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A Genomic and Transcriptomic Approach to Understanding Cold Acclimation in *Pseudomonas fluorescens* HK44

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I am submitting herewith a thesis written by Abby Ellen Smartt entitled "A Genomic and Transcriptomic Approach to Understanding Cold Acclimation in *Pseudomonas fluorescens* HK44." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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A Genomic and Transcriptomic Approach to Understanding Cold Acclimation in *Pseudomonas fluorescens* HK44

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ABSTRACT

Bacterial response to cold shock and cold adaptation is not fully understood. While several cold inducible genes have been identified in mesophilic orangisms, the roles they play in cold acclimation remain unclear. Few studies emphasize cold acclimation of psychrotrophic or psychrophilic bacteria. Available cold acclimation studies look at a limited number of genes involved in cold stress, and fewer studies compare genes involved in cold shock and cold adaptation. As "omics" technologies become more readily available, looking at whole cell response to cold stress is more achievable. This study uses genomic and transcriptomic approaches to advance the understanding of cold shock and cold adaptation in *Pseudomonas fluorescens* HK44. Genomic mining was performed using the RAST online database to identify genes that may be used during cold shock and cold adaptation. After defining temperature growth ranges for *P. fluorescens* HK44, RNA was harvested from cells grown at 4˚C, 25˚C and cells shifted from 25˚C to 4˚C for 30 minutes during exponential growth to generate transcriptomic libraries. The Illumina High Seq was used to generate raw sequencing reads, before analysis was carried out in CLC Bio. Growth studies indicate that *P. fluorescens* HK44 is a psychrotroph with a growth range between 4˚C and 32˚C. Genomic mining of the HK44 genome identified multiple copies of the *cspA* gene, where phylogenetic analysis suggests the number of *cspA* gene copies present in a genome is indicative of the temperature classification of *Pseudomonas* species. Genes surrounding cold shock protein genes were investigated for their potential role in cold acclimation and indicate that cryoprotectants and/or membrane modifications may occur in response to cold stress in HK44. Transcriptomic data indicated that two *cspA* genes were differentially expressed; *cspA* 1 during the cold adapted vs cold shock experiment, and *cspA* 2 during the cold shock 1 experiment. Expression of the genes surrounding

cold shock protein genes indicate that cells respond to low temperature by regulating genes that may allow for a) the accumulation of metabolites that can be transformed into cryoprotectants and b) the production of alternative phospholipids to be incorporated into the membrane in order to increase fluidity.

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LIST OF ABBREVIATIONS

bp, base pairs

FDR, false discovery rate

GOLD, Genomic OnLine Database

HK44, Pseudomonas fluorescens HK44

LB, Luria Bertani

NCBI, National Center for Biotechnology Information

ND, not detected

PAH, polycyclic aromatic hydrocarbon

PCR, polymerase chain reaction

PGPR, plant growth promoting rhizobacteria

q-PCR, quantitative polymerase chain reaction

qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

RAST, Rapid Annotation using Subsystem Technology

RPKM, reads per kilobase per million sequenced reads

SNK, Student Newman Keuls

CHAPTER I: INTRODUCTION

Microorganisms are widespread in the environment and encounter stresses on a regular basis including nutrient availability, pH, osmolarity, and temperature. The ability of an organism to overcome temperature stress is important because an environmental setting often has rapidly fluctuating temperatures or stays at constant high or low temperatures for a significant period of time. For example, over 20% of the Earth's surface is frozen, and approximately 90% of the oceans experience temperatures at or near 5˚C year round [2]. Additionally, the National Oceanic and Atmospheric Administration reported the average ambient temperature during the 20^{th} century was ~14˚C [2, 3]. Considering that so much of the environment is exposed to low temperatures, elucidating mechanisms of cold acclimation and distinguishing the role of organisms capable of growing at low temperatures is important for several fields of study. Cold adapted organisms are important in environmental settings because of the large role bacteria play in nutrient cycling, plant interaction, and their potential for biotechnology applications, such as bioremediation. These organisms also play a role in the food industry due to the use of refrigeration and freezing as means for food preservation and the microbes that cause food spoilage at these temperatures.

While bacterial response to heat shock has been well studied, less is known about an organism's ability to adapt during cold shock and prolonged periods of exposure to a less than optimum growth temperature. Over the last 25 years, studies have been conducted to evaluate bacterial response to cold shock and cold adaptation. Cold shock is defined as the period of time immediately following a temperature drop of more than 10˚C, until the organism to reenters an exponential stage of growth. Cold adaptation refers to a prolonged period of exposure to low

temperatures, either continuous growth of an organism at low temperatures, or the period of resumed exponential growth after a cold shock, depending on the experiment [4, 5].

A decrease in the environmental temperature an organism inhabits can result in loss of membrane fluidity and inhibited transcription and translation, three major determinants in cell survival during cold acclimation [6, 7]. Cellular response to cold shock and cold adaptation is not limited to a specific temperature classification of organism. Mesophiles, psychrotrophs, and psychrophiles all experience cold shock and cold adaptation. Differences in cold acclimation between classifications are with respect to temperatures used to induce cold shock and adaptation. Mesophiles show optimum growth near 37˚C, suggesting that cold shock or adaptation begins to occur when the organism is exposed to temperatures in the mid 20˚C range. Psychrotrophs and psychrophiles are both capable of growing at 5˚C, but psychrotrophs have an optimum temperature generally near 25-30˚C and psychrophiles grow optimally near 10-15˚C. Therefore, cold shock for psychrotrophs and psychrophiles occurs near 15˚C and 0˚C, respectively. Most studies have focused on response to cold shock and cold adaptation in mesophilic organisms.

In this study, the environmental bioreporter, *Pseudomonas fluorescens* HK44, was evaluated in response to cold shock and continuous long-term cold adaptation. *P. fluorescens* HK44 was chosen as a model organism because it has been well characterized as a naphthalene bioreporter, has a sequenced and annotated genome, and is capable of growing at low temperatures [8, 9]. In environmental applications the decline of the organism over time was postulated to be due to competitive dominance by other organisms better suited to the environment, perhaps temperature [10]. Genomic analysis of *P. fluorescens* HK44 reveals that it is taxonomically representative of other *Pseudomonas fluorescens* strains and groups with other

psychrotrophic Pseudomonads. Herein, the expression levels of genes identified during genome mining as having potential for being expressed at low temperatures are explored in addition to non-predicted genes that are significantly expressed.

