the culture. The Q_{10} in the temperature range 17°C to 0°C and 0°C to -6°C were 6.2 and 8.4, respectively. Change in growth rates of cultures grown at 0°C and -6°C were not continuous with changes in growth rates of cultures grown at higher temperatures. The observed inflection in growth rate decline at T < 4°C indicates that some changes in *P. arcticus* physiology have occurred resulting from incubation at 0°C or -6°C.

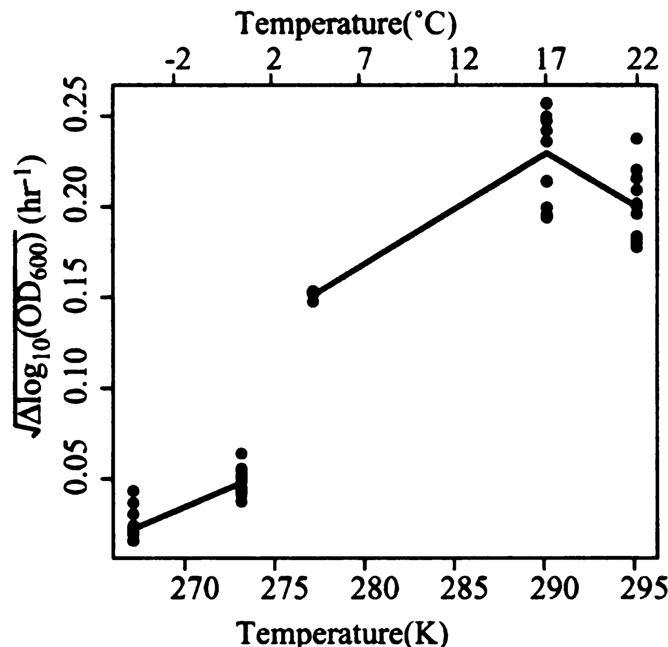

Figure 4.2. Square-root transformed of growth rates plotted over temperature. *P. arcticus* was grown at 22°C, 17°C, 4°C, 0°C, and -6°C. Lines are the result of fitting the square-root growth rates to a Belehradek-type model.

Microarray Results. Microarray analysis of growth in acetate medium at -6°C, 0°C, 17°C, and 22°C was performed to elucidate differences in gene expression between growth at the optimal growth temperature of 17°C and 22°C, cold acclimated growth at 0°C, and near the limits of *P. arcticus* growth at -6°C (Fig. 4.1). One-thousand-eighty-

five coding sequences (CDS) were differentially expressed during growth at one or more temperatures with $P < 0.01$. LIMMA analysis identified 92.9% of those genes identified by R/MAANOVA, but R/MAANOVA identified only 73.1% of the genes identified by LIMMA as differentially expressed. This highlights the more conservative nature of the ANOVA analysis, which models sources of variance in expression values (1). Temperature comparison plots of the microarray results demonstrate that transcriptomes at 0°C and -6°C are more similar to each other than to the trancriptomes at 22°C and 17°C (Fig. 4.3). The largest observed number of differentially expressed genes, 572 (27% of the *P. arcticus* genome), were in the comparison of -6°C vs. 17°C. Only 88 genes were differentially expressed between cells growing at 22°C vs. 17°C, and 24 genes were differentially expressed between cells grown at 0°C vs. -6°C. The trends in number of differentially expressed genes with temperature show that the *P. arcticus* transcriptome can be classified into the high temperature transcriptome at 17°C and 22°C and the low temperature transcriptome at 0°C and -6°C. When grouped by functional category, hypothetical and conserved hypothetical genes accounted for 28% of the differentially expressed genes (Fig. 4.4). For ease of results communication, all fold-changes are expressed relative to growth at 17°C unless otherwise noted.

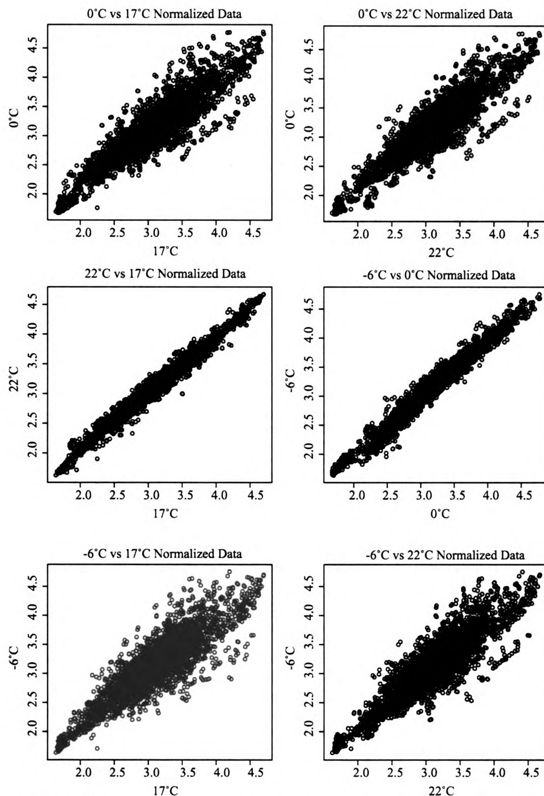

Figure 4.3. Plots of normalized average fluorescence values across 15 cDNA populations hybridized from each temperature. Fluorescence data are $\log_{10}$ transformed.

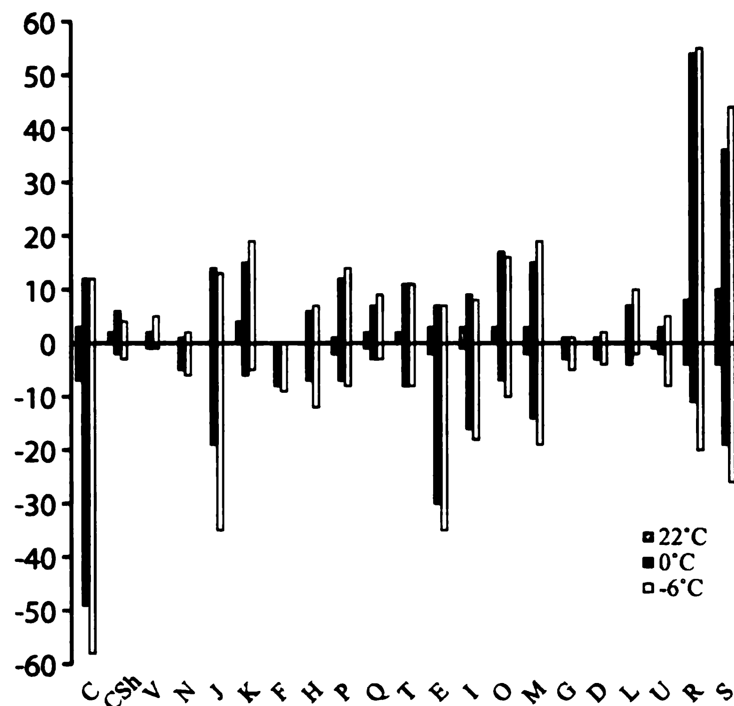

Figure 4.4. Counts of differentially expressed genes compared with 17°C by COGS category. Up-regulated genes are plotted above the zero line, down-regulated genes are plotted below the zero line. C energy conversion, CS previously reported cold shock genes, V defense mechanisms, N cell motility, J translation, ribosomal structure and biogenesis, K transcription, F nucleotide transport and metabolism, H coenzyme transport and metabolism, P inorganic ion transport and metabolism, Q secondary metabolite biosynthesis, transport and metabolism, T signal transduction, E amino acid transport and metabolism, I lipid transport and metabolism, O posttranslational modification, protein turnover and chaperones, M cell wall, membrane and envelope biogenesis, G carbohydrate transport and metabolism, D cell cycle control, cell division, and chromosome partitioning, L DNA replication, recombination and repair, U intracellular trafficking, secretion, and vesicular transport, R conserved hypothetical proteins, S hypothetical protein.

Substrate uptake and activation. Genes for central metabolism of acetate (TCA cycle with glyoxylate shunt) were down-regulated at low temperatures at least 2-fold. However, concurrent 2-fold up-regulation of acetyl-CoA synthetase and down-regulation of acetate kinase and phosphotransacetylase were observed at low temperatures (Fig. 4.7A). Likewise, there was concurrent up-regulation of glutamate dehydrogenase and down-regulation of glutamate synthase subunits at low temperatures (Table 4.2). These

data indicate that the exchange of expression of pathways for acetate activation and glutamate biosynthesis differentiates low temperature and high temperature growth. *Psyc_2044,* for the uptake of ammonium and inorganic phosphate uptake transporters, *Psyc_1926-1930*, were not differentially expressed at low temperatures. Genes for the uptake of sulfate, *Psyc_1070* the periplasmic subunit for an ABC sulfate transporter and *Psyc_2041* the sulfate uptake transporter associated with the *isc* operon in *P. arcticus,* were up-regulated 2.1-fold and 2.3-fold respectively at -6°C. Phosphate and ammonium starvation were probably not significant in the cold, but sulfate uptake was being compensated.

mRNA and Protein Turnover. Twelve peptidases and five ribonucleases were up-regulated at low temperatures at least 2-fold (Fig. 4.5A). Genes for targeting RNAs for degradation were up-regulated at low temperatures including *pcn*B at 2-fold, and *ssr*A at 1.6-fold. Only two RNases were down-regulated at 0°C and -6°C: *Psyc_0292*, a cold shock RNA binding domain containing ribonuclease (2.1-fold at -6°C), and polynucleotide phosphorylase (PNPase) (1.8-fold at -6°C).

Oxidative Stress Response. The oxidative stress response was up-regulated at low temperatures (Fig. 4.5B). In *P. arcticus* this included an ABC transporter for zinc uptake *Psyc_2033-2035*, *ahp*C, *hsp*33, the *isc* operon *Psyc_1477-1482*, and peptide methionine sulfoxide reductases *Psyc_1043* and *Psyc_1950.* Genes for Fe^{2+} uptake transporters, *Psyc_1546-1547*, were down-regulated, consistent with the oxidative stress responses. Likewise, aconitase A was upregulated 2.2-fold at -6°C whereas aconitase B was down-regulated 5.6-fold at -6°C (Fig. 4.7B).

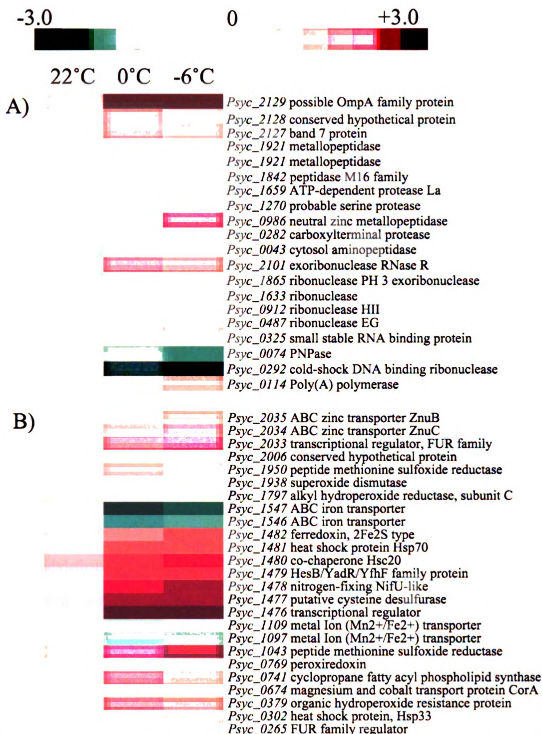

Figure 4.5. Heat map of selected differentially expressed genes. A) Peptidases and RNases. B) Oxidative stress response. Temperature comparisons with 17°C are mapped in columns , genes are mapped in rows. Values are expressed as $\log_2$ ratio of expression. All genes were differentially expressed in at least one contrast with $P < 0.01$. Images in this dissertation are presented in color.

Energy Conversion and Metabolism. All NADH dehydrogenase, ATP synthase and Na^+-translocating NADH-ubiquinone oxidoreductase subunits were down-regulated at low temperatures (Fig. 4.6A). *Psyc_1515* cytochrome bd ubiquinol oxidase was up-regulated at low temperature with a 1.8-fold induction. C-type cytrochromes were either down-regulated or not differentially expressed in the cold except *Psyc_0260* which was upregulated 2.3-fold in the cold. All TCA cycle and glyoxylate shunt genes were down-regulated in the cold (Fig. 4.6A). Succinate and lactate dehydrogenase cytochromes, *Psyc_0098-0101* and *Psyc_1654*, respectively, were down-regulated approximately 2-fold and 1.4-fold at -6°C.

Membrane Lipid and Cell Wall Response. Fatty acid biosynthesis pathways were down-regulated at low temperatures (Fig. 4.6B). Only acyl-carrier protein, *Psyc_0522*, was up-regulated 3.8-fold at -6°C, indicating that this central protein in fatty acid chain growth is being compensated. Fatty acid desaturase, *Psyc_1365*, was up-regulated 2.7-fold at low temperatures and cyclopropane fatty acid synthase was up-regulated 2.8-fold at -6°C. Phosphatidylserine decarboxylase, *Psyc_1925*, which encodes phosphatidylethanolamine biosynthesis, was up-regulated 3.5-fold at -6°C. Up-regulation of cardiolipin synthase at 0°C and -6°C was also observed at 1.6-fold. This change in phospholipids head-group biosynthesis may be indicative of modifications to increase membrane interactions with cytoplasmic water.

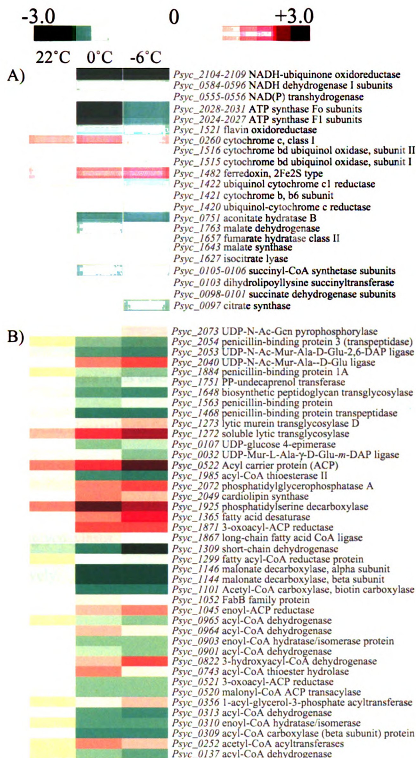

Figure 4.6. Heat map of selected differentially expressed genes. A) Energy conversion. B) Fatty acid and peptidoglycan metabolism. C) Transcription. Temperature comparisons with 17°C are mapped in columns , genes are mapped in rows. Values are expressed as $\log_2$ ratio of expression. All genes were differentially expressed in at least one comparison with $P < 0.01$. Figure continued on next page. Images in this dissertation are presented in color

Figure 4.6 Continued. Images in this dissertation are presented in color.