Role of *Pseudomonas fluorescens* in the Environment

Pseudomonas fluorescens are important environmental bacteria found in soil, water, and on plant surfaces. The rhizosphere is a zone of soil in which *P. fluorescens* plays a large role. First defined in 1904, the rhizosphere is the zone of soil surrounding plant roots that is inhabited by microorganisms; these microorganisms interact with each other and the plant roots based on the availability of nutrients present in the soil [11-13]. As rhizosphere studies became more prevalent, the definition has evolved to reflect the zones of interaction with plant roots to include the endorhizosphere, rhizoplane, and the ectorhizosphere. Within the rhizosphere there is a large population of prokaryotes and eukaryotes that interact with each other to maintain a homeostatic environment favorable for both communities to flourish.

Plant Growth Promoting Bacteria

The microbial community found within the rhizosphere is vast and diverse, with an estimated 10^9 microbes per gram of soil [14]. Plant-microbe interactions within the rhizosphere can result in positive or negative effects on the plant. Amongst the microbial population with positive effects on plants are a group of bacteria known as plant-growth-promoting rhizobacteria (PGPR), which can promote plant growth, alter soil chemistry and protect plants from pathogens. *Pseudomonas fluorescens* are plant-growth-promoting rhizobacteria and provide a variety of benefits, including increasing plant yields, altering nutrient uptake, and acting as biocontrol agents [15]. They act as biocontrol agents by different mechanisms, including the production of antimicrobials or antifungals, or they may alter nutrient availability [16-18]. Numerous studies

have shown a variety of antimicrobials or antifungals are produced by *P. fluorescens* strains that are effective in deterring pathogen colonization of plants, allowing plant production to continue [15-21]. *P. fluorescens* can also act as a biocontrol agent by limiting nutrient availability to eliminate harmful organisms. *P. fluorescens* can limit iron availability to pathogenic organisms through the production of siderophores, with limited availability of iron in the environment, pathogenic microbes or fungi cannot survive and therefore, cannot harm plants in the root vicinity [16, 18, 22-24]. Due to the biocontrol properties of *P. fluorescens*, plant root systems that are colonized by *P. fluorescens* have seen larger root systems, more plant biomass, and increased fruit production.

Hydrocarbon Utilization

Hydrocarbon contamination is widespread within the environment, and usually results from petroleum or byproducts spillage during production, transportation, or use. Unregulated, heavy environmental contamination occurred prior to the rules and regulations set forth by U.S. Environmental Protection Agency. The Resource Conservation and Recovery Act of 1976 addresses the generation, storage and use of hazardous materials, and was amended in 1986 to address the problems arising from underground storage of petroleum and other hazards. Once a hydrocarbon is introduced to the environment, the length of time it remains in the environment is determined by an array of factors, including the ability of the compound to be volatilized, photooxidized, chemically oxidized, or absorbed by the soil [25]. In order to expedite the removal of these harmful chemicals, some microorganisms can be used to metabolize hydrocarbons and reduce them to usable carbon forms. Both naturally occurring and genetically modified organisms can be used for hydrocarbon removal.

Several strains of *Pseudomonas fluorescens* have been shown to degrade a variety of hydrocarbons found in the environment. Many of these isolates have come from soil samples contaminated by hydrocarbons and are able to degrade hydrocarbons such as naphthalene, pyrene, ethylbenzene and phenanthrene [26-29]. Other strains of *P. fluorescens* have been genetically modified to degrade hydrocarbons or enhance their capability of degradation [30, 31]. In addition, the plasmids that carry degradation pathways found in Pseudomonads, such as *P. putida,* have been extensively studied and used to transform other bacteria in order to confer hydrocarbon degradation to organisms [32-34].

***Pseudomonas fluorescens* HK44**

The role *Pseudomonas fluorescens* plays in hydrocarbon degradation and promoting plant growth makes these bacteria excellent candidates to develop into bioreporters. A bioreporter is a living microorganism engineered to produce a signal in response to a stimulant. A well-studied bioreporter is *P. fluorescens* strain HK44, which produces bioluminescent light in response to chemical stimulants, and can serve as a model organism for evaluating phenotypic response to environmental factors.

Pseudomonas fluorescens HK44 is a Gram negative rhizobacterium, originally isolated from hydrocarbon contaminated soil, then later engineered with a *luxCDABE* cassette to function as a bioreporter for hydrocarbon availability and degradation. It is one of the most evaluated bioreporters that has been used for the detection and degradation of bioavailable naphthalene, substituted naphthalenes, and salicylate [35]. Due to extensive background information regarding the HK44 strain, including construction, detection limits, long-term environmental field applications, and genome sequence, *P. fluorescens* HK44 is an excellent model organism to use to evaluate the affects of low temperature on transcriptional activity. Furthermore, in order to

evaluate the effectiveness of HK44 as a bioreporter in an environmental setting at a temperature less than the optimum growth temperature, it is necessary to elucidate mechanisms that are utilized during cold shock and cold adaptation.

Construction and Naphthalene Detection

Pseudomonas fluorescens HK44 was constructed from a parent strain isolated from hydrocarbon-contaminated soil surrounding a manufactured gas plant. *P. fluorescens* HK44 harbors the pUTK21 plasmid containing the upper naphthalene pathway (*nahABCDEF*), the regulator gene *nahR*, and the lower pathway gene *nahG* fused to the *luxCDABE* cassette from *Vibrio fischeri* [30]. King et al. [30] demonstrated light response to naphthalene at a concentration of 200 µg/L after 15 minutes in a chemostat culture. *P. fluorescens* HK44 has been exposed to a variety of pure chemical compounds and has been tested on environmental samples ranging from soil to water. A thorough review of the applications of HK44 as a bioreporter has been reported by Trögl et al. [8].

Environmental Field Study

A unique aspect of the *P. fluorescens* HK44 bioreporter is that it was used in a contained field release study spanning almost two decades. In 1996, *P. fluorescens* HK44 was released in lysimeters constructed of large galvanized steel pipes filled with soil and rock layers similar to those found in East Tennessee in order to monitor the ability of a genetically modified organism to detect and degrade bioavailable naphthalene *in situ* [36]. The lysimeter facility was monitored for two years after the release of *P. fluorescens* HK44. Over the two year time span, regular sampling was performed to track the amount of HK44 present in the soil, as well as the amount of bioluminescence produced, which was indicative of naphthalene concentration and the presence of actively degrading organisms present. *P. fluorescens* HK44 persisted in both PAH

contaminated soils and non-PAH contaminated soils and decayed at similar rates as indicated by culture-based methods [36, 37]. Bioluminescence was also successfully detected after supplemental PAHs were added to all lysimeters on day 135 [36]. In another experiment to measure HK44 presence in the lysimeter soil, Ripp et al. [10] compared selective plate counts confirmed by colony hybridization to a bioluminescent most-probable-number assay. Findings showed that plate counts overestimated the number of HK44 colonies from PAH contaminated soil by 50%, as confirmed by lack of hybridization to a *luxA* probe. Bioluminescent response from the *lux*-based most-probable-number assay predicted a more accurate depiction of HK44 presence.

After 12 years of dormancy, the lysimeters were revisited in 2010 with the hopes of finding the reporter still present in the soil. While the strain was not culturable from the samples taken in 2010, metagenomic and quantitative PCR analysis of soil samples identified signature genes of *P. fluorescens* HK44 [1]. In addition to determining the persistence of *P. fluorescens* HK44 in this study, two metagenomic profiles of lysimeter soil were evaluated for population diversity and abundance. When soil samples were first harvested in 2010, DNA was extracted and pooled from lysimeter 2 to generate a metagenomic library. Analysis of 16S rRNA genes identified 95% of sequences belonged to Burkholderia and <0.1% of sequences belonged to *Pseudomonas* [1]. Soil samples were stored for 1 year at 4˚C before another metagenomic library was generated using DNA extracted from the same lysimeter soils. The number of sequences representing *Burkholderia* decreased to 2.6%, where the sequences representing *Pseudomonas* increased to 18.7% [1]. This observation gave preliminary evidence that environmental *Pseudomonas* were capable of dealing with cold stress.

The field release of *P. fluorescens* HK44 was successfully showed the survival of a genetically modified organism in an environmental setting over the course of several years with temperature fluctuations. Bioluminescent detection on site demonstrated *P. fluorescens* HK44 could be used *in situ* for near real time detection of naphthalene. During the field study, temperatures were not reported at sampling points; therefore, temperature stress cannot be correlated with the organism's ability to perform.

HK44 Genome

In 2010, the genome of *P. fluorescens* HK44 was sequenced using Roche 454 GS FLX Titanium system [9]. From this sequencing data it was determined that *P. fluorescens* HK44 is 6.1Mbp in size and has a GC content of 58.73%. The annotated genome has been uploaded to NCBI (National Center for Biotechnology Information) under the accession number NZ_AFOY00000000.2 (contigs AF0Y02000001 – AF0Y02000032) and the RAST (Rapid Annotation using Subsystem Technology) server. Comparison between *P. fluorescens* HK44 and other *P. fluorescens* strains reveals that 30% of the genes found in strain HK44 are unique to this strain [9]. The availability of annotated sequences allows for genome mining, which is useful for inferring phenotypic characteristics, including identification of genes that may suggest whether an organism is mesophilic, psychrophilic, or psychrotrophic.

Bacterial Physiological Classification

Psychrophiles, Psychrotrophs, and Mesophiles

Many stresses in the environment can affect the bacterial population; temperature is one such stress that affects the function and survival of bacteria. While extensive research has been completed on evaluating the effects of warming on bacteria, a more recent endeavor has been undertaken to understand bacterial response to cooling. A large portion of the earth's climate is

subject to low temperatures for extended periods of time or constantly throughout the year. Some bacteria grow optimally at low temperatures, while others are capable of growing at low temperatures, but are optimally grown at higher temperatures. The term psychrophile was first used in 1902 to describe any bacterium that was capable of growing at low temperatures, however as more organisms capable of growing at low temperatures were discovered, the definition of the word needed modification. Eddy [38] suggested that in order for a bacterium to be classified as a psychrophile, it must optimally grow at a low temperature, 10-15˚C. Psychrophiles are also unable to survive temperatures above 20˚C [39]. Bacteria classified as psychrotrophs are capable of growing at low temperatures, at or below 5˚C, but grow optimally at a higher temperature [38]. Expanding the terminology helps to more accurately classify bacteria and their growth capability across a range of temperatures. Mesophiles prefer growing at warmer temperatures, and optimally near 37˚C, where most mesophiles are not able to withstand temperatures lower than 10˚C. Because mesophiles are more frequently studied, initial findings related to cold acclimation were identified in mesophilic organisms, such as *E. coli.* However, much less is known about cold acclimation in organisms that preferentially grow at lower temperatures.

Cold Shock and Cold Adaptation

All organisms can experience shifts in temperature that require modifications within the cell to overcome the stress caused by temperature change. Therefore, cold shock and cold adaptation are not discussed based on defined temperatures, but rather periods of time after a temperature shift occurs. Cold shock and cold adaptation are not limited to psychrophiles and psychrotrophs.

Cold Shock vs. Cold Adapted

When a microorganism is exposed to a temperature lower than its optimum growth temperature, it must adapt to the new environment to survive. Experimentally, two terms are used to describe the period of time during which an organism is acclimating to a lower temperature. Cold shock is most often used to describe the response of an organism during the period of time immediately following a temperature decrease of 10 or more degrees Celsius [40]. However, there is no standardized length of time that defines a period of cold shock and various studies have considered organisms to be in a state of cold shock from minutes to hours after exposing the organism to a lower than optimum temperature [41-43]. Thus, cold shock is more appropriately characterized by cellular function, a lag in growth and significant changes in transcriptionally active genes are typically seen.

Whereas cold shock is the initial introduction of an organism to lower than optimum temperatures, cold adapted organisms have been held at a low temperature for an extended period of time. These organisms are generally capable of growing at a rate that is slightly lower than the growth rate seen at the optimum growth temperature. Cold adapted organisms may have normalized transcriptionally active genes that are required for growth at low temperatures, and thus may not show as significant changes in fold induction as seen in cold shock. Like organisms undergoing cold shock, there is not a defined length of time that can be used to describe an organism classified as cold adapted. Studies have classified organisms to be cold adapted after the lag time seen during cold shock and during continuous growth at low temperatures. Understanding cold adaptation is of great importance because most psychrotrophic environmental organisms introduced to low temperatures (~5˚C or lower) will experience these temperatures for extended lengths of time, and thus become cold adapted.

Bacterial Response to Low Temperatures

Bacteria must employ a host of genes in response to low temperatures to overcome cellular limitations, such as loss of membrane fluidity, inefficient transcription and translation due to stabilized secondary formation of nucleic acids, and inefficient folding of proteins. Mesophilic organisms were first used to investigate bacterial response to temperature downshifts. These studies involving cellular responses to temperature downshifts are discussed below.