Peptidoglycan biosynthesis for murein disaccharides, transglycosylation reactions, and transpeptidation of peptidoglycan strands were down-regulated (Fig. 4.6B). In contrast, genes for the elastic growth of the cell wall including soluble and membrane-bound lytic transglycosylases, *Psyc_1272* and *Psyc_1273*, were up-regulated 3.5-fold and 1.6-fold respectively.

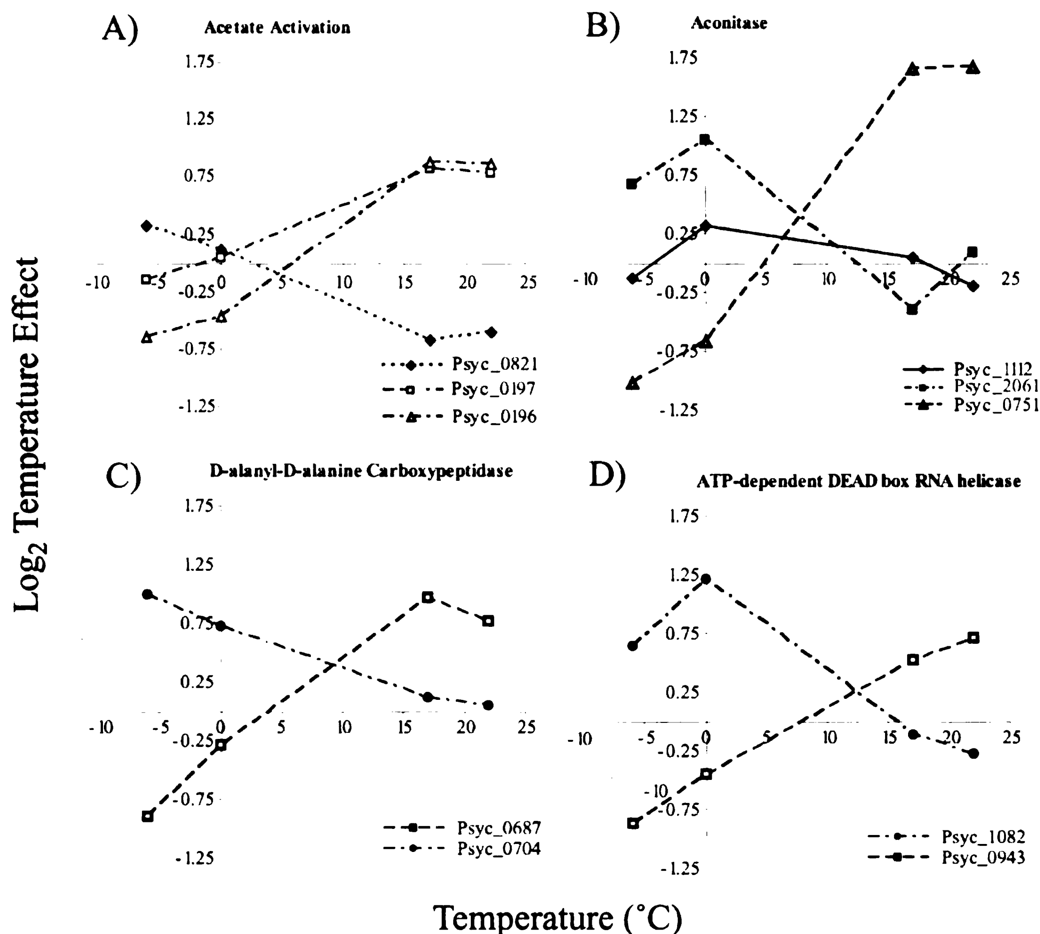

Figure 4.7. Exchange of gene expression over temperature. Low temperature upregulated genes are depicted with closed symbols and high temperature upregulated genes are depicted with open symbols. A) Acetate activation pathways. B) Aconitase, C) D-alanyl-D-alanine carboxypeptidase. D) ATP-dependent DEAD-box RNA helicase. In total, eleven pairs of homologous genes were found to switch expression with temperature. Presented genes have high confidence annotations in the *Psychrobacter arcticus* genome database.

Also related to growth of the cell wall, two putative D-alanyl-D-alanine carboxypeptidase isozymes were differentially expressed with temperature. *Psyc_0704* (*dac2*) was up-regulated 1.9-fold at -6°C and *Psyc_0687* (*dac1*) was down-regulated 3.2-fold at -6°C (Fig. 4.7C). *dac2* and *dac1* are 27% identical and 50% similar in their amino acid sequence from amino acid positions 42-273 of *dac1* by use of BLAST homology searches against the genome (2). Primary structure disorder was computed using DisEMBL 1.5 with the default parameters (41). To compare the results of DisEMBL

analysis from the two genes, the disorder predictions for each peptide were manually aligned by adjusting for gaps in BLAST alignments of the two amino acid sequences. *dac2* features a predicted coil of 64 amino acid residues near the N-terminal end of the DD-peptidase domain that is not present in *dac1*. Two additional coils are predicted in the DD-peptidase domain of *dac2* which are not present in *dac1* from positions 210-225 and positions 354-361 of the aligned disorder data. *dac2* contained 155 disordered amino acid residues in the aligned sequence region whereas *dac1* contains only 128. Only 32 amino acids positions were predicted to reside in hot loops, i.e. highly disordered amino acid positions, in the aligned region *dac1* sequence, but DisEMBL predicted 125 amino acids to reside in hot loops in the *dac2*. *P. arcticus* may exchange the expression of these two isozymes with temperature because the more disordered *dac2* probably has higher activity rates in the cold but is likely more thermolabile.

Nucleotide metabolism and transcription. Purine, pyrimidine and nucleotide biosynthesis were downregulated in the cold or not differentially expressed. *Psyc_2011*, thymidylate synthase and *Psyc_1263*, ribonucleotide reductase, were down-regulated most strongly at 1.9-fold and 1.8-fold at -6°C.

The alpha and beta subunits of RNA polymerase were down-regulated in the cold. Sigma 70 was not differentially expressed, but a sigma 32 homolog was up-regulated 2.6-fold at low temperatures, and 1.5-fold at 22°C. The omega subunit of RNA polymerase, *rpoZ*, was 5-fold up-regulated in the cold. Nine transcription factors out of 17 in the genome were up-regulated at low temperature, though the role of many of these factors is unclear from their annotation and genome context (Fig. 4.6C).

Table 4.2. Amino acid biosynthesis genes that are differentially expressed (P < 0.01) in fold change from 17°C.

Amino Acid	ORF	Gene Name	Annotation	Fold Change[a] 22°C	0°C	-6°C
Arg	Psyc_0815	*carA*	putative carbamoyl-phosphate synthase, small subunit	-1.13	-2.46	-3.27
	Psyc_0814	*carB*	carbamoyl-phosphate synthase, large subunit	-1.25	-3.12	-3.29
	Psyc_0957	*argF*	probable ornithine carbamoyltransferase	-0.98	-1.84	-1.79
Glu	Psyc_1062	*gdh*	probable bacterial NAD specific-glutamate dehydrogenase	-1.06	2.14	2.03
	Psyc_1873		putative glutamate synthase (NADPH-dependent), small subunit	-1.20	-1.96	-1.92
	Psyc_1874		putative glutamate synthase (NADPH-dependent) large chain	-1.37	-1.84	-1.91
Gly	Psyc_0798	*gcvP*	glycine cleavage H protein	-1.24	-3.05	-3.23
	Psyc_0796	*gcvH*	glycine dehydrogenase	-1.15	-2.46	-3.07
	Psyc_0799	*gcvT*	putative glycine cleavage T protein (aminomethyl transferase)	1.09	-1.34	-1.42
Ala	Psyc_0177	*agxT*	possible serine-pyruvate aminotransferase	-1.18	-2.20	-2.36
Ile, Leu, Val	Psyc_0951	*ilvA*	probable threonine dehydratase I	-1.29	-2.16	-2.62
	Psyc_0527	*ilvG*	putative acetolactate synthase, large subunit	-1.16	-2.73	-2.87
	Psyc_0528	*ilvM*	putative acetolactate synthase, small subunit	-1.25	-2.57	-2.77
	Psyc_0529	*ilvC*	Ketol-acid reductoisomerase	-1.21	-2.16	-2.30
Leu	Psyc_1410	*leuB*	3-isopropylmalate dehydrogenase	-1.21	-2.36	-2.28
	Psyc_1413	*leuC*	3-isopropylmalate dehydratase large subunit	-1.35	-2.20	-2.01
	Psyc_1412	*leuD*	3-isopropylmalate dehydratase small subunit	-1.27	-2.11	-2.08
Lys	Psyc_0028	*dapB*	probable dihydrodipicolinate reductase	-1.27	-1.83	-2.38
	Psyc_1523	*asd*	putative aspartate semialdehyde dehydrogenase	-1.16	-2.17	-2.66
	Psyc_0200	*dapF*	probable diaminopimelate epimerase	1.06	1.42	1.45
Pro	Psyc_1391	*ocd*	possible ornithine cyclodeaminase	-1.30	1.44	1.27
	Psyc_0293	*proA*	putative gamma-glutamyl phosphate reductase	1.01	2.19	2.41
Trp	Psyc_0973	*trpG*	probable anthranilate phosphoribosyl transferase	1.28	1.33	1.48
	Psyc_0974	*trpD*	putative anthranilate synthase component II	1.36	1.33	1.52
Met	Psyc_0792	*metZ*	probable O-succinylhomoserine sulfhydrylase	-1.04	1.87	1.41
His	Psyc_1902	*hisD*	putative histidinol dehydrogenase	1.06	1.36	1.29
Ser	Psyc_0369	*serA*	putative D-3 phosphoglycerate dehydrogenase	-1.38	-1.41	-2.53

[a] Data are expressed in fold change compared to 17°C.

Amino Acid Biosynthesis and the Stringent Response. Among regulators, a *relA* homolog was detected as differentially expressed with 2-fold up-regulation at -6°C vs 17°C (Fig. 4.6C). Such a result suggests that *P. arcticus* may be responding to deficits in amino-acylated tRNAs (27). Genes for the biosynthesis of proline, methionine, histidine and tryptophan were up-regulated during low temperature growth (Table 4.2). Genes for the biosynthesis of branched-chain amino acids, arginine, and lysine were down-

regulated in the cold. The glycine cleavage system genes *gcv*H and *gcv*P were down-regulated 3-fold at low temperature, suggesting glycine limitation. Differential expression of amino acyl tRNA synthetases was also observed suggesting that cytoplasmic pools of amino acids are different between low temperature and high temperature growth (Table 4.3).

Table 4.3. tRNA synthetase genes that are differentially expressed (P < 0.01) in fold change from 17°C.

			Fold Change[a]		
Amino Acid	ORF	tRNA Synthetase	22°C	0°C	-6°C
Gln	*Psyc_0694*	glutaminyl-tRNA synthetase	-1.06	1.68	1.58
Gly	*Psyc_1774*	glycyl-tRNA synthetase, alpha subunit	1.32	1.46	1.67
Gly	*Psyc_1773*	glycyl-tRNA synthetase, beta subunit	1.40	1.39	1.89
Tyr	*Psyc_1970*	tyrosyl-tRNA synthetase	-1.53	-3.89	-5.46
Phe	*Psyc_1995*	phenylalanyl-tRNA synthetase, beta subunit	-1.36	-2.97	-3.94
Phe	*Psyc_1996*	phenylalanyl-tRNA synthetase, alpha subunit	-1.31	-2.36	-2.99
Cys	*Psyc_1361*	cysteinyl-tRNA synthetase, class Ia	-1.21	-1.60	-2.35
Trp	*Psyc_1568*	tryptophanyl-tRNA synthetase, class Ib	-1.27	-1.78	-2.17
Ala	*Psyc_1460*	alanyl-tRNA synthetase, class IIc	-1.37	-2.38	-2.46
Pro	*Psyc_0429*	prolyl-tRNA synthetase, class IIa	-1.20	-1.93	-2.20
Ile	*Psyc_0364*	isoleucyl-tRNA synthetase, class Ia	-1.01	-1.71	-1.84
Ser	*Psyc_1618*	seryl-tRNA synthetase, class IIa	-1.15	-1.68	-1.43
His	*Psyc_0683*	histidyl-tRNA synthetase, class IIa	-1.04	-1.59	-1.33
Arg	*Psyc_1946*	arginyl tRNA synthetase	1.15	-1.42	-1.17
Met	*Psyc_1432*	methionyl-tRNA synthetase	-1.09	-1.04	1.06

[a] Data are expressed in fold change compared to 17°C.

Compatible Solute Uptake and Biosynthesis. Proline, glutamate and glycine are potential compatible solutes for which data were presented in the previous section. *P. arcticus* also contains genes for the biosynthesis and uptake of carnitine, betaine and choline. *Psyc_0728*, choline dehydrogenase was up-regulated 2.1-fold at 22°C, and *Psyc_0729*, betaine aldeyde dehydrogenase was upregulated 2.3-fold at 22°C and down-regulated 1.4-fold at -6°C. The *P. arcticus* genome contains three annotated betaine/carnitine/choline type transporters: *Psyc_0727* was not differentially expressed with temperature, *Pysc_0826* was down-regulated 1.5-fold at -6°C, and *Psyc_1301* was upregulated 1.8-fold at -6°C.

Table 4.4. Expression profiles of *P. arcticus* genes involved in protein folding and disaggregation. Differentially expressed genes have a P-value < 0.01[a].

ORF	Gene Name	Annotation	Fold Change[b] 22°C	0°C	-6°C
Psyc_0034	*dsbB*	possible disulfide bond formation protein	1.27	1.41*	1.67*
Psyc_0254	*dsbC*	possible thiol:disulfide interchange protein	1.27	1.26	1.56*
Psyc_1560	*dsbE*	probable periplasmic protein thiol:disulfide oxidoreductase DsbE	-1.12	-2.27*	-2.14*
Psyc_0552	*groES*	putative Chaperonin HSP10	-1.18	-1.09	-1.41
Psyc_0553	*groEL*	putative Chaperonin HSP60 family	1.04	1.04	-1.28
Psyc_0027		probable chaperone protein dnaJ	-1.21	-2.38*	-2.33*
Psyc_0039	*dnaJ*	probable heat shock protein DnaJ	-1.01	1.15	1.06
Psyc_2132	*dnaK*	chaperone protein dnaK	1.19	1.18	1.04
Psyc_2133	*grpE*	heat shock protein GrpE, hsp70 family	1.09	-1.16	-1.35*
Psyc_0823	*clpB*	putative chaperonin clpA/B	1.82*	1.40	1.93*
Psyc_1941	*clpP*	putative ATP-dependent ClpP protease, proteolytic subunit	-1.06	-1.06	-1.61*
Psyc_1942	*clpX*	putative ATP-dependent ClpX protease	-1.11	-1.27	-1.66*
Psyc_1719	*htpG*	probable chaperone protein htpG (Hsp90)	1.07	-1.91*	-2.41*
Psyc_1940	*tig*	trigger factor	-1.36	-1.67*	-2.22*
Psyc_0857		peptidyl-prolyl isomerase	-1.22	-1.13	-1.09
Psyc_0693	*ppiB*	peptidyl-prolyl cis-trans isomerase, cyclophilin type	-1.29	1.12	-1.58*
Psyc_0283	*slyD*	putative peptidyl-prolyl cis-trans isomerase, FKBP-type	-1.04	1.08	1.05
Psyc_0358		probable peptidyl-prolyl cis-trans isomerase protein, FKBP-type	-1.01	1.10	-1.08
Psyc_1473	*ppiC*	possible PpiC-type peptidyl-prolyl cis-trans isomerase	-1.17	-1.79*	-1.67*

[a] P-values are estimated from an across six contrasts in the experiment. Three contrasts are presented in the table.