Major Cold Shock Proteins

The first major cold shock protein was identified in *Escherichia coli* after a temperature shift from 37˚C to 10˚C or 15˚C [43]. Upon shifting *E. coli* cells to a lower temperature, Goldstein et al. [43] noticed an induced set of genes including *cspA*, encoding a 7.4 kDa protein that was undetectable at 37˚C, but accounted for 13% of the total protein synthesized within one hour of temperature shift. High levels of CspA occurred within 30 minutes of the temperature shift and returned to baseline levels after 1.5 hours [43]. CspA is composed of five anti-parallel β strands that form a β barrel and contains two highly conserved binding domains, RNP-1 and RNP-2 [40]. CspA is 43% homologous to the eukaryotic Y-box protein family that is known to contain a cold shock domain [44]. The Y-box protein interacts with mRNA and is involved in the regulation of transcription and translation [45]. Jiang et al. [44] showed that CspA is capable of binding to ssRNA of at least 74 bases in length and has low sequence specificity. CspA also acts as an RNA chaperone causing destabilization of secondary RNA structures. The destabilization of secondary structures allows for the cell to overcome the stress from low temperatures and continue transcription. Bae et al. [46] showed that when CspA is added to an immobilized transcription complex consisting of DNA with a promoter followed by a rho-independent terminator at bp 105, and stalled at bp 20, read through efficiency increased, therefore, CspA acts

as a transcription anti-terminator. Increased read through efficiency indicates that CspA is able to negate the effects of the terminator and allow for transcription to continue. Due to its ability to reduce transcription termination, it is suggested that CspA may explain the increased expression of *nusA, infB,* and *rbfA*, among other genes seen during cellular cold shock [40, 46]

Since *cspA* was first identified in *E. coli*, eight additional homologous cold shock proteins have been identified in *E. coli, cspB- cspI* [6, 40, 47]. Along with CspA, genes encoding CspB, CspG, and CspI are cold inducible [40]. The *cspB* gene from *Bacillus subtilis* has over 60% homology to the *cspA* gene of *E. coli* and reportedly increased levels of expression was seen after cultures were shifted to lower temperatures from 37˚C [48]. Lopez et al. [49] showed that CspB acts as an RNA chaperone, similar to CspA described in *E. coli.* Cold shock genes *cspC* and *cspE* are constitutively expressed at 37˚C, where *cspD* is induced during stationary phase and glucose starvation in *E. coli* [40]. CspC and CspE also act as transcription anti-terminators [46], and *cspE* has some overlapping properties to *cspA*, as shown in a quadruple deletion mutant where *cspE* is over expressed in response to cold shock [40]. The cellular functions of *cspF* and *cspH* are still unknown.

Cold Acclimated Proteins

Similar in sequence and function to cold shock proteins, cold acclimation proteins are a group of nucleic acid binding proteins that destabilize secondary DNA and RNA structures at low temperatures. The genes encoding cold acclimation proteins are homologous to the *cspA* gene that is transiently expressed in organisms undergoing cold shock. Unlike the cold shock proteins, the cold acclimation protein genes in psychrotrophic and psychrophilic bacteria are continuously expressed throughout low temperature incubations. These homologous genes encode cold acclimation proteins, or Caps [50, 51]. Cold acclimation protein expression is more

stable than cold shock proteins, which are highly expressed at the point of temperature shift before their expression returns to a pre cold shock state. Michel et al. [50] showed that there is 60% homology between the CspA protein of *E. coli* and the cold acclimation proteins of *Pseudomonas fragi.* In fact, the binding motifs RNP-1 and RNP-2 that are found in CspA are almost identical in both organisms, with a single amino acid change from valine to leucine in RNP-2. Due to the similarities between Caps and Csps, it is suggested that they have similar function [50, 51].

Membrane Modification

A decrease in temperature affects the fluidity of bacterial membranes in a negative manner. Low temperatures often cause membranes to lose fluidity by altering the arrangement of fatty acid chains, forming a more crystalline structure [52]. In order to overcome membrane rigidity at low temperatures bacteria modify their membranes, most frequently by increasing the ratio of unsaturated fatty acids or decreasing fatty acid chain length [53]. Several early studies examined the membrane composition of *P. fluorescens* grown in low and moderate temperatures. Farrell and Rose [54] compared mesophilic pseudomonad *P. aeruginosa* to a "psychrophilic" Pseudomonad grown at 30˚C and 10˚C. Their results indicated that the fatty acids differed between mesophilic and psychrophilic Pseudomonads, with the psychrophilic Pseudomonad containing a larger proportion of unsaturated fatty acids at both 30˚C and 10˚C than the mesophilic Pseudomonad. However, there was an increase in the percent of unsaturated fatty acids present when both Pseudomonads were grown at 10˚C compared to 30˚C [54]. In the same study, Farrell and Rose showed that organisms grown at 10˚C had an increase in shorter chain fatty acids not seen when grown at 30˚C. In another study of psychrotrophic *Pseudomonas* species isolated from marine sediments, there were no significant changes in the fatty acid

composition or the amount of saturated and unsaturated fatty acids grown between 20˚C and 0 ˚C [55]. In a study of four marine isolated Pseudomonads, temperature change did not cause significant changes in the fatty acid composition [52]. Differences may have been more noticeable in the Farrell and Rose study because of the side-by-side comparison of mesophilic and psychrotrophic Pseudomonads. However, results from the other studies indicate that psychrotrophic bacteria may not need to significantly alter their membranes in response to temperature change.

Modifying fatty acids in the membrane can be done either by synthesizing new fatty acids or by altering fatty acids already in place in the membrane. In *Bacillus subtilis*, the latter is carried out by a desaturase gene *des* [7]. Fatty acids within the membrane are modified with double bonds to create unsaturated fatty acids. Due to the space created by the double bond, the bacterial membrane becomes more fluid and allows for cellular survival at low temperatures. Alternatively to altering preexisting fatty acids within the membrane, an organism alter fatty acid biosynthesis to increase fluidity, such is the case for *E. coli*. Lipopolysaccharide is a component of the outer membrane of Gram negative bacteria, that consists of lipid A, the core sugar region, and the O-antigen [56]. Under normal growth conditions for *E. coli* (37˚C), saturated fatty acids are attached to lipid A; however, when *E. coli* is stressed with low temperatures, unsaturated fatty acids take the place of saturated fatty acids. Carty et al. [56] identified an *lpxP* gene encoding palmitoleoyl acyltransferase inducible at 12˚C. When *lpxP* was induced, an unsaturated fatty acid, palmitoleate, was preferentially attached to the lipid A precursor, Kdo_2 lipid $IV_{A,}$ in place of laurate [56]. .Similar to *E. coli*, *Leigonella pnuemophila* contains a homologous *lpxP* gene, responsible for altering fatty acid biosynthesis at low temperatures [57].