[b] Data are expressed in fold change compared to 17°C.

*A significant fold change (P < 0.01).

Protein Folding Response: Peptidyl-prolyl *cis-trans* isomerases (PPIases), trigger factor and the major heat shock associated chaperones were down-regulated or not differentially expressed at 22°C, 0°C and -6°C (Table 4.4). Only chaperones associated with iron-sulfur cluster biosynthesis, oxidative protein damage, and a *clpB* chaperone homolog were up-regulated at low temperatures (Fig. 4.5B and Table 4.4). Genes with functions in disulfide bridge formation were differentially expressed with temperature. *dsbB* and *dsbC* were up-regulated approximately 1.6-fold at low temperature, but the periplasmic enzyme gene *dsbE* was down-regulated (Table 4.4).

Cold acclimation and cold shock gene expression. *P. arcticus* possesses two major cold shock protein homologs. One *csp*A homolog, *Psyc_531*, was expressed at high levels at all temperatures. The other cold shock protein, *Psyc_942*, and the adjacent DEAD box helicase gene, *Psyc_0943*, are down-regulated 3.1-fold and 2.4-fold respectively at -6°C. A second DEAD-box helicase, *Psyc_1082*, was up-regulated 2-fold at cold temperatures (Fig. 4.7D).

Psyc_1082 (*csdA*) and *Psyc_0943* are annotated as homologous ATP-dependent DEAD-box RNA helicases with high scoring alignments to the TIGRfam DEAD-box helicase equivalog. These genes are predicted to encode proteins of 396 and 567 amino acids respectively. The *csdA* amino acid translation has a C-terminal extension rich in glycine, serine and arginine, supporting its function at low temperature similar to mesophilic and psychrophilic cold associated DEAD box helicases (36). The amino acid sequences of the two genes were 40% identical over a 375 amino acid homologous region which included the DEAD-box RNA helicase domain of each gene by BLAST alignment. While these DEAD box helicases may act on different RNA substrates, we hypothesized that the amino acid sequence of the low temperature up-regulated *csdA* would be more disordered owing to putative activity in the cold. DisEMBL 1.5 was used to predict disordered regions in the two genes in the conserved DEAD box helicase domains. *csdA* contained two predicted coils in a sequence region from positions 272-336 which were not in *Psyc_0943* and another predicted coil in positions 180-200 of the aligned sequences. In contrast, *Psyc_0943* contained only one predicted coil from positions 109-127 which was not present in *csdA.* Over the aligned length of the sequences, *csdA* contained 213 amino acid positions in predicted coils versus 195 in the -

6°C down-regulated *Psyc_0943*. Hot loop predictions by DisEMBL revealed a larger difference in protein disorder over the aligned sequences, with *csdA* containing a predicted 120 hot loop amino acid positions versus only 70 in *Psyc_0943*. These results are consistent with cold adaptation of *csdA*, the DEAD-box helicase up-regulated at -6°C.

Transcription and translation factors associated with cold shock were observed to be up-regulated at -6°C including *rbf*A ribosomal binding factor at 1.8-fold, *nusB* and *nusA* at 1.7-fold and 2.6-fold respectively, and translation initiation factor IF-2 at 1.8-fold (Fig. 4.6C). However, no previously reported cold shock associated genes involved in recombination or carbon catabolism were up-regulated in the cold. The anti-aggregation chaperone *clpB* was the only previously identified cold expressed chaperone to be up-regulated, at 1.9-fold, during -6°C growth in *P. arcticus*.

Global model and knockout mutants. Considering the overall differential expression trends in the data, a global conceptual model differentiating high temperature growth from low temperature growth was developed (Fig. 4.8). Fast growth rate was the simplest explanation for all of the up-regulated genes at 22°C and 17°C. Heat stress is a possible explanation for up-regulation of chaperone and PPIase genes at high temperature. At low temperature, explanations are more diverse as cells must respond to the multiple stresses associated with low temperature growth at 0°C and -6°C.

Figure 4.8. Conceptual model of the temperature response in the transcriptome of *P. arcticus*. Low temperature induced functions are depicted on the left and high temperature induced functions are depicted on the right.

Based on this conceptual model and to test the role of exchanged putative isozymes in the different temperature regimes, knockout mutant strains were generated using the suicide vector pJK100. Catalase (*Psyc_0570*, *katE*) and methionine sulfoxide reductase (*Psyc_1043, msrA*) knockout mutants were generated to examine the effect of loss of function of these genes on growth under increased oxidative stress at low temperature. To examine the effects of gene expression exchange with temperature, mutants were constructed in the two DD-peptidases, *Psyc_0687* (*dac1*) and *Psyc_0704* (*dac2*), which were respectively up-regulated at high and low temperature. The cold up-regulated DEAD box helicase (*Psyc_1082*, *csdA*) was also deleted with the hypothesis that loss of this gene would result in reduced growth rates at low temperature.

No mutant growth rates were statistically different from wild-type under optimal growth conditions at 17°C or 4°C (Fig. 4.9B and C). At 0°C and -2.5°C, Δ*katE*, Δ*dac2*, and Δ*csdA* grew slower than wild-type as predicted (Fig. 4.9D and E, $P < 0.05$). Δ*msrA* also grew significantly slower than wild-type at -2.5°C (Fig. 4.9E, $P < 0.05$). At 17°C, growth was negatively effected in Δ*dac1* and Δ*msrA* (Fig. 4.9A, $P < 0.05$). Conversely, Δ*dac2* exhibited improved growth over wild-type at 22°C (Fig. 4.9A, $P < 0.05$). Whereas Δ*dac2* grew more slowly than the wild-type *P. arcticus* at low temperature, Δ*dac1* exhibited no statistical difference in growth rate versus wild-type at low temperature. This switch in growth rate phenotype between Δ*dac2* and Δ*dac1* with temperature is consistent with the both expression data and bioinformatics data suggesting the *dac2* activity is favored at low temperature and *dac1* activity is favored at high temperature. The observation of improved growth over wild-type at high temperature in Δ*dac2* coupled with wild-type growth of Δ*dac1* at low temperature suggests that these two DD-

peptidases may interact competitively. This competition may be relieved by deletion of one participant.

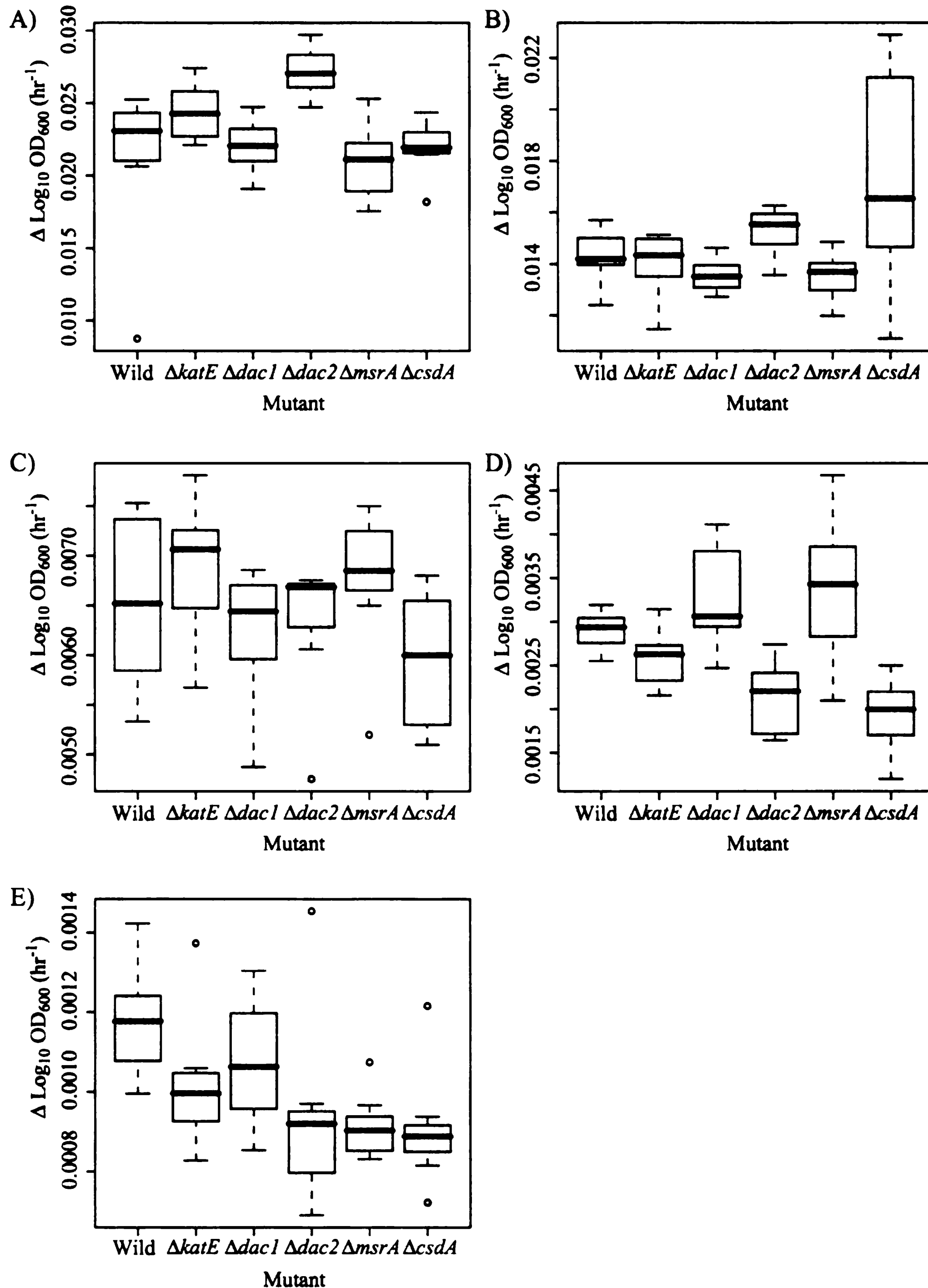

Figure 4.9. Growth rate distributions for knockout mutants and wild-type *P. arcticus* controls. Panels contain data from different growth temperatures: A) 22°C, B) 17°C, C) 4°C, D) 0°C, and E) -2.5°C. Bars represent the population medians ($n = 8$). Boxes are plotted from the 25th to 75th percentiles, and whiskers are plotted from to the 10th to the 90th percentiles. Outliers are plotted as dots.

Discussion

This study assessed the temperature effect on *P. arcticus* of gene expression during growth under nutrient poor, briny conditions as expected in cryo-environments including permafrost (22, 23). Our data demonstrate several important aspects of *P. arcticus* adaptation for function in the cold. The threshold temperature at which *P. arcticus* demonstrates acclimation to the cold is lower than 4°C under the conditions used for this experiment. Exchange of isozyme expression during growth in high versus low temperatures was not widespread in the genome, but it did occur as did up-regulation of expression of energy efficient substrate incorporation pathways at low temperature. Multiple adjustments to gene expression were employed to respond to growth in the cold. Based on our array data, oxidative stress, amino acid biosynthesis, and cell wall and membrane dynamics appeared to be the growth rate limiting factors for which compensation was necessary instead of energy conversion, transport, translation or peptide chaperoning. Clearly these modes of acclimation to low temperature life warrant further investigation.

A growth rate inflection point was reported at 6°C for *P. arcticus* during growth in nutritionally complex and less saline ½ TSB broth with or without 5% NaCl added (47). Our medium elicited no such response, indicating that the elevated salt concentration in our media may have shifted the cold acclimation threshold substantially downward. Our growth experiments show *P. arcticus*, a eurypsychrophile, grows under a similar physiology at 4°C as at 17°C. Cold shock and cold acclimation studies of psychrotolerant microbes have typically been carried out in the comparisons of various warm temperatures to 4°C (7, 8, 29). The threshold for low temperature response in *P.*

arcticus, in this experiment, is lower than the threshold for other organisms such as *Shewanella oneidensis* MR-1 at 8°C, *Acinetobacter* HH1 at 4°C, *Methanococcoides burtonii* at 4°C, and *Bacillus psychrotrophicus* at 4°C (7, 20, 29, 61). The reduced threshold of the acclimation response calls into question the usefulness of the benchmark 4°C temperature in studies of low temperature physiology of psychrophiles. The shift in growth temperature response in *P. arcticus* seems dependent on medium composition, and so one should consider the scientific question at hand before deciding on how to treat cells for low temperature growth.

Numerous proteome studies have been performed on cold shocked organisms. It is known that posttranscriptional control is an important factor in both *E. coli* and *B. subtilis* during cold shock and cold acclimated growth (10, 24, 32). Only a few proteome studies have been performed on cold acclimated growth of psychrophiles (6, 30). A transcriptome approach offers the strength of analyzing data for 93% of the genes in *P. arcticus* during cold acclimated growth across statistically relevant numbers of replicates (1). The thoroughness of this approach has allowed us to identify the complex metabolic responses to prolonged low temperature exposure. While transcriptome analyses can provide insight into coarse metabolic changes, they do not take into account translational and post-translational regulation. Thus, we have directed our discussion toward outcomes resulting from complex gene expression responses such as resource and energy efficiency, biosynthetic pathways, and regulation of molecular dynamic motion at low temperature. Our transcriptome data show that transcription control plays a role in differentiating cold-acclimated growth state at 0°C and -6°C from that at higher temperatures.

Proteome studies in Psychrobacteria

Two proteome studies have been carried out with *Psychrobacter* species which examined temperature effects. Zheng *et al* (2006) reported a small number of proteins, 14, were detected as differentially expressed between 22°C and 4°C in ½ TSB + 5% NaCl (64). This culture condition was most similar to the conditions of our experiment. Alkyl-hydroperoxide reductase, *ahpC*, was strongly upregulated in the comparison, and we observed upregulation of its transcript at low temperature. Downregulated genes included the chaperone HSP60 and peptidyl-prolyl *cis-trans* isomerases whose transcripts were also observed to be downregulated in our experiment.

Bakermans *et al* (2006) carried out proteome analysis of *Psychrobacter cryohalolentis*, a related permafrost isolate from cryopegs in the Kolyma permafrost (6). In addition to up-regulation of energy production enzymes, proteins for enhancing translation including CspA, EF-Ts and EF-Tu were upregulated during growth at -4°C in *P. cryohalolentis*. Our trascriptome data suggest that *P. arcticus* has unique adaptive features for low temperature growth among permafrost *Psychrobacteria* with basal energy and translation machinery that function well at low temperature due to a combination of cold adaptation of the enzymes and constitutive expression of the genes. Alternatively, it is possible that a degree of posttranscriptional control is allowing genes that are transcriptionally downregulated to be translated more efficiently at low temperature. Other instances of posttranscriptional control have favored translational down-regulation of transcriptionally highly expressed genes at high temperature, e.g. *cspA* and *pnp* (25, 45).