Cryoprotectants and Anti-freeze Proteins

Psychrophiles and psychrotrophs are found at temperatures where water is at or near its freezing point. If a bacterial cell does not have a way to utilize water at low temperatures or prevent water present in the cell from freezing, cell death may occur. Therefore, psychrophiles and psychrotrophs are able to produce compounds or proteins that keep the bacterial cell from freezing, such as cryoprotectants and anti-freeze proteins. Cryoprotectants are chemical compounds that are produced or imported into the cell in response to environmental stress and have been postulated to prevent protein denaturation, protein aggregation, and maintain membrane fluidity [6, 58, 59]. Some common prokaryotic cryoprotectants include glycerol, trehalose, glycine betaine, and sorbitol [58]. Extracellular polysaccharides (EPS) are suggested to play a role as a cryoprotectant. The presence of EPS is known to cause biofilm formation and as a result allow bacterial adherence to surfaces. The aggregation of cells and EPS also allows for retention of water, protection against extracellular enzyme degradation, and offers potential nutrient storage [60]. While some bacteria are capable of producing cryoprotectants, others produce ice nucleating or anti-freeze proteins. Anti-freeze proteins inhibit ice crystal growth and recrystalization by creating an unfavorable state for water to turn to ice, which in turn causes the freezing temperature of water to decrease [61]. Decreasing the temperature at which water freezes allows for the cell to remain fluid and access the water in its surrounding. Whereas antifreeze proteins decrease the freezing point of water to limit ice formation, ice nucleation proteins create ice crystals above the freezing point of water [62]. Increasing the temperature at which ice forms may also limit damage that can occur at lower temperatures.

Psychrophilic Enzymes

As temperatures decrease, the activity of enzymes generally decreases as well. Without the appropriate enzymatic activity, cellular processes cannot occur. In order to overcome decreased enzymatic activity, psychrophilic organisms produce enzymes that are less temperature dependent [63]. Enzymes produced in psychrophilic organisms are highly active, up to an order of magnitude greater than comparable enzymes in mesophiles. This activity is attributed to the increased flexibility of the enzyme from an increase in glycine clusters and decrease in proline and arginine, that allows for low energy binding of the substrate [39, 63]. Maintaining cellular function at low energy costs is important for low temperature survival and means energy can be spent on more critical cellular processes.

Whole Cell Response to Cold Acclimation

Several studies have evaluated targeted mechanisms for adapting to the cold. However, examining complete genomic and transcriptomic profiles of organisms that adapt to lower than optimum growth temperatures may be used to elucidate the whole cell response during cold shock or cold adaptation. As technology has advanced, a more in depth look at an organism's transcriptomic profile after cold shock or cold adaptation may identify other genes being induced or repressed in parallel to the cold shock proteins that have been documented. Several different techniques have been employed to evaluate transcriptomic responses to cold acclimation including DNA microarrays, continuous monitoring via bioluminescent gene fusions, and RNA-seq of mRNA transcripts.

DNA Microarray Transcriptomic Analysis

Polissi et al. [64] evaluated the changes of the *E. coli* transcriptome during cold shock and after cold adaptation. To evaluate gene expression levels, a high density DNA microarray

was used that contained probes for all of the known open reading frames in *E. coli*. Using this technique, Polissi et al. [64] was able to identify 20 genes with a 2-fold or greater change in transcript abundance when *E. coli* was transferred from 37°C to 16°C. Genes that were induced upon shift from 37°C to 16°C included *cspA, cspB, cspG,* and *cspI,* which were previously identified as cold inducible. In addition to the four induced cold shock protein genes, eight genes not previously associated with cold shock or cold adaptation were induced. These genes included four genes induced at initial cold shock, *rhlE, rpoE, rseA,* and *sfa* [64]. The RhlE protein encodes the DEAD box ATP-dependent RNA helicase, RpoE and RseA proteins are involved in stress response associated with misfolded proteins in the periplasm or outer membrane, and the function of the Sfa protein is unknown. Two other genes identified were permanently expressed at low temperatures, where one gene, *proX,* is involved in glycine betaine import, the other gene, *ycgF,* has unknown protein function. Glycine betaine is a cryoprotectant that can be used to maintain membrane fluidity and prevent protein degradation. The final two genes that were identified in the microarray as being induced at low temperatures have unknown function, but were seen in the late stages of cold acclimation. In addition to identifying new genes associated with cold adaptation, this study shows evidence that PNPase is an important regulator of cold shock proteins. PNPase is part of the RNA degradosome and in important for the maturation of stable RNA and degradation of mRNA [65]. The rate at which PNPase degrades mRNA under environmental conditions effects gene expression [66]. During transcriptomic analysis of the *E. coli* mutant lacking the *pnp* gene encoding PNPase, overexpression of the cold shock protein genes continued past the initial cold shock phase and lasted well into the cold adapted phase of growth [64]. While this study did identify a role for PNPase in cold acclimation, several known cold induced genes were not identified, suggesting that this method of transcriptomic analysis is

not sensitive enough to provide a clear picture of the transcriptomic profile during cold shock and cold adaptation.

Continuous Transcriptomic Analysis

Yersinia enterolitica is a Gram-negative bacterium that is pathogenic in humans, causing fever and diarrhea [67]. Assessing the transcriptional response of this organism may provide insight to new environmental reservoirs that harbor pathogenic *Y. enterolitica.* The transcriptional response of *Yersinia enterolitica* was continuously evaluated over long term exposure to low temperature using transposon mutants with *luxCDABE* promoter fusions [5]. A transposon library consisting of 5700 mutants were screened for a greater than five-fold change in gene expression, of which 109 were identified, and 42 genes were identified with changes in promoter activity. There were 37 genes upregulated and 5 genes downregulated at 10°C compared to 37°C [5]. Analysis of genes was divided into three groups based on time of expression and further grouped into categories based on gene function including regulation, motility/chemotaxis, virulence, and metabolism. As expected, cold shock protein genes were expressed during the acclimation phase of growth. Also identified during the acclimation phase was a gene known to be a thermoregulator of virulence factors, *ymoA* [5]. The majority of genes with increased expression at 10˚C were identified during early to mid exponential growth. The most marked increase in expression during exponential growth was response of genes involved in motility and chemotaxis, including *cheA* and the flagellin operon. Motility genes have been linked to virulence and allow for colonization of new habitats at low temperatures. Other genes induced during exponential growth included genes homologous to virulence genes *tcaA* and *tcaB*, insecticides of *Photorhabadus luminescens,* and a homologous gene involved in magnesium uptake found in *Salmonella* and associated with virulence [5]. During late

exponential and early stationary growth, several genes involved in metabolism were upregulated, including genes for nitrogen and glycogen utilization.