Resource efficiency responses

Glutamate dehydrogenase is known to be a slow, energy efficient pathway to produce glutamate from 2-oxoglutarate and ammonium. Glutamate synthase, in contrast, is dependent on glutamine synthetase which requires ATP and glutamine (34, 35). Acetyl-CoA synthetase is also potentially an energy saving pathway for the activation of acetate. Acetyl-CoA synthethase performs the same function as both acetate kinase and phosphotransacetylase which were up-regulated at warm temperatures. The differential expression of these two gene systems has been observed in *E. coli* which utilizes *ackA* and *pta* during fast growth to excrete acetate and acetyl-CoA synthetase to metabolize acetate when other carbon sources have been depleted (39). Our data suggest that the regulation of these two pathways has been coupled to temperature changes in *P. arcticus* under acetate-utilizing conditions. So, *P. arcticus* may save energy by inducing acetate metabolism via acetyl-CoA synthetase and nitrogen incorporation via glutamate dehydrogenase in the cold. Under cold conditions, where generation times are long, energy efficiency is likely more important than growth at slightly faster rates than competitors.

Increased expression for energy production and ATP synthesis were observed in two psychrophiles growing at low temperature, *Psychrobacter cryohalolentis* and *Methanococcoides burtonii* (6, 30). In the *P. arcticus* transcriptome, genes for energy conversion and metabolism were most often down-regulated at low temperature, suggesting that NADH and ATP production are not the limiting metabolic pathways at low temperature. An exception to energy conversion expression in *P. arcticus* was cytochrome bd ubiquinol oxidese (*Psyc_1515-1516*). Cytochrome bd is a high affinity

oxidase, and *E. coli* knockout mutants of cytochrome bd oxidase are sensitive to hydrogen peroxide (26). Therefore, the upregulation of cytochrome bd in *P. arcticus* was interpreted to be responding to higher steady state concentrations of reactive oxygen species. The *P. arcticus* response for energy metabolism suggests that this psychrophile is differently adapted for growth at low temperature in comparison to other psychrophiles examined to date.

TCA cycle genes for the catabolism of acetate and regeneration of biosynthetic intermediates were down-regulated in the cold. The down-regulation of carbon catabolic reactions is different from previous findings during cold acclimation in *E. coli*, *S. oneidensis* MR-1, *P. cryohalolentis*, and *M. burtonii* in which increased expression of genes especially in glycolysis and some TCA cycle genes was detected (6, 20, 30, 46, 56). Down-regulation of catabolic pathways was observed in *B. subtilis*, where it was concluded that this is probably a result of reduced growth rates (10).

Down-regulation of thymine biosynthesis, and ribonucleotide reductase genes suggests that *P. arcticus* is modifying nucleotide metabolism to support more RNA synthesis than DNA replication. The result is consistent with the observation of increased synthesis of RNA and protein incorporation rates normalized per cell division with no change in DNA synthesis rates during growth at subzero temperatures (5). *P. arcticus* also exhibited high levels of RNase and peptidase expression in general during growth at low temperature. RNase R and PNPase of *E. coli* are induced during cold shock (11, 46). PNPase is also essential for the growth of *Yersinia entercolitica* at low temperature (31). RNase PH, PNPase and RNase R were reported as induced during growth at 15°C in *B. subtilis* (10). In constrast, PNPase was one of two RNases down-

regulated at low temperature in *P. arcticus* during cold acclimated growth. Increased expression of RNases and peptidases during cold acclimated growth would result in increased turnover RNA and proteins to address the need for transcriptome and proteome flexibility during growth with generation times on the order of days or weeks at temperatures where inherent half-lives of transcripts and peptides will be longer.

Differential expression of *hisJ* and *trpG* was detected in *L. monocytogenes* growing at 10°C (42). Genes for arginine and aromatic amino acid biosynthesis are up-regulated during cold shock and cold acclimated growth in both *E. coli* and *B. subtilis* (10, 46). Growth arrest in *E. coli* induced the arginine and histidine biosynthetic pathways, but not those of other amino acids in a starvation experiment (13). The observed down-regulation and energy metabolism, translation and transcription machinery with concurrent up-regulation of *rpoZ* and *relA* is consistent with the stringent response for growth arrest (13). Up-regulation was also observed in *Vibrio cholerae* in a Q-RT-PCR comparison between the VBNC state induced at 4°C and a starvation stressed growth state at 15°C (28). Simultaneous examination of amino acid biosynthetic genes and tRNA synthetase genes showed that in *P. arcticus* genes for arginine, the branched chain amino acids and lysine were down-regulated at all temperatures relative to 17°C. However, genes for proline, histidine, methionine and tryptophan were up-regulated. This suggests that *P. arcticus* attempts to compensate for hypothesized reductions in amino acid biosynthesis activity by up-regulating specific genes in a pathway. Down-regulation of the glycine cleavage system is consistent with reduced cytoplasmic concentration of glycine. Up-regulation of the stringent response may be involved in

slowing the growth of *P. arcticus* in order to allow the organism to maintain an adequate supply of amino acids and nucleotides.

Comparisons to cold acclimated growth in mesophiles

In addition to the major cold shock proteins and RNA helicases, the cold shock response in *E. coli* leads to up-regulation of pyruvate dehydrogenase complex, *rec*A, chaperones, *rbf*A, translation initiation factors, and trehalose biosynthesis (32, 46). Cold inducible genes like *rbf*A, IF-2, and *nus*A were up-regulated during growth in the cold in *P. arcticus*. *P. arcticus* expresses one cold shock protein homolog constitutively with temperature. Differential expression of membrane fatty acid desaturase and the oxidative stress response are also commonly observed during cold acclimated expression studies (46, 64). The genes shared with mesophilic cold acclimation responses are those involved in the initiation and stable function of transcription and translation but not those in the basal machinery such as RNA polymerase subunits and ribosomal proteins.

DEAD box RNA helicases function to unwind duplex RNA and possibly to displace RNA-protein complexes. These enzymes exhibit functions as diverse as ribosome biogenesis, RNA degradation and translation initiation (49). CsdA from *E. coli* has roles in all of the former processes whereas other DEAD box RNA helicases have specific RNA targets (36). Comparison of inversely expressed DEAD box RNA helicase genes *csdA* (*Psyc_1082*) and *Psyc_0943* showed that the low temperature up-regulated *csdA* is predicted to be more disordered, i.e. more flexible, than *Psyc_0943*. The reduced growth rate of our *P. arcticus csdA* mutant further solidifies the role of this DEAD box helicase in cold acclimated growth. While the two alleles of DEAD box RNA helicase in *P. arcticus* may not target the same RNA populations, it was clear from our analyses that

csdA (*Psyc*_1082) encodes a gene important to the low temperature growth of *P. arcticus*. Another interesting feature of this *P. arcticus csdA* is that it possesses a highly disordered C-terminal extension rich in glycine, serine and arginine. A similar C-terminal extension was observed in the cold regulated DEAD box helicase of the psychrophilic methanogen *Methanococcoides burtonii* (40). The structural similarity of these two cold adapted RNA helicases from disparate microbes isolated from opposite hemispheres of the planet highlights that some specific alleles can contribute significantly to psychrophily. Perhaps such a protein can be transformed into more cold sensitive organisms to improve cold tolerance as was observed in *E. coli* expressing a cold adapted Cpn60/10 chaperone (56).

Protein misfolding and aggregation are thought to lead to the induction of chaperones at low temperature and PPIase at low temperature (6, 10, 30, 46, 56, 58). However, PPIases and heat-shock associated chaperones were down-regulated or not differentially expressed during growth in the cold in *P. arcticus*. This result suggests that in *P. arcticus*, protein folding stress is only minimal at low temperature. Only *clpB* chaperone and *hsp*33 were up-regulated in our experiment. The *hsp*33 chaperone is activated by oxidation and responds transcriptionally to heat shock and oxidation stress (14). It is most likely playing a role in the oxidative stress response in *P. arcticus*. The *clpB* chaperone is thought to play a role in protein disaggregation and it is expressed highly in *B. subtilis* and *E. coli* and *L. monocytogenes* during cold acclimated growth at 15°C or 10°C (10, 42, 46). ClpB induction occurs during cold acclimation of *Synecchococcus* sp. PCC 7942, and deletion of *clpB* reduced survival and photosynthesis during cold acclimated growth at 25°C in this cold sensitive bacterium (48).

Conservation of *clpB* up-regulation in *P. arcticus* demonstrates the universal nature of protein aggregation stress at low temperature.

Oxidative Response

Differential expression in response to reactive oxygen species was one of the strongest magnitude changes in the transcriptome with temperature. Genes involved were consistent with those observed in the oxidative stress stimulon in *E. coli* (19). Aconitase isozymes A and B were also exchanged with temperature in *P. arcticus*. Aconitase A and B in *E. coli* were exchanged in response to superoxide (59). Knockout of two oxidative stress genes decreased growth rates of *P. arcticus* at subzero temperatures.

Cell Wall Response

DD-peptidases control the degree of cross-linking between peptidoglycan strand in the cell wall by catalyzing the removal of one D-alanine residue from peptidoglycan peptides before cross-linking can occur via transpeptidase activity (44, 52). Lytic transglycosylases are involved in the dynamic insertion of new peptidoglycan strands into the cell wall. The peptidoglycan cell wall is a elastic structure whose elasticity is a function cross-bridging between peptidoglycan strands (63). Low temperature decreases elasticity, so differential expression of genes with functions in cross-bridging control could adjust the elasticity of the cell wall at low temperature. Consistent with reduced growth rates, we observed down-regulation of peptidoglycan strand biosynthesis genes. In contrast, lytic transglycosylases were upregulated in the cold. Interestingly, isozyme exchange of DD-peptidases was also observed. If these isozymes yield different levels

of cross-linking in the cell wall, they may contribute to control of elasticity in the cell wall with temperature. Our observation of isozyme exchange and differential effects of knockout of these isozymes reveals a new role for control of cell wall elasticity as a mechanism of cold adaptation.

Conclusion

We summarize our conceptual model of the low temperature response in *P. arcticus* in Fig. 4.8. We attribute up-regulation of genes at warm temperatures solely to growth rate effects or possibly heat in the case of chaperone up-regulation at high temperature. The transcriptome at 0°C and -6°C was attributed to a conglomerate of effects of life in the cold including increased dissolved oxygen concentrations in our cultures, acclimation of metabolic pathways for resource and energy efficiency, biosynthetic demand or reduced supply of specific amino acids, and expression to increase the dynamic behaviors of the cell wall and cytoplasmic membrane. Altogether, these data suggest the concept that *P. arcticus* is evolutionarily adapted for function in both temperature ranges in genes for central cellular processes and components as diverse as membrane and peptidoglycan biosynthesis, energy metabolism, carbon catabolism, nucleotide biosynthesis, amino acid biosynthesis, RNA polymerase, and the ribosome. Compensation was reserved for specific processes such as cell wall and membrane dynamics, differential expression of a small number of isozyme pairs, and specific biosynthetic steps in amino acid and macromolecular turnover at low temperature.

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CHAPTER 5

TEMPERATURE-INDUCED DIFFERENTIAL EXPRESSION IN GLYCINE, METHIONINE AND LEUCINE METABOLISM CORRESPOND TO BIAS IN AMINO ACID CONTENT OF GENES HIGHLY EXPRESSED BELOW 0°C BY *PSYCHROBACTER ARCTICUS* 273-4

Abstract

Growth at ≤ 0 °C induces numerous changes in the transcriptome of the eurypsychrophilic permafrost microbe *Psychrobacter arcticus* 273-4. Among the changes previously observed was induction of the stringent response concomitant with gene expression patterns consistent with metabolism to increase abundance of six amino acids. A Δ*relA* knockout mutant exhibited decreased growth rates during mid-late exponential phase relative to the wild-type *P. arcticus* strain. We examined the relationship between amino acid biosynthesis expression, the effect of supplementation with amino acids on growth rate and the biases in amino acid content predicted from genes highly expressed in the transcriptome at subzero temperatures. Results for gene expression and amino acid supplementation experiments are consistent with increased demand for glycine and methionine in cells growing at subzero temperatures. The subzero proteome had the strongest biases for reduced glycine and methionine content and increased leucine content. Based on these data, we hypothesize that glycine and methionine biosynthesis are negatively impacted by subzero temperature growth whereas leucine biosynthesis may have been favored over valine and isoleucine in the same condition. Metabolic flux analysis may yield further insights into magnitude and causes of these hypothesized changes in amino acid pool.

Introduction

Eurypsychrophilic (i.e. "broad cold-loving") microbes can commonly grow in a 30-40°C temperature range by adjusting their physiology to cope with growth at both at sub- and supra-optimal temperatures (12). Growth rates of microbial cultures exhibit exponential decline as temperatures depart from the optimal growth temperature (T_{opt}) except near the low temperature limit of growth, where an inflection is observed in the growth rate trend (21). The temperature at which this growth rate inflection occurs is known as the critical temperature (T_{crit}); the temperature at which optimal growth physiology is exchanged for low temperature acclimated physiology. Below T_{crit}, transcriptomic, proteomic, and enzymatic regulation contribute to adaptations for low temperature growth such as increased membrane fluidity, differential expression of biosynthesis pathways, and changes to energy metabolism (6, 15, 18). While energy consumption and rates of macromolecular synthesis decrease below T_{crit}, these processes represent a larger investment of energy per cell division than at temperature above T_{crit} (3, 5).

Transcriptome analysis of the eurypsychrophilic permafrost bacterium *Psychrobacter arcticus* growing at temperatures on either side of T_{crit} revealed the expected expression increases in genes for homeoviscous adaptation of membranes and oxidative stress response genes in addition to downregulation of transcription, translation and nucleotide biosynthesis (Chapter 4). Surprising results included up-regulation of genes for dynamics of the cell wall, increased expression of ribonucleases and peptidases, and down-regulation of chaperones. A mineral medium with no amino acids was utilized in the transcriptome experiment to enable discovery of challenges to biosynthesis below

0°C. *P. arcticus* exhibited increased expression of six species of amino acids: proline, glycine, tryptophan, histidine and sulfur-containing amino acids.

The stringent response defines a regulon devoted to energy and carbon conservation under nutrient limited conditions. Differential expression of genes under stringent response control was also observed in *P. arcticus* during growth at 0°C and -6°C, corroborating the potential importance of the amino acid biosynthesis response at low temperatures. When the stringent response is induced by the presence of uncharged-tRNAs, RelA, a ribosome associated protein, catalyzes the synthesis of the stringent response "alarmone" (p)ppGpp (7). In *Escherichia coli* and *Bacillus subtilis*, (p)ppGpp represses expression of tRNAs, rRNAs, ribosomal proteins, RNA polymerase subunits, DNA replication and ATP synthase (8, 10). These modifications of gene expression are not observed in *relA* deletion strains (10). Induction of the stringent response transiently arrests growth and is thought to permit carbon and energy conservation under short term nutrient-limited conditions while avoiding entry into stationary phase (or sporulation in *Bacillus*), a comprehensive dormancy state that includes the expression of several damage resistance responses.