RNA-seq Transcriptomic Analysis

Mesophilic *Pseudomonas putida* KT2440 is known to thrive in the environment as a saprophyte and has been used for biotechnology purposes due to its metabolic diversity [68]. Transcriptional analysis of *P. putida* KT2440 was performed to determine the whole genome response to cold adaptation when shifted from 37°C to 10°C; cells were held at 10˚C for 2 hours before RNA was harvested [4]. RNA-seq analysis showed 1478 genes were upregulated during cold adaptation and 859 genes were down regulated. The genes with the greatest fold of induction at 10°C were hypothetical proteins. Of the annotated genes that were upregulated, the most abundant genes were involved in transcription and translation efficiency, including the *rbfA-nusA-infB* operon that has been shown in *E. coli* to be upregulated during cold stress [4]. Other genes upregulated are affiliated with membrane fluidity. The *bkd* operon is known to increase branch chained fatty acids that allow for membranes to remain fluid at low temperatures [4]. Not surprisingly, and similar to what was found in *Y. enterolitica,* genes that were most downregulated at 10°C were heat shock genes. The use of RNA-seq provides a more in depth look at whole cell response to cold acclimation than DNA arrays, however due to the large proportion of proteins that are indicated as hypothetical and have no proven function, complete cellular response to cold acclimation remains unknown.

One of the most recent studies evaluating cold shock response using RNA-seq analysis was carried out by Spaniol et al. [41] on mesophilic *Moraxella catarrhalis*, a human pathogen that colonizes the mucosal surface in the nose. Identifying genes upregulated during cold shock is important because disease caused by *M. catarrhalis* has been shown to increase during cold

months [69]. In order to evaluate the cold shock response of *M. catarrhalis*, cells were grown to mid exponential phase at 37˚C before being shifted to 26˚C and incubated for an additional three hours prior to RNA extraction [41]. Transcriptomic analysis revealed that the transcriptional activities of many genes were significantly altered in response to cold shock. Unlike most cold shock studies, Spaniol et al. [41] only identified a moderate increase (1.4 fold) in the transcript levels of *cspA* at 26˚C compared to 37˚C. This may be due to the length of time *M. catarrhalis* was exposed to 26˚C before cells were harvested for RNA extraction, as *cspA* is reported to have the highest level of expression within 1.5 hours of exposure to low temperature in *E. coli* [43]. However, other genes involved in transcription and translation were upregulated including those previously described, such as *rpoA, nusA, infB,* and *rho.* Genes involved in membrane modification were upregulated at 26˚C including *lpxB* and *lpxX*, which are involved in the synthesis of unsaturated fatty acids. Gene products that may play a role in virulence were upregulated at 26˚C include type IV pili genes involved in adhesion, biofilm formation, and motility. In addition genes encoding transporters and binding proteins that allow for nutrient acquisition, including iron, nitrogen, and phosphate were upregulated [41]. The increases in expression of genes associated with virulence factors may elucidate some of the mechanisms allowing for a heightened number of illnesses due to *M. catarrhalis* during cold months.

As technology continues to advance, more organisms have sequenced genomes and "omics" studies are becoming more popular. This provides the ability to evaluate global cellular response to environmental stress. Comparative genomic and transcriptomic studies may elucidate cold adaptation and cold shock response and identify functions for the large number of hypothetical proteins involved in cold acclimation.

CHAPTER II: SIGNIFICANCE AND RESEARCH OBJECTIVES

Evaluation of cold shock and cold adaptation of mesophilic microorganisms is documented in the literature, and suggest mechanisms that allow for acclimation during cold shock and cold adaptation. However, studies evaluating cold acclimation of psychrotrophic bacteria are less common. In addition, many of the cold shock and/or cold adaptation studies examine a limited number of genes, and fewer studies compare genes involved in cold shock and cold adaptation. Here, the temperature classification of *Pseudomonas fluorescens* HK44 will be deterimined before evaluating the genomic profile to identify genes present in the genome that may be involved in cold shock and cold adaptation. In addition, transcriptomic profiles for cells exposed to cold shock and cold adaptation will be generated to identify genes with significantly changed levels of expression in response to cold shock and/or cold adaptation. This study evaluates the mRNA transcripts 30 minutes after shifting a mid-exponential culture from 25˚C to 4˚C to mimic cold shock, and transcripts from cultures that have been grown continuously at 4˚C until mid exponential phase is reached. These results may help elucidate how *P. fluorescens* HK44 adapts to the environment at low temperatures and will further evaluate its potential for use as a bioreporter. Results of this study will be used to address the hypotheses:

H_1: *Pseudomonas fluorescens* HK44 is a pyschrotroph.

H_2: There are unique genes in the *P. fluorescens* HK44 genome responsible for psychrotrophic phenotypes.

$H_{3:}$ The *cspA* and surrounding gnese will be differentially expressed during low temperature conditions.

H_4: Genes involved in the production of cryoprotectants and/or membrane modification will be expressed in response to cold shock and/or cold adaptation.

CHAPTER III: MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Pseudomonas fluorescens HK44 (HK44) was the model organism used for these studies. Fresh cultures of HK44 were started from an -80˚C freezer stock in 100 mL of Luria Bertani (LB) broth with 12.5 µg/mL tetracycline at 28˚C, shaking at 200 rpm overnight. To grow cells for transcriptomic assays, overnight cultures were used to start 100 mL cultures in LB broth with 12.5 µg/mL tetracycline at an OD_{600} ~ 0.01 and incubated at either 25˚C or 4˚C, shaking at 200 rpm. All 100 mL cultures were grown in 250 mL flasks to provide adequate aeration.

Growth Kinetics

To determine the range of temperatures *P. fluorescens* HK44 is capable of growing, two growth methods were implemented to calculate growth rates: microplate assays and small batch culture.

Microplate Method

Microplate assay growth curves were generated using, clear, 12 well polypropylene microplates in the Synergy2 (Biotek). Each plate was loaded with 3 wells of LB broth containing tetracycline (control) and 3 wells of an overnight HK44 culture diluted to an OD_{600} ~ 0.01. For temperatures of 20˚C and above, the Synergy2 was left at room temperature and the internal temperature of the instrument was controlled with the software. The Synergy 2 cannot reduce internal temperatures, therefore the instrument was moved to a temperature-controlled room to evaluate growth at temperatures below 20˚C. Furthermore, the lowest temperature at which the Synergy would function was 10˚C. Microplates were loaded into the Synergy 2. Plates were programmed to shake and the OD_{600} was read every 30 minutes until the growth curve was

determined to be complete (OD_{600} ~ 1.0). Growth rates were calculated for the following temperatures: 10˚C, 15˚C, 20˚C, 28˚C, 30˚C and 32˚C.