Up-regulation of amino acid biosynthesis genes has been observed in several bacteria growing at low temperatures, but the relevance of these expression responses to low temperature physiology has not been examined. Amino acid biosynthesis is a major fraction of energy cost to the cell during growth under energy and nutrient limiting conditions. Indeed, metabolic costs of amino acid biosynthesis have been shown to influence the amino acid content of microbial proteomes (1). Therefore, to understand

life in cold, energy and nutrient limited environments like permafrost, both the effect of biosynthetic hurdles and strategies for coping with such stress must be understood.

We hypothesized that up-regulation of amino acid biosynthesis is indicative of an increased metabolic cost on *P. arcticus* during growth at low temperatures. Induction of the stringent response at low temperatures could help moderate that cost by slowing growth and resource consumption by *P. arcticus*. We examined the effect of deletion of the stringent response signal gene *relA* on growth at low temperature and found that growth rate was decreased at temperatures < 4°C. Wild-type *P. arcticus* cultures were supplemented with extracellular pools of amino acids to test for increased growth rates, some of these results indicated relief from the cost of biosynthesis of key amino acids. Highly expressed genes from our transcriptome comparison of subzero growth and that at T_{opt} were compiled into predicted proteomes specific for those temperatures. The amino acid content of predicted proteomes at T_{opt} and subzero temperatures were analyzed to detect amino acid content biases corroborating increased cost of synthesis of the six low temperature favored amino acids.

Materials and Methods

Construction of a Δ*relA* mutant *P. arcticus* strain. A knockout mutant of *relA* (*Psyc_0343)* was generated essentially as described in Denef *et al* (9). Primers, plasmids and strains for this study are described in Table 5.1. Flanking regions of each gene were PCR amplified with primers containing restriction sites for insertion into pJK100. Upstream flanking regions were amplified with primers containing *Bgl*II and *Not*I restriction sites on the forward and reverse primers respectively. Downstream flanking

regions were amplified with *Sac*II and *Sac*I site containing primers on the forward and reverse primers respectively.

Table 5.1. Strains, primers and plasmids used in this study.

Strains	Description[a]	Source
Psychrobacter arcticus		
273-4	Wild type	
PB0343	*ΔrelA ::kan*	This study
Escherichia coli WM3064	*thrB1* 004 *pro thi rpsL hsdS lacZ ΔM15* RP4-1360 Δ(*araBAD*)*567 ΔdapA* 1341::[*erm pir*]	9
Primers		
del343UF	AGCTAGATCTgcttatcgatccaccacgtt	This study
del343UR	AGCTGCGGCCGCttgcgtggtttgtccatcta	This study
del343DF	AGCTGAATTCctggcgcatttatccaagtt	This study
del343DR	AGCTCATATGctcgtttgatgggttgcttt	This study
kanF	GAAGATCTctcaaaatctctgatgttacattgc	This study
kanR	GAAGATCTtggttgatgagagctttgttgtaggt	This study
Plasmids		
pJK100	Allelic Exchange Vector	9
p343U	pJK100 with *Psyc_0343* upstream	This study
p343UD	pJK100 wth *Psyc_0343 up- and downstream*	This study

[a] Capital letters include a 4 nucleotide extension on the 5' end of primers and a restriction site for insertion into pJK100.

P. arcticus 273-4 cultures for conjugation were grown in LB broth at 22°C for 36 hours. *E. coli* WM3064 containing the suicide vector for knockout was grown at 37°C in Luria Bertani broth (LB) supplemented with 25 μg ml^{-1} kanamycin (Kan), 20 μg ml^{-1} tetracyclin (Tet) and 100 μg ml^{-1} diaminopimellic acid (DAP) overnight. Cells were combined in a ratio of 200 μl *E. coli* donor and either 200 μl or 800 μl *P. arcticus* 273-4 recipient. The mixtures were pelleted by centrifugation for 2 min at 4,000 x g in an Eppendorf 5415D tabletop centrifuge. Pellets were resuspended in 100 μl LB and the full volume was spot plated onto LB agar supplemented with 100 μg ml^{-1} DAP. Plates were incubated at 22°C for 24 hours. Cells were then harvested and resuspended in 500 μl LB. This cell suspension was decimally diluted 10^2-fold and 100 μl of each dilution was plated on LB agar supplemented only with 25 μg ml^{-1} Kan. Conjugants were allowed to

grow for 72 hours at 22°C. Putative knockout mutant strains were isolated on LB with 25 μg ml^{-1} Kan and screened for a Kan^r Tet^s phenotype. PCR screening of knockouts revealed amplicons of the expected gene deletion product size and these strains were used for the temperature growth experiment.

Mutant Growth Experiment. A *P. arcticus* Δ*relA*::*kan* strain, PB0343, was grown in marine broth supplemented with 25 μg ml^{-1} Kan for 48 hr at 17°C. Two successive cultures were carried out to acclimate the mutant to acetate mineral medium containing 7.5% sea salts with 25 μg ml^{-1} Kan for 72 hr at 17°C. One liter acetate mineral medium contained 75 g l^{-1} sea salts, 50 mM MOPS, pH 7.0, 20 mM sodium acetate, 5 mM NH_4Cl, 1 mM K_2HPO_4, 1X Wolfe's vitamins and 1X trace minerals (Chapter 3).

Growth rates were assayed using 96 well plates. Eight replicates of each mutant were inoculated into 200 μl 7.5% sea salts acetate medium in each well. Sixteen replicates of wild-type *P. arcticus* 273-4 were present on each plate and 12 blank wells. Plates were incubated at 22°C, 17°C, 4°C, 0°C, and -2.5°C. Blank subtracted OD_{600} data were collected using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were compiled using a Perl script to parse data by growth temperature, mutant strain and time point. T-tests and plotting of data were carried out using R 2.4.0. The script calculated average growth rates for each replicate of each mutant for optical density increases between OD_{600} values 0.03 and 0.4. Two-tailed t-tests were performed to determine if growth rates were statistically different between wild-type *P. arcticus* and PB0343 within a temperature. Growth rates were considered statistically different if $P < 0.05$.

Amino acid supplementation experiment. In a 96 well plate, 200 μl cultures were prepared in mineral medium with 20 mM acetate (see Chapter 4). Treatments consisted of cultures supplemented with 1 mM concentrations of each of the following amino acids: phenylalanine, tryptophan, proline, cysteine, alanine, glycine, leucine, histidine, and methionine. Additional control cultures included 1% casamino acids and a defined mixture composed of 1 mM concentrations of phenylalanine, tryptophan, proline, cysteine, alanine, glycine, and methionine. A 1% inoculum from stationary phase culture of wild-type *P. arcticus* 273-4 grown through three passages in acetate medium was introduced to each medium. Four replicates of each nutrient supplementation condition were examined during growth at 22°C, 17°C, 4°C, 0°C, and -2.5°C. Growth data were parsed using a custom Perl script. Growth rates were calculated in the range $0.04 < OD_{600} < 0.4$. Two-tailed t-tests were performed comparing growth rates of amino acid supplemented cultures versus the unsupplemented cultures at each temperature. Growth rates were significantly different if $P < 0.05$.

Computational analysis of predicted proteomes from T_{opt} and subzero transcriptomes. Low temperature and optimal temperature proteome gene contents were computed from the transcriptome at -6°C and 17°C or T_{opt} (see Chapter 4). Genes exhibiting high levels of transcript expression and a high magnitude of differential expression were grouped into the predicted subzero and T_{opt} proteomes (Supp. Tables 5.1 and 5.2). Cutoffs for inclusion in these groups were selected as $A > 10$ and $-1 \geq M \geq 1$ where $A = \log_2\sqrt{(-6°C \cdot 17°C)}$ and $M = \log_2(-6°C / 17°C)$ because these genes were on the extremes of the *P. arcticus* transcriptome data and represent a large hypothetical amino acid cost to the cell (Supp Table 5.1). The 17°C predicted proteome consisted of 105

genes and the -6°C predicted proteome consisted of 88 genes. Percent amino acid content was calculated for each amino acid sequence to normalize for length of sequences using a custom Perl script. Distributions of percent amino acid data were plotted for each proteome in R 2.4.0. Amino acid bias in the proteomes was determined by $\log_2$ ratio of medians. If a median amino acid proportion differed from the total median of all amino acids by more than 1 standard deviation, then we considered the difference between the proteomes to be significant.

Results

Knockout mutation of *relA*. The *P. arcticus relA* (*Psyc_0343*) homologue increased expression during growth at 0°C and -6°C compared to warmer temperatures in transcriptome analysis. Energy, translation and transcription gene expression responses of *P. arcticus* at low temperature were indicative of stringent response induction. Analysis of gene expression indicated that genes for methionine, tryptophan, proline, glycine, alanine and histidine accumulation were up-regulated in the cold (Chapter 4). Branched chain amino acids, lysine and arginine biosynthesis were down-regulated at low temperatures. The up-regulation of biosynthesis genes for six amino acids at 0°C and -6°C led us to hypothesize that a depleted cytoplasmic pool of at least one amino acid contributed to stringent response induction.

To examine the role of the stringent response during growth at low temperatures, we generated a knockout mutant of *relA* (*Psyc_0343*) and compared the growth of this mutant to the wild-type *P. arcticus* 273-4 (Fig 5.1). We hypothesized that the *relA* knockout, designated PB0343, would exhibit either reduced growth rate or reduced

growth yield at low temperature. PB0343 exhibited a shorter lag phase of growth at all temperatures similar to the relaxed control of growth in a *B. subtilis* Δ*relA* strain (Fig 5.1A) (10). Our mutants were pre-cultured at 17°C and no effect on growth rate was observed at 17°C (Fig 5.1A), so reduced lag time at low temperatures is possibly the result of residual amino acid pools carried over in the inoculum. Growth of PB0343 at the two temperature extremes, 22°C and -2.5°C, was characterized by a decrease in growth rate before stationary phase between OD_{600} 0.3 and 0.4 followed by an extended period of growth rate reduction leading to growth cessation as compared to wild-type where growth arrest occurred over a shorter time interval (Supp. Fig. 5.1 and Fig 5.1A); stationary phase was observed at $OD_{600} \sim 0.6$ at 17°C and 4°C. Growth rates did not differ at 22°C and 17°C between wild-type and PB0343 with median rates $\mu_{Wild} = 0.021$ and $\mu_{PB0343} = 0.025$ at 22°C and $\mu_{Wild} = \mu_{PB0343} = 0.0143$ at 17°C. Growth of PB0343 ($\mu_{PB0343} = 0.0061$) at 4°C was slower on average than wild-type ($\mu_{Wild} = 0.0065$), and the variability in PB0343 growth rate was large ($S.D._{PB0343} = 0.003$) at this temperature. Mutant growth was significantly slower than wild-type at 0°C and -2.5°C with $P < 0.01$ (Fig 5.1B). At 0C, $\mu_{Wild} = 0.0029$ and $\mu_{PB0343} = 0.0022$, a 25% decrease in growth rate. At -2.5C, $\mu_{Wild} = 0.0012$ and $\mu_{PB0343} = 0.0010$, a 20% decrease in growth rate. No differences between wild-type and PB0343 were observed in growth yield at any temperature, demonstrating that PB0343 was able to achieve biomass similar to wild-type even though the starvation response was impacted by loss of the *relA*. Thus, the temperature specific effect of *relA* deletion was observed at low temperatures as a growth rate reduction.

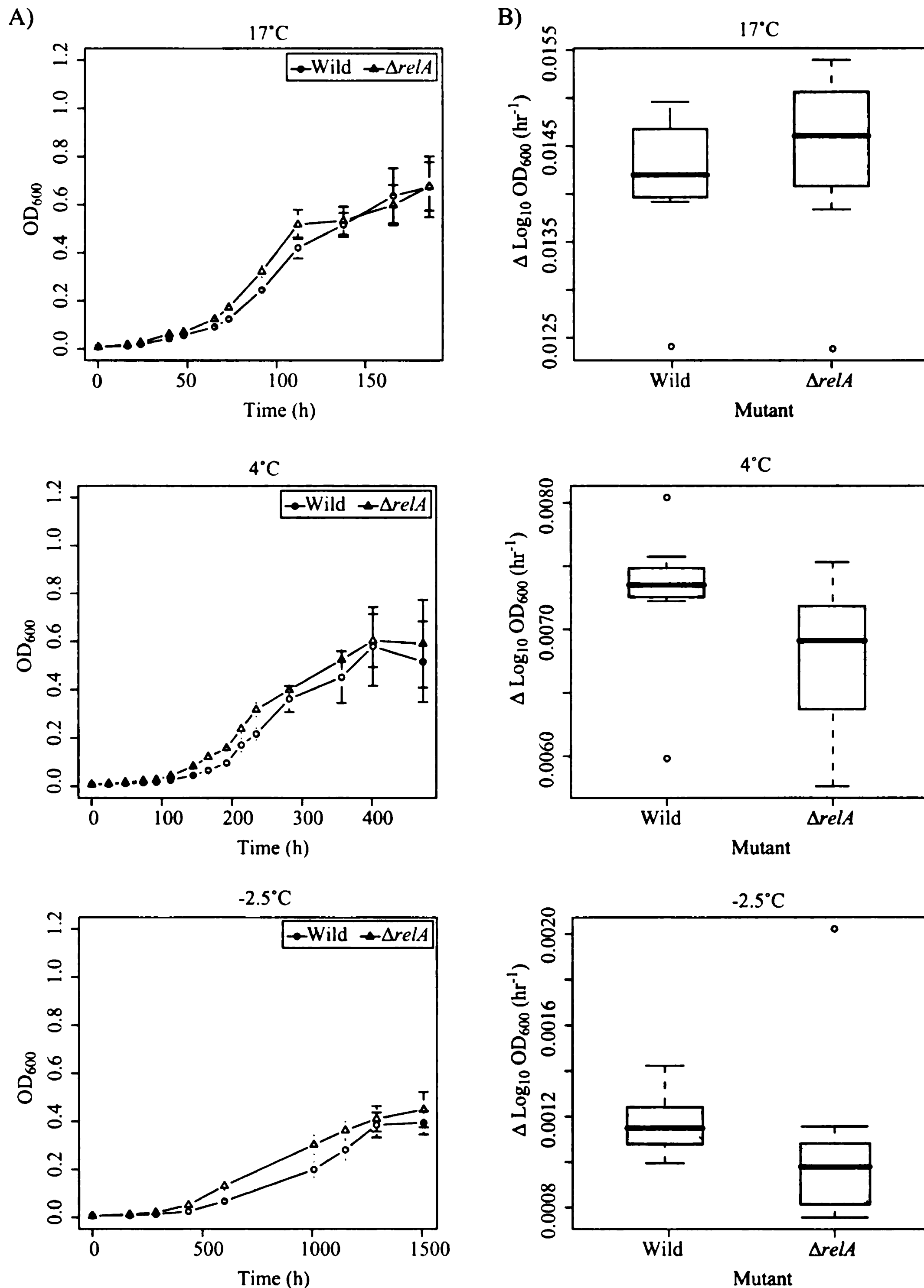


Figure 5.1. Growth characteristics of *P. arcticus* 273-4 wild-type strain and Δ*relA* knockout strain PB0343. A) Growth curves of the two strains, error bars represent one standard deviation from the mean ($n = 8$). B) Box plots of growth rates calculated for the two strains. Bars indicate sample medians, boxes are plotted from the 25th to the 75th percentiles, and whiskers are plotted to the 10th and 90th percentiles. Dots represent outliers which resulted primarily from pipettor error during inoculation.

Amino acid effects on growth rate. We hypothesized that reduced growth rates at 0°C and -2.5°C in the Δ*relA* mutant were the result of loss of stringent response control of growth in PB0343. In the wild-type strain, presence of *relA* yielded longer lag phase and constant growth rates at 22°C and -2.5°C (Supp. Fig. 5.1A and Fig. 5.1A). Those results corroborated our hypothesis that increased expression of amino acid biosynthesis genes resulted from challenges to production of those amino acids specifically resulting from incubation at subzero temperatures. The challenges of biosynthesis of the six amino acids might be alleviated by supplying those amino acids in the growth medium. Observation of temperature specific effects of an amino acid on growth rates would clearly support the hypothesis that *P. arcticus* is starved for those amino acids at low temperature.

P. arcticus was grown in the presence of individual amino acids and mixtures thereof (Table 5.2). Glycine, proline, casamino acids and a defined mixture of amino acids, designated "Complex", increased growth rates at both optimal and sub-optimal temperatures. Fewer significant changes were observed at 4°C than at the other temperatures. This may be due to variability in the response to incubation at these temperatures, which reside on either side of T_{crit}. Interestingly, casamino acids did not improve growth at -2.5°C compared to unsupplemented cultures. Phenylalanine and methionine increased growth rates exclusively at -2.5°C. Leucine and tryptophan decreased growth rates at -2.5°C. Histidine and alanine did not significantly increase growth rates at any temperatures.

Table 5.2. Ratios of average growth rates in cultures supplemented with amino acids to unsupplemented cultures[a,b].

Amino acid	Temperature 17°C	4°C	0°C	-2.5°C
Phe	1.17	1.10	1.04	2.18*
Trp	NG[c]	-1.19	-2.04*	NG[c]
Pro	3.38*	1.46	1.68*	1.30*
Cys	1.68	1.57	1.89	-1.03
Ala	1.67	-1.01	1.13	1.24
Gly	2.48*	1.40	1.50	1.72*
Leu	-1.14	1.00	1.05	-1.22*
Met	2.31	1.19	-1.05	1.58*
Casamino Acids	3.85*	2.11*	1.51*	1.16
His	1.24	1.13	1.25	1.29
Complex	2.64*	1.00	1.71*	1.71*

[a] Positive ratios are ratio of growth rate in supplemented over unsupplemented culture. For conditions in which unsupplemented cultures grew faster, ratios are displayed as the negative inverse of supplemented to unsupplemented.

[b] Statistically significant ratios ($P < 0.05$) are denoted by '*'.

[c] NG = No Growth

Analysis of amino acid bias in the predicted low temperature and high temperature proteomes. If biosynthesis of key amino acids was limiting for translation, then we expected that highly expressed proteins at low temperatures would contain lower proportions of those amino acids to improve efficiency of growth in the cold. Therefore, a negative correlation between ratio of amino acid content and ratio of gene expression was expected between the predicted proteomes. Analysis of the predicted proteome was performed to detect biases in protein amino acid content for genes highly expressed at low temperature as compared to genes highly expressed at T_{opt}. Genes were divided into low and optimal temperature expressed proteomes based on their average intensity, A, and their log-ratio of expression, M. Genes in the -6°C up-regulated proteome were selected with A-value > 10 and $M \geq 1$ (Supp. Tables 5.1). Genes with $M \leq -1$ and $A > 10$

were placed in the 17°C up-regulated transcriptome (Supp Table 5.2). Genes annotated as membrane bound proteins were removed from the analysis, because the interactions of membrane bound proteins with lipid rather than aqueous solutions could introduce biases in amino acid composition due purely to evolution for function in different fluid matrices. Such biases would appear temperature associated in our analysis because a much greater proportion of 17°C up-regulated genes were membrane bound than those at low temperature. Percent amino acid content of each predicted sequence was determined and population distributions for each amino acid were compared (Fig. 5.2). Ratios of median amino acid content in the -6°C to the 17°C proteome were calculated. Amino acid contents were considered significantly different between the two populations if the $\log_2$ ratio of medians was greater than one standard deviation away from the median for all $\log_2$ ratios: 0.01 ± 0.18. Glycine, methionine, arginine and valine were reduced in proportion in the -6°C proteome while tryptophan, cysteine, and leucine increased in proportion.

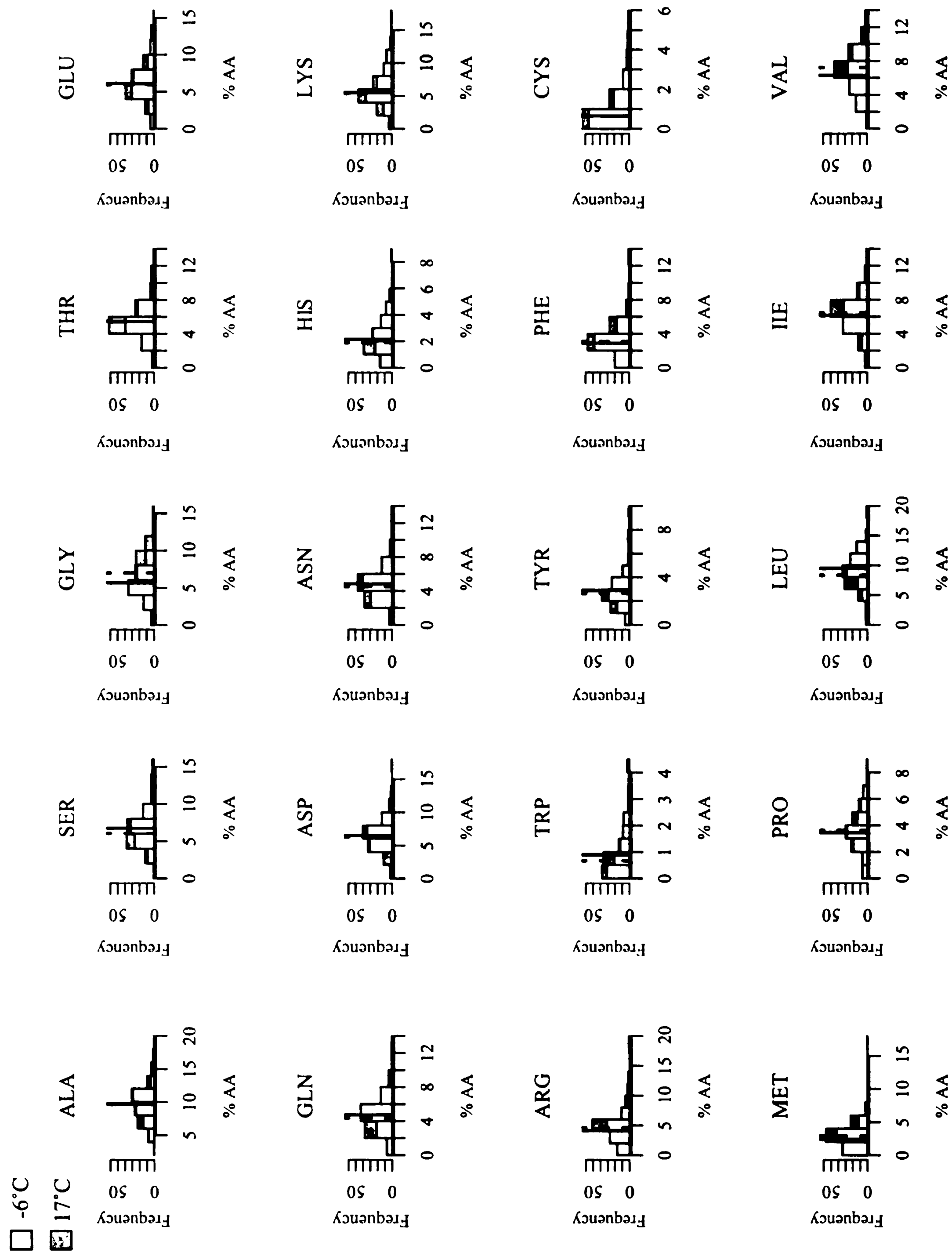


Figure 5.2. Overlaid histograms of amino acid proportions in the -6°C (□) and 17°C (■) proteomes. Bin boundaries are defined by percent amino acid content (% AA) values. Histograms have been overlaid with -6°C amino acid content in the foreground. Vertical lines represent median amino acid proportions for -6°C (solid) and 17°C (dashed) proteomes. Frequencies are representative of the number of proteins in each predicted proteome whose percent content of an amino acid fell within a bin.

Table 5.3. Compiled data from expression of amino acid biosynthesis genes, their proportion in -6°C vs. 17°C proteomes and the effect on growth of supplementation with those amino acids.

Amino Acid	Fold-Change Expression (6°C/17°C)[a,b]	-2.5°C Growth Rate Ratio (AA added/no AA)	Ratio of Amino Acid Medians (-6°C/17°C)[c]
Gly	3.23[d]	1.72	-1.23
Pro	1.27	1.30	-1.06
Met	1.41	1.58	-1.21
Phe	1.00	2.18	-1.07
Cys	1.41	-1.03	1.20
Trp	1.48	NG[e]	1.34
Ala	-2.36	1.24	1.00
His	1.29	1.29	1.05
Leu	-2.28	-1.22	1.13

[a] If more than one gene was differentially expressed in an amino acid pathway, then the gene closest to the pathway product was used to represent fold-change behavior in the pathway.

[b] Results from Chapter 4, Table 4.2

[c] Untransformed ratios of medians

[d] Inverse of fold-change for glycine cleavage protein, *gcvH*

[e] NG = No growth was observed in this trial.

Ratios of median percent amino acid content comparing the two proteomes were calculated and compiled with data from the amino acid supplementation experiment (Table 5.3). Principal components analysis (PCA) was performed on these data to group amino acids based on data trends in these three experiments (Fig. 5.3). PCA delineated the amino acid data into groups with similar trends. Those amino acids whose biosynthesis genes increased greater than 1.4-fold also exhibited substantial changes in proportion between 1.2 and 1.4-fold in the proteome comparison. Both methionine and glycine biosynthesis are increased, and these amino acids increased growth rate when supplemented into acetate medium. Both amino acids decrease their proportion 1.2-fold in the low temperature proteome which supported our hypothesis that increases in expression and growth rate would be correlated with bias against these amino acids in the

low temperature proteome. Thus, compensation by increasing transcription of glycine and methionine biosynthesis appears insufficient to support translation of highly expressed proteins at low temperature. In contrast, the biosynthesis of the least abundant amino acids, cysteine and tryptophan, was up-regulated at low temperature, but the proportions of these amino acids increased 1.2 to 1.3-fold in the low temperature proteome. It may be that compensation by increased expression of biosynthesis genes is sufficient to support translation, because these residues are rare in peptide sequences. Alanine and histidine did not significantly effect growth at any temperature and their proportion did not change in the low temperature proteome. Leucine increases in proportion in the low temperature proteome while expression of leucine biosynthesis genes decreases. This indicates that the biosynthesis of leucine is sufficient at low temperatures. It is interesting to note that leucine proportion increased while valine and isoleucine proportions decreased in the low temperature predicted proteome. The balance of these inter-related biosynthesis pathways may have shifted at subzero temperatures.

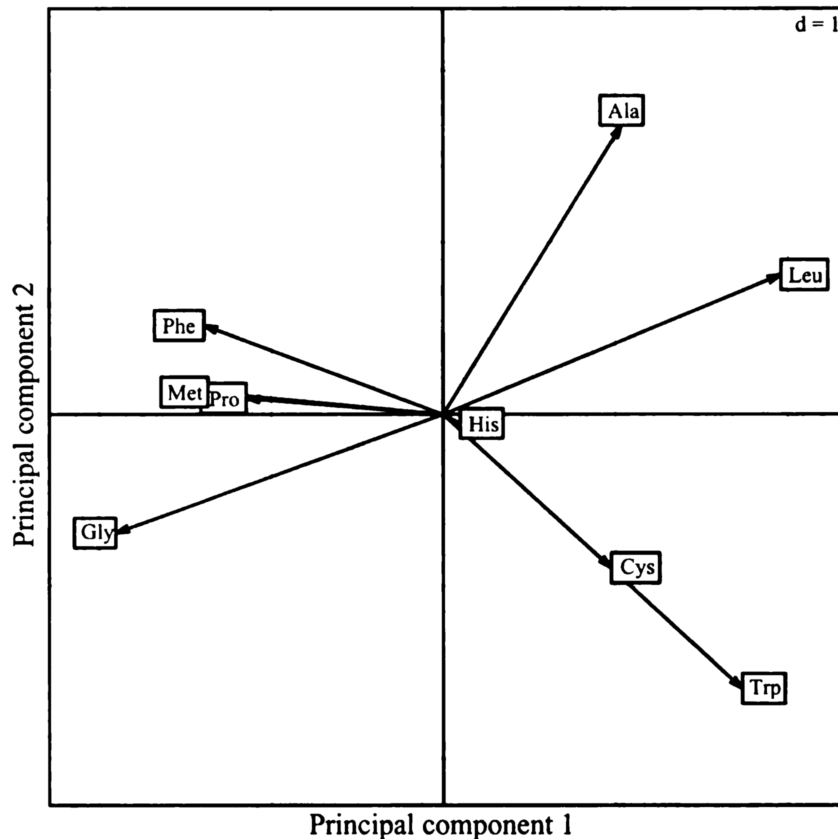


Figure 5.3. PCA comparing data trends in amino acid content, growth rate effect and gene expression. Amino acid loading on principal component 1 is plotted on the horizontal axis and explains the most variation across the three experiments. Principal component 2 explains the second most variation and is plotted on the vertical axis.

Discussion

Growth at low temperatures has been reported to induce genes for various combinations of arginine, tryptophan, glutamate, glutamine, asparagines, cysteine and leucine in diverse mesophiles and psychrophiles (4, 6, 15, 18, 20). The biosynthetic cost of amino acids as a function of both molecular weight and ATP have been shown to act as a selective force on proteome evolution across taxa (1, 23). Here we expand this hypothesis and search for evidence of effects of amino acid cost on the predicted proteome specific to a physiologic condition, in this case temperature specific

metabolism. If an amino acid was limiting for growth at low temperatures, we expected that i) low temperature expression for accumulation of that amino acid would increase, ii) growth rates in cultures supplemented with that amino acid would increase as is observed in auxotrophs which are also limited for an amino acid (13, 22), and iii) if this change in metabolism is ecologically significant to *P. arcticus*, then selection against this amino acid would be observed in the genes highly expressed at low temperature. This relationship was observed for glycine and methionine. And the inverse was observed for the branched chain amino acid leucine. These observations suggest that the metabolism of glycine and methionine is limiting at low temperature and of leucine at optimal growth temperature. Consequently, the production of these amino acids probably effects *P. arcticus* fitness in the permafrost. The presented work is the first synthesis of global gene expression data, growth experiments, and proteome prediction and analysis to elucidate the relationships between gene expression at low temperatures and adaptations for metabolism across the critical temperature.