Small Batch Culture Method

Small batch cultures were used to generate a second compilation of growth data. Small batch cultures contained 100 mL cultures grown in LB broth supplemented with 12.5 mg/mL tetracycline in 250 mL Erlenmeyer flasks. Cultures were shaking at 200 rpm. As in the microplate growth curves, cultures were started at an OD_{600} ~0.01. The OD was measured every 30 minutes using 1 mL aliquots for cultures grown at 25˚C, and every 4 hours at 4˚C. Due to the amount of time it took to complete the 4˚C growth curve, multiple sets of cultures were used to avoid depleting the culture volumes to a point that would cause a change in growth rate.

RNA Extraction Methods

Three RNA extraction methods, including the FastRNA Pro Soil Direct (MP Biomedicals), RNeasy Mini Kit (QIAGEN), and a hot phenol method previously described[70, 71], were compared (Table A1). Extraction efficiency was measured based on quantification and purity determined by Nanodrop 2000 readings and agarose gel imaging.

FastRNA Pro Soil Direct

The FastRNA Pro Soil Direct kit protocol was modified slightly from the manufacturer's recommendations to accommodate pure culture extractions. This kit is optimized for soil samples, therefore it was determined 1.5 mL of culture should be pelleted in a 1.5 mL centrifuge tube at max speed (14,000 rpm) for 1 min. The supernatant was discarded and the pellet was resuspended in 1.0 mL Lysis Buffer provided in the kit. The mixture was then transferred to the Lysing Matrix E tube provided in the kit and extractions proceeded per the manufacturer's

protocol. Total RNA was divided into two aliquots and stored at -80˚C. One for initial examination of extraction efficiency and the other was kept frozen for downstream applications.

RNeasy Mini Kit

The QIAGEN RNeasy mini kit was used according to the manufacturer's protocol for enzymatic cell lysis. The enzymatic protocol (Protocol 1) was used for cell lysis, followed by purification of total RNA using Protocol 7. The protocol was altered for the combination of enzymatic and mechanical cell lysis. Lysing Matrix E tubes (MP Biomedicals) were used in lieu of the suggested 100-600 µm diameter glass beads. Mechanical disruption was performed on the Fast Prep 24 instrument (MP Biomedicals). After mechanical disruption, Protocols 1 followed by 7 was resumed. Total RNA extracted was divided as described above and stored at -80˚C.

Hot Phenol Extraction

Modifications were made to the hot phenol extraction method previously published by Fozo et al. [71]. At the desired OD_{600} 10 mL of culture was harvested in 50 mL conical tubes and centrifuged at 4700 rpm for 5 minutes at either 25˚C or 4˚C, depending on the temperature the sample was grown. The supernatant was discarded and 600 µL of Solution GP (50mM Tris-HCl, 10mM EDTA, 1% SDS, 30mM Sodium Acetate) was added to the cells. This mixture was transferred to Lysing Matrix E tubes containing 650 µl phenol:chloroform pH 7.9 and mechanical disruption was performed with a bead beater at 4˚C for 2 minutes followed by icing for 2 minutes followed by centrifugation at 10000 rpm for 10 minutes at 4˚C. The supernatant was transferred to 500 µL acid phenol:chloroform pH 4.5 preheated to 65˚C, vortexed and incubated at 65˚C for 10 minutes. The sample was centrifuged at 13000 rpm for 10 minutes before supernatant was transferred to 400 µl phenol:chloroform pH 7.9, vortexed and centrifuged at 13000 rpm for 5 minutes. The supernatant was transferred to 400 µl chloroform and

centrifuged at 13000 rpm for 5 minutes. To precipitate total RNA, supernatant was transferred to 400 µl 99% ethanol and incubated overnight at -80˚C, followed by centrifugation at 4˚C for 20 minutes at max speed. RNA was washed with 70% ethanol and again centrifuged at 4˚C for 20 minutes at max speed. Pellets were allowed to air dry, then resuspended in 100 µl RNase free water. Samples were divided into two aliquots and stored at -80˚C.

Genomics

RAST

The *Pseudomonas fluorescens* HK44 genome was previously sequenced [9] and uploaded to the RAST (Rapid Annotation using Subsystems Technology) server for annotation. The HK44 genome was investigated and compared to other sequenced and available genomes using the RAST server. Target genes were identified by evaluating function tables in RAST and corresponding protein sequences were collected.

Phylogenetic Analysis

Phylogenetic analysis was completed using an online database, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA 5.2.2 software [72]. Protein sequences downloaded from the RAST server were uploaded to an alignment program and aligned by the ClustalW algorithm (MEGA) or Clustal Omega algorithm (Clustal Omega). Using the protein alignments, maximum likelihood trees were generated based on the percent similarities calculated.

Transcriptomics

Sample Collection

To evaluate gene expression of acclimated *P. fluorescens* HK44 cells at 4˚C and 25˚C, cells grown in biological triplicate were harvested during the lag, exponential, and stationary

phases of growth (Figure 1a) at approximately equal OD_{600} for each growth phase between the two temperatures (Table A2). Cells were harvested and immediately underwent RNA extractions using the hot phenol extraction protocol to eliminate any change in gene expression that may occur from storage at -80˚C. The cells harvested during exponential growth at 4˚C were labeled TA4 A, TA4 B, and TA4 C, and serve as the cold adapted samples. In order to evaluate gene expression during cold shock, cells were grown at 25˚C until mid exponential phase, upon which three aliquots were harvested per temperature regime (Figure 1b). One aliquot was immediately sacrificed for RNA extraction and serve as the 25˚C adapted cells (TA25 A, TA25 B, and TA25 C). The second aliquot was shifted to 4˚C for 30 minutes before RNA was extracted, and serve as the cold shock samples (S4 A, S4 B, and S4 C). The third sample was kept at 25˚C for 30 minutes to serve as a 25˚C shock control (S25 A, S25 B, and S25 C). After the total RNA was extracted, it was treated with Turbo DNase (Ambion) according to the manufacturer's protocol to remove any DNA contamination. The quality of RNA was checked after DNase treatment with an Agilent RNA Bioanalyzer kit.