The stringent response induces gene expression for growth rate reduction under starvation conditions. The stringent response signal, (p)ppGpp, decreases the expression of stable RNAs and consequently decreases growth rates and down-regulates central and energy metabolism (7). Up-regulation of amino acid biosynthesis, in particular histidine and arginine biosynthesis, has been linked to the stringent response, but recent global transcriptome analysis confirmed that these responses are complicated and are probably under metabolic control by feedback from cytoplasmic pools of these amino acids rather than direct stringent response control (8). Reduced thermotolerance to supra-optimal temperatures during growth on minimal media has been reported in *E. coli ΔrelA* mutants

and these mutants also exhibited a long period of growth rate reduction before growth arrest (26). A *B. subtilis* Δ*relA* strain exhibited relaxed growth rate reduction and decreased lag phase length compared to a wild-type strain (10), effects which were also observed in *P. arcticus* Δ*relA* growing at low temperature. Nutrient limitation is a constant condition in permafrost and other cryo-environments (14), so association of the stringent response with low temperature growth as hypothesized from our microarray data is consistent with conditions to which *P. arcticus* is evolutionarily adapted. Our observation that wild-type *P. arcticus* had a growth advantage compared with the Δ*relA* mutant PB0343 suggests that the stringent response plays a major role in regulating growth rate at 0°C and -2.5°C. This advantage was negated at warmer temperatures suggesting either that the stringent response is not as important in the optimal temperature range from 22°C to 4°C, or that the growth rate effect of loss of *relA* can be compensated by *spoT* activity under optimal growth conditions. SpoT is the primarily involved in (p)ppGpp degradation, but can also act in its synthesis (7). However, the former explanation seems most likely in the context of our microarray observation that the stringent response is induced in the cold.

Not surprisingly, casamino acid supplementation and that with a defined mixture of amino acids improved growth at all temperatures. Clearly, provision of a mixed population of amino acids alleviated some of the energetic cost of growth in the acetate medium. Leucine exhibited a small negative effect on growth at 17°C and -2.5°C. Excess leucine can induce starvation for other amino acids including tryptophan (13), and this may explain decreased growth rates in the presence of excess leucine. Biosynthesis of leucine was down-regulated in the subzero transcriptome indicating that biosynthesis

of leucine is sufficient to support growth at subzero temperatures. Furthermore, the proportion of leucine in the subzero predicted proteome was higher than in the optimal temperature highly expressed proteome corroborating the conclusion that leucine biosynthesis is efficient at low temperature relative to optimal temperatures where competition between translating ribosomes for leucine may lead to decreased proportion of leucine per protein. Position of the methyl-group in branched chain amino acids may have effects on flexibility of low temperature adapted enzymes, thus shifting the balance between valine, isoleucine and leucine pools whose biosynthesis is interrelated.

Proline can act as a compatible solute and provision of up to 1 mM extracellular proline can allow *E. coli* to grow in salt concentrations up to 1 M NaCl (16). Compatible solute uptake and biosynthesis genes are up-regulated in bacteria following cold shock and improve survival of psychrotolerant organisms at low temperatures where these compounds can benefit both growth rate and protein stability (2, 19). Compatible solutes provide the cell with a mechanism to maintain both protein interactions with intracellular water and to maintain the turgor pressure which drives cell growth (16, 25). The medium used in our growth experiments was 7.5% sea salts, a moderately high salinity. Supplementation of *P. arcticus* cultures with 1 mM proline enhanced growth rates at all temperatures and no significant change in proline proportion was observed in the proteomes in spite of increased expression of two proline biosynthesis genes in our transcriptome analysis. Proline is most likely serving a role as a compatible solute for growth at low temperatures.

P. arcticus may be limited for production of methionine and glycine at low temperatures. Glycine is produced either via glycine hydroxymethyltransferase (*glyA*) or

serine-glyoxylate transaminase (*sgaA*) in this organism. During growth on acetate, *P. arcticus* should produce substantial amounts of glyoxylate as part of the glyoxylate bypass to regenerate amino acid precursors. Based on the genome sequence, the glyoxylate bypass in *P. arcticus* could be completed via either malate synthase or via the β-hydroxyaspartate pathway which produces glycine as an intermediate substrate (17). As was observed in *P. arcticus* at subzero temperatures, expression of the glycine cleavage system is repressed in response to glycine limitation in *E. coli* (24). Neither *glyA* nor *sgaA* were differentially expressed *P. arcticus* transcriptome. It seems likely that glycine generation is significantly dictated by the flux of acetyl-CoA through the TCA cycle, glyoxylate bypass, and β-hydroxyaspartate pathway. A consequence of life over long generation times under limiting conditions is that the ratio between energy production and structural precursor biosynthesis is likely shifted towards ATP. For example, flux of acetyl-CoA through the complete TCA cycle may be increased at subzero temperatures to produce energy, because more energy is required per generation at low temperatures (5). Flux analysis with labeled acetate as the growth substrate will shed light on this model of growth at low temperature.

Alternatively, glycine can also play a role in the cell as a compatible solute by conversion to glycine-betaine (16). Utilization of glycine as a compatible solute is consistent with growth rate ratios in glycine supplemented cultures and down-regulation of glycine cleavage particularly if C1-metabolism activities are decreased at low temperatures. The proteome data for glycine could be explained under this compatible solute alternative if the ribosome is at some competitive disadvantage for glycine versus the betaine biosynthesis genes *betAB*. However, *betAB* expression was down-regulated at

low temperatures in our microarray experiment whereas glycyl-tRNA synthetases (*glySQ)* were up-regulated at low temperatures. Furthermore, the overall abundance of *glySQ* transcript was 4-fold higher than *betAB* in that transcriptome analysis. For these data to be resolved in the compatible solute model, betaine biosynthesis genes would have to be much more efficient at subzero temperatures than the more central function of the glycyl tRNA synthetases. Therefore, reduced glycine biosynthesis at low temperature due to changes in acetate flux seems to be the most parsimonious explanation for the results of gene expression, growth rate and subzero proteome glycine content.

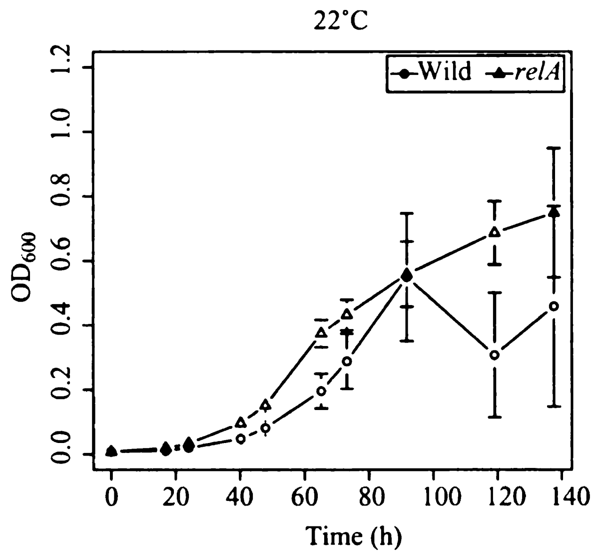
The limitation of growth based on methionine is most likely related to sulfur metabolism at low temperatures. Cysteine and methionine increased growth rates at all temperatures, but effects were only statistically significant under methionine supplementation at -2.5°C (Table 5.2). Low rates of biosynthesis would then lead to decreased rates of reduced sulfur incorporation into coenzyme A, S-adenosylmethionine, cysteine, glutathione, iron-sulfur clusters, and others thereby seriously impacting the function of the cell. Sulfur incorporation rates at low temperature are particularly critical in light of results indicating increased methionine biosynthesis. There was also increased expression of genes for oxidative stress response (including iron sulfur cluster biosynthesis), sulfur incorporation for coenzyme A production and the periplasmic binding protein for an ABC sulfate uptake transporter in our microarray experiment. Addition of methionine to cultures of *P. arcticus* grown at -2.5°C could reduce the energy investment required for sulfur incorporation at subzero temperatures and enable increased growth rates. The observed decrease in methionine proportion in the subzero

predicted proteome may be the result of competition between sulfur metabolism enzymes and the ribosome for methionine.

The rarest amino acids in both the high and low temperature proteomes are cysteine and tryptophan. Both amino acids increased significantly in proportion in the low temperature up-regulated proteome. Cysteine biosynthesis is linked with methionine metabolism. The increased proportion of oxidative stress proteins in the subzero predicted proteome may explain the higher proportion of cysteine present in these proteins, because cysteine plays a reactive role in many oxidation repair and resistance functions (11). In contrast, tryptophan is the most energetically expensive amino acid to synthesize and has the bulkiest side chain, so it would be expected represent a smaller proportion of amino acid positions. Tryptophan supplementation would be expected to increase growth rates. Our results for this study seem perplexing in light of this model. It is possible that increased expression of anthranilate synthase compensates for decreased rates of tryptophan synthesis. Tryptophan comprises only 0.9% of the amino acid positions in proteins even in the subzero temperature proteome, so small increases in gene expression might balance the cellular tryptophan budget. Another aromatic amino acid, phenylalanine, did increase growth rates at low temperature and likely alleviated the high cost of aromatic amino acid biosynthesis, but this result cannot be linked to potential bias in the proteome.

Our results demonstrate that glycine, methionine and leucine metabolism gene expression at temperatures is likely related to limitation for those amino acids due to changes in cellular physiology below the critical temperature. Combined genome, transcriptome and growth analysis have enabled exploration of the link between the

challenges to biosynthesis in the cold and the ecology of a permafrost microbe adapted to exploit a niche near its lower thermal limit of growth and under nutrient limitation. *P. arcticus* appears unable to compensate for putative reduced glycine and methionine production at low temperature, but can most likely compensate for changes in tryptophan and cysteine production. Analysis of the predicted highly expressed subzero temperature proteome indicates that limitation for glycine and methionine has likely been a selective factor in the evolution of the *P. arcticus* genome leading to modification of peptide sequences to compensate for reduced cellular pools of these amino acids at subzero temperatures. Metabolic flux analysis could confirm that the cellular pools of glycine and methionine change at subzero temperatures and reveal the changes in metabolism that account for depletion of these key amino acids in central and sulfur metabolism respectively.



Supplementary Figure 5.1. Growth of *P. arcticus relA* knockout compared with wild-type *P. arcticus* 273-4. Growth rates are not statistically different. The *relA* knockout exhibits decreased lag time and relaxed growth arrest.

Supplementary Table 5.1. Genes included in the proteome amino acid content analysis. Genes were differentially up-regulated > 2-fold at the indicated temperature and highly expressed based on transcriptome analysis.

-6°C Predicted Proteome			
Psyc_0021	conserved hypothetical protein	*Psyc_1062*	probable glutamate dehydrogenase
Psyc_0036	conserved hypothetical protein	*Psyc_1080*	glutathione S-transferase family
Psyc_0042	possible DNA polymerase III, chi subunit	*Psyc_1118*	probable C-factor protein
Psyc_0043	probable cytosol aminopeptidase	*Psyc_1228*	hypothetical protein
Psyc_0044	probable isochorismatase hydrolase	*Psyc_1240*	putative glucose inhibited division A
Psyc_0079	conserved hypothetical protein	*Psyc_1241*	hypothetical protein
Psyc_0081	conserved hypothetical protein	*Psyc_1256*	conserved hypothetical protein
Psyc_0112	conserved hypothetical protein	*Psyc_1257*	23S RNA pseudouridylate synthase
Psyc_0114	PcnB, poly(A) polymrase	*Psyc_1264*	conserved hypothetical protein
Psyc_0124	probable RpoS regulator, TraR/DksA family	*Psyc_1270*	probable serine protease
Psyc_0128	hypothetical protein	*Psyc_1274*	hypothetical protein
Psyc_0130	conserved hypothetical protein	*Psyc_1303*	hypothetical protein
Psyc_0132	conserved hypothetical protein	*Psyc_1317*	possible biopolymer transport protein
Psyc_0135	conserved hypothetical protein	*Psyc_1325*	probable potassium channel, VIC family
Psyc_0151	hypothetical protein	*Psyc_1331*	conserved hypothetical protein
Psyc_0154	hypothetical protein	*Psyc_1342*	probable glycerate kinase
Psyc_0156	conserved hypothetical protein	*Psyc_1349*	hypothetical protein
Psyc_0207	putative Sigma 32 (RpoH)	*Psyc_1353*	ThiJ/PfpI family protein
Psyc_0250	conserved hypothetical protein	*Psyc_1354*	hypothetical protein
Psyc_0251	conserved hypothetical protein	*Psyc_1365*	probable fatty acid desaturase
Psyc_0260	probable cytochrome c, class I	*Psyc_1366*	conserved hypothetical protein
Psyc_0302	possible heat shock protein, Hsp33	*Psyc_1423*	starvation protein A
Psyc_0343	putative RelA/SpoT family protein	*Psyc_1433*	hypothetical protein
Psyc_0360	conserved hypothetcial protein	*Psyc_1476*	possible transcriptional regulator
Psyc_0379	probable organic hydroperoxide resistance	*Psyc_1477*	putative cysteine desulfurase
Psyc_0396	methionine aminopeptidase, type 1	*Psyc_1478*	nitrogen-fixing NifU-like
Psyc_0405	conserved hypothetical protein	*Psyc_1479*	HesB/YadR/YfhF
Psyc_0487	putative ribonuclease EG	*Psyc_1480*	probable co-chaperone Hsc20
Psyc_0522	probable Acyl carrier protein (ACP)	*Psyc_1481*	heat shock protein Hsp70
Psyc_0523	conserved hypothetical protein	*Psyc_1482*	ferredoxin, 2Fe-2S type
Psyc_0551	conserved hypothetical protein	*Psyc_1506*	hypothetical protein
Psyc_0664	probable Glycosyl transferase, group 1	*Psyc_1581*	hypothetical protein
Psyc_0698	conserved hypothetical protein	*Psyc_1658*	5-formyltetrahydrofolate cyclo-ligase
Psyc_0702	conserved hypothetical protein	*Psyc_1659*	ATP-dependent protease La
Psyc_0715	conserved hypothetical protein	*Psyc_1697*	metal-dependent phosphoesterases
Psyc_0766	possible RNA methylase	*Psyc_1712*	hypothetical protein
Psyc_0770	hypothetical protein	*Psyc_1724*	RNA polymerase, omega subunit
Psyc_0801	hypothetical protein	*Psyc_1762*	conserved hypothetical protein
Psyc_0841	monoamine oxidase	*Psyc_1775*	putative PspC
Psyc_0918	conserved hypothetical protein	*Psyc_1777*	conserved hypothetical protein
Psyc_0921	Hfq family protein	*Psyc_1033*	hypothetical protein
Psyc_0962	bis(5'-nucleosyl)-tetraphosphatase	*Psyc_1043*	peptide methionine sulfoxide reductase
Psyc_0985	DNA binding protein , HTH	*Psyc_1056*	transcriptional regulator, LysR family
Psyc_0986	neutral zinc metallopeptidases		

Supplementary Table 5.2. Genes included in the predicted 17°C (Topt) proteome. Genes were selected for analysis based on differential up-regulation > 2-fold and were highly expressed transcripts.

17°C Predicted Proteome			
Psyc_0027	chaperone protein dnaJ	*Psyc_1175*	putative acetyl-CoA hydrolase/transferase
Psyc_0097	citrate synthase	*Psyc_1309*	probable short-chain dehydrogenase
Psyc_0099	succinate dehydrogenase anchor	*Psyc_1326*	probable outer membrane protein
Psyc_0101	succinate dehydrogenase subunit	*Psyc_1337*	ribosomal protein S6
Psyc_0104	dihydrolipoamide dehydrogenase	*Psyc_1338*	ribosomal protein S18
Psyc_0105	succinyl-CoA synthetase beta subunit	*Psyc_1339*	ribosomal protein L9
Psyc_0106	succinyl-CoA synthetase alpha subunit	*Psyc_1358*	putative pyridoxamine 5'-phosphate oxidase
Psyc_0127	conserved hypothetical protein	*Psyc_1359*	phosphoglucomutase/phosphomannosemutase
Psyc_0137	probable acyl-CoA dehydrogenase	*Psyc_1360*	inosine 5'-monophosphate dehydrogenase
Psyc_0171	ribosomal protein L25	*Psyc_1361*	cysteinyl-tRNA synthetase, class Ia
Psyc_0172	ribose-phosphate pyrophosphokinase	*Psyc_1363*	hypothetical protein
Psyc_0177	serine-pyruvate aminotransferase	*Psyc_1410*	3-isopropylmalate dehydrogenase
Psyc_0196	phosphate acetyltransferase	*Psyc_1412*	3-isopropylmalate dehydratase small subunit
Psyc_0259	conserved hypothetical protein	*Psyc_1413*	3-isopropylmalate dehydratase large subunit
Psyc_0292	ribonuclease, cold-shock domain	*Psyc_1460*	alanyl-tRNA synthetase, class IIc
Psyc_0319	cell division protein	*Psyc_1468*	penicillin-binding protein transpeptidase
Psyc_0369	D-3 phosphoglycerate dehydrogenase	*Psyc_1487*	ribosomal protein S20
Psyc_0370	rhodanese-related sulfurtransferase	*Psyc_1492*	hypothetical protein
Psyc_0429	prolyl-tRNA synthetase, class IIa	*Psyc_1493*	histone-like bacterial DNA-binding protein
Psyc_0453	hypothetical protein	*Psyc_1521*	NADH-dependent flavin oxidoreductase
Psyc_0505	ribosomal protein L18	*Psyc_1523*	aspartate semialdehyde dehydrogenase
Psyc_0506	ribosomal protein S5	*Psyc_1534*	ribosome recycling/releasing factor (RRF)
Psyc_0507	ribosomal protein L30	*Psyc_1535*	Uridylate Kinase
Psyc_0519	ribosomal protein L32	*Psyc_1560*	periplasmicthiol:disulfide oxidoreductase DsbE
Psyc_0527	acetolactate synthase, large subunit,	*Psyc_1561*	cytochrome c-type biogenesis protein
Psyc_0528	acetolactate synthase, small subunit	*Psyc_1562*	hypothetical protein
Psyc_0529	Ketol-acid reductoisomerase	*Psyc_1566*	ribosomal protein S9
Psyc_0556	NAD(P) transhydrogenase, alpha subunit	*Psyc_1572*	ribosomal protein L27
Psyc_0557	NAD(P) transhydrogenase, beta subunit	*Psyc_1573*	ribosomal protein L21
Psyc_0649	tyrosine kinase	*Psyc_1577*	outer membrane efflux protein, OprM
Psyc_0672	hypothetical protein	*Psyc_1588*	Beta-lactamase-like superfamily protein
Psyc_0673	tetrahydrofolate cyclohydrolase	*Psyc_1592*	conserved hypothetical protein
Psyc_0678	nucleoside diphosphate kinase	*Psyc_1594*	hypothetical protein
Psyc_0687	D-alanyl-D-alanine carboxypeptidase	*Psyc_1598*	hlyD family secretion protein
Psyc_0708	fructose-bisphosphate aldolase class II	*Psyc_1599*	ABC drug efflux transporter
Psyc_0709	Holliday junction DNA helicase RuvA	*Psyc_1600*	outer membrane efflux protein
Psyc_0717	aminotransferase	*Psyc_1604*	Pyridoxal phosphate-dependent deaminase
Psyc_0751	aconitate hydratase B	*Psyc_1624*	phosphoribosylformylglycinamidine cycloligase
Psyc_0797	nitroreductase family protein	*Psyc_1627*	isocitrate lyase
Psyc_0798	glycine cleavage H-protein	*Psyc_1642*	porin, gram-negative type
Psyc_0828	s-adenosylmethionine synthetase	*Psyc_1643*	malate synthase
Psyc_0883	hypothetical protein	*Psyc_1657*	fumarate hydratase class II
Psyc_0884	hypothetical protein	*Psyc_1670*	NADP-specific glutamate dehydrogenase
Psyc_0928	possible rare lipoprotein A family	*Psyc_1700*	hypothetical protein
Psyc_0942	Cold-shock protein	*Psyc_1719*	chaperone protein htpG (Hsp90)
Psyc_0943	ATP-dependent RNA helicase	*Psyc_1742*	conserved hypothetical protein
Psyc_0956	ribose 5-phosphate isomerase	*Psyc_1763*	malate dehydrogenase
Psyc_0968	S-adenosyl-L-homocysteine hydrolase	*Psyc_1814*	MCP methyltransferase, CheR-type
Psyc_1066	3'-phosphoadenosine sulfotransferase	*Psyc_1815*	chemotaxis sensory transducer
Psyc_1067	conserved hypothetical protein	*Psyc_1816*	type IV pili signal transducer, CheW
Psyc_1102	acetyl-CoA carboxylase	*Psyc_1817*	two-component response regulator
Psyc_1103	conserved hypothetical protein	*Psyc_1818*	two component reponse regulator

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CHAPTER 6

SUMMARY AND FUTURE PERSPECTIVE

Psychrophilic response to low temperature and its implications for Psychrobacter ecology

This work has addressed the phenomenon of growth in ice-like conditions in a heterotrophic psychrophile isolated from Siberian permafrost. The strength of our model organism is that it is capable of growth both at moderate and subzero temperatures in salt concentrations roughly equivalent to twice that in seawater. I have demonstrated that *Psychrobacter arcticus* exhibits a low temperature response directed towards slowing metabolic rates with continuing growth at low temperatures as opposed to the alternative of growth cessation with a generalized stress response to resist damage. Unlike all mesophiles and some psychrophiles, *P. arcticus* does not exhibit an increased resistance to freeze/thaw or DNA damage after low temperature incubation. *P. arcticus* was also shown to exhibit only a narrow (44 genes) transcriptome response to incubation at 4°C compared to 22°C, indicating that *P. arcticus* does not primarily regulate its response to 4°C by transcription control. Rather, the response to 4°C may be a fine-tuning response involving the regulation of enzyme activity. Subzero transcriptome analysis indicates that true to theories of functional enzyme adaptation to multiple temperature ranges (2), energy metabolism, most anabolic pathways and the basal machinery of transcription and translation decreased in expression suggesting that their function is adequate in the cold.. Some compensation was observed including expression for exchange of metabolic pathways for carbon and nitrogen incorporation, increased expression of membrane desaturase, increased expression for oxidative stress resistance, and a newly discovered response to adjust cell wall dynamics. These observed changes in metabolism may be of ecological relevance to *P. arcticus*. In the case of amino acid metabolism, differential

expression for glycine, methionine and leucine biosynthesis can be correlated to differences in amino acid content of highly expressed genes at low temperature compared to those highly expressed at T_{opt}.

The strategy of adaptation for resource efficiency in *P. arcticus* is likely central to its ecological strategy in cryo-environments. Indeed, *P. arcticus* is able to tolerate a 79-fold decrease in average growth rate between T_{opt} and -6°C without entering a dormant state. Why would this strategy of slow growth near the physical limits of life be favored in *P. arcticus* evolution? Presumably this allows *P. arcticus* to exploit a niche unavailable to other heterotrophs. Entry into dormancy, with all of its advantages for damage resistance, requires major changes in the expression of proteins and such adjustments must be undone when conditions again become favorable for growth. Perhaps, *P. arcticus* has specialized to live at the energy limits by continuing growth with very low energy inputs. This might permit success over other organisms that will not grow in an energy limited situation such as that expected in cryo-environments. However, it is important to note that tolerance to energy limited growth and dormancy strategies are both viable adaptations for survival in permafrost as indicated by the isolation of *Bacillus* and *Clostridium* species, known spore-forming genera, from permafrosts of various ages.

If such adaptation is critical to *P. arcticus* survival in cryo-environments, then why has this species retained the ability to grow at warmer temperatures? We have an extremely limited sense of the history of *P. arcticus* before it entered the permafrost. It is unlikely that *P. arcticus* could have evolved under stably subzero temperatures previous to incorporation into the permafrost community, because such stably cold environments

tend to be ephemeral on geological time scales longer than 10^5 yr. Furthermore, *Psychrobacter* species have never been isolated from permafrost older than 110,000 yr. While it is clear that aspects of *P. arcticus* adaptation have allowed substantially long term survival in permafrost, continuously frozen soils may not represent the niche to which *Psychrobacter* species are primarily adapted (1).

Perhaps *P. arcticus* is primarily adapted for success in environments that undergo annual freezing cycles such as the active layer of permafrost soils or aquatic surface waters which exhibit yearly freezing cycles and remain frozen for 9 months of the year in many polar habitats. In the active layer of permafrost, freezing occurs slowly over a period of several months. Such a freezing process can create stable temperature conditions at about 0°C until the freezing process is complete. Consumption of carbon derived from soil microbiota increases in freezing tundra presumably due to death of microbial populations during freezing (4). *P. arcticus* adaptations for growth over a range of temperatures in variable water activities, and the displayed preference of oxidized organic acids is consistent with an ecology supported by freeze-thaw cycles. Thus, *P. arcticus* may represent only a small proportion of microbial populations particularly in high activity soils during the summer, but be adapted to make substantial gains in resources during the winter months while other microbial populations are merely surviving or even dying. Such an ecological strategy is consistent with the lack of anaerobic respiration pathways in the genome versus adaptation to the highly reducing conditions of Siberian permafrosts which may contain no free oxygen (6).

Future perspectives for research on Psychrobacter arcticus

My experiments have not addressed the direct measurement of changes in metabolic reactions with temperature. *P. arcticus* decreases growth rate 79-fold at -6°C versus 17°C, but transcriptome analysis showed decreased in energy metabolism genes of only 2 to 8-fold in the same comparison. The degree of down-regulation of transcription at low temperature was, on average, higher than the degree of up-regulation (Fig. 4.3). Undoubtedly, some of the discrepancy between growth rate decrease and transcriptome changes can be explained by underestimation of gene expression changes with temperature, a common problem in high throughput expression analyses which require global normalization and estimation of the effects of multiple sources of variance over thousands of data points. Another contributor to this discrepancy is certainly that the cell is functioning more slowly at low temperature, so growth at low temperatures with concomitant decreases in metabolic rates might lead to this skewing in the distribution of gene expression changes. The relationship between transcription and magnitude of compensation remains unclear and only physiological characterization of psychrophilic growth at low temperature can elucidate this relationship. Nevertheless, my growth experiments in both knockout mutants and wild-type cells, and the results of my genomic analyses demonstrate that the hypotheses based on differential expression in the transcriptome are testable.

The observed relationship between amino acid metabolism expression, growth rate response to amino acid supplementation and proteome content suggests changes in metabolism with temperature in our laboratory experiments may be of ecological significance to *P. arcticus*. Thus, experiments constructed to understand the changes in

flux of nutrients through metabolic pathways and how changes in gene expression contribute to that flux will yield important insights into the metabolism of *P. arcticus* in cryo-environments. Specific experiments include analysis of how acetate is shunted between energy production and biosynthesis with incubation at different temperatures and how sulfur metabolism is regulated at low temperatures. The analysis of sulfur metabolism at low temperatures is particularly interesting, because $MgSO_4$ is the second most abundant salt in our acetate medium and there was no reason to expect any sulfur starvation to occur.

While gene expression and mutant data support a model of isozyme exchange to maintain elasticity of the cell wall, many questions remain unanswered. First, is the elasticity of cell walls from low temperature cultured cells greater than that from warm temperature cultured cells, and how does this correlate to the degree of cross-linking? How does cross-linking of peptidoglycan strands change in the isozyme mutants that we generated? Based on mutant growth results, I propose that the DD-peptidase isozyme deletion leads to increased cross-linking of the murien sacculus at the respective functional temperatures of the isozymes. Biochemical characterization of the regulatory process underlying transcriptional regulation of the DD-peptidases would enable us to differentiate temperature regulation versus regulation by some property of the cell wall controls their expression. Outer membrane lipoproteins are covalently anchored in the cell wall and might contribute to sensing cell wall characteristics.

The model of *P. arcticus* as an organism adapted for annually frozen environments leads to testable hypotheses. *P. arcticus* in frozen microcosms with other organisms should be able to incorporate carbon including that derived from microbial

biomass. *P. arcticus* should be able to outcompete other microbes in soils for carbon under frozen conditions. Such hypotheses can be tested with stable isotope probing combined with specific probes for *P. arcticus* cells and any competitor we might choose. Additionally, because *P. arcticus* does not enter a dormant state, it may have an advantage over competitors in acquiring resources immediately following the spring thaw.

The ability to grow at very slow rates makes *P. arcticus* a candidate model organism for metabolic analysis. Does metabolism at slow rates occur continuously or in a stepwise fashion? There is a high probability that the metabolism of *P. arcticus* at low temperatures exhibits interesting spectral qualities associated with discontinuous flux through metabolic pathways. All biological systems from whole cells to enzymes exhibit periodic behaviors. For instance, an enzyme in ice exhibits periodic fluctuations in activity as substrate is locally depleted more quickly than it is replenished through diffusion (5). The advantage of a model organism with very slow growth rates is that such periodic shifts in the activity of the metabolic network could be analyzed at high temporal resolution relative to the biological development of a cell. Spectral analyses also permit the analysis of noise in biological behavior (3). Understanding the contribution of noise to biology is one of the next great frontiers in biological science and has implications for phenomena ranging from population diversity to behavioral control in the single cell.

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