Library Preparation

Transcriptomic libraries were prepared for 12 of the samples harvested as shown inTable 1. The process for library preparation involved several steps, including rRNA depletion, mRNA enrichment, fragmentation, cDNA synthesis, adapter ligation, and library enrichment.

In order to achieve rRNA depletion and mRNA enrichment, the MICROBExpress mRNA enrichment kit (Life Technologies) was used according to the provided protocol. The enriched mRNA was checked for quality using the Agilent RNA Bioanalyzer kit. Once the mRNA quality was sufficient for downstream use, library preparation continued using the Illumina TruSeq RNA Preparation Version 3 chemistry. The Illumina TruSeq RNA Sample Preparation version 2 guide

was followed starting from the fragmentation step (step 12 of the "Make RFP" directions in the "Purify and Fragment mRNA" section). The only modification to the protocol was exchanging the AMPure XP magnetic beads for SPRI magnetic beads; the rest of the protocol was followed as provided. Briefly, enriched mRNA was fragmented to approximately 200 bp in size before constructing the first strand of cDNA (ss cDNA). Double stranded cDNA is then generated from the ss cDNA. In order to prepare the ds cDNA for the addition of indexes, hanging ends from the fragments are converted to blunt ends and the 3' end is adenylated to prevent the fragments from ligating to each other downstream. Index adapters are then ligated to the fragments. Each sample has a unique index (Table 2) so that sequences can be separated during analysis. Once index adapters have been added, the sequences are enriched to increase copy numbers within the library; however, the number of PCR cycles is limited in order to avoid skewing the number of transcripts that are present due to the original sample treatment.

Library Sequencing

The libraries generated for the 12 samples were checked for quality and quantified before being sequenced using the Illumina HiSeq 2000. Quality of the libraries was assessed with the Agilent Bioanalyzer DNA assay. Libraries were quantified using KAPA qPCR (KAPABiosystems), a quantification assay that is specifically designed to determine the quantity of sequences that are in the library. The quantification of libraries is needed to determine the volume of sample library to load during the sequencing process. Paired end sequencing of the libraries was performed in two lanes of an Illumina HiSeq flow cell. Table 2 shows the samples that were loaded into wells 7 and 8.

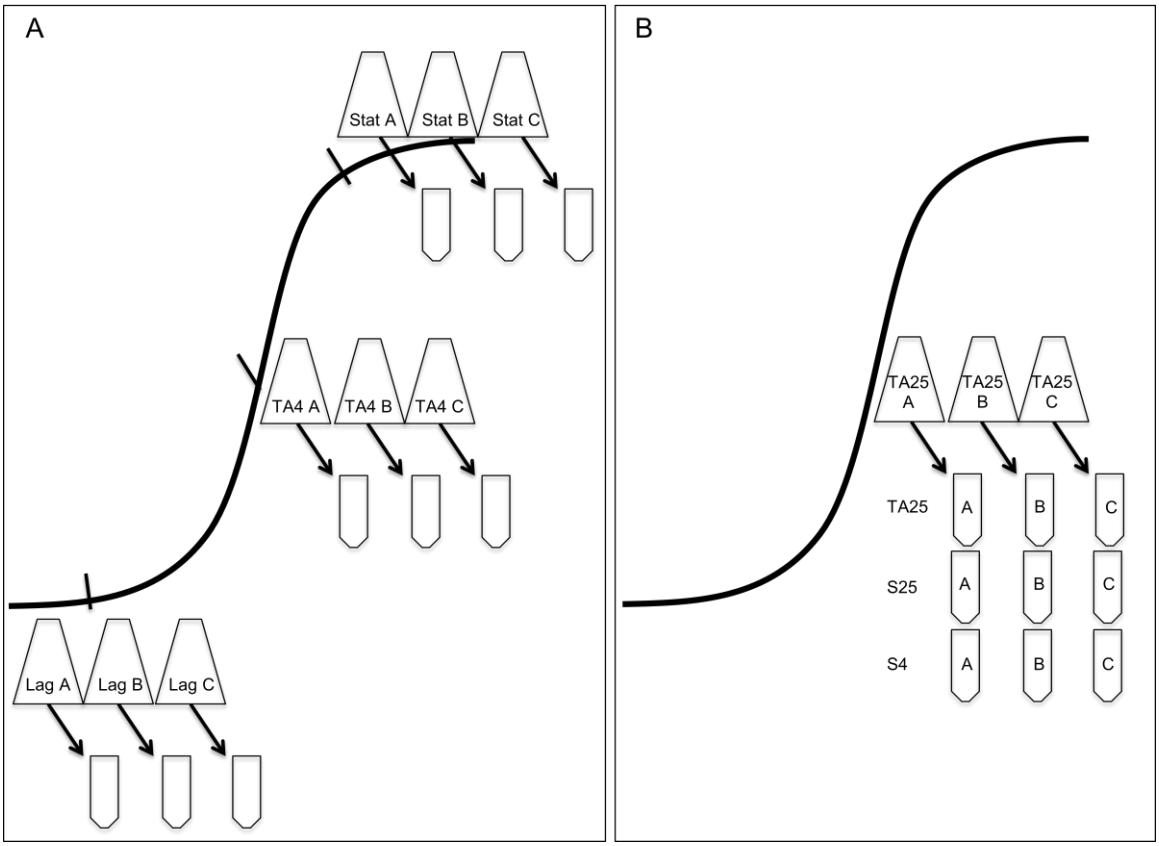

Figure 1. Sample Collection.

1a. Sample collection for temperature adapted cultures was done in biological triplicate at the three phases of growth, lag, exponential, and stationary. Samples TA4 A, TA4 B, and TA4 C were collected during exponential growth. 1b. Sample collection for cold shocked cultures. Three 10 mL aliquots were taken from each TA25 flask for immediate RNA isolation, to be transferred to 4˚C for 30 minutes before RNA isolation (S4), and to be kept at 25˚C for 30 minutes before RNA isolation (S25).

Table 1. Samples Used for Transcriptomic Library Preparation

	25˚C	4˚C
Temperature Adapted (TA)	TA25 A, TA25 B, TA25 C	TA4 A, TA4 B, TA4 C
Shocked (S)	S25 A, S25 B, S25 C	S4 A, S4 B, S4 C

Data Analysis

Transcriptomic data analysis was performed using CLC Bio Genomics Workbench. Raw reads were uploaded, where adapter sequences were removed and sequences smaller than 75 bp were discarded. Quality, trimmed sequences were aligned to the reference genome, *Pseudomonas fluorescens* Pf0-1 (NC_007492), allowing for a similarity fraction of 0.8. After the twelve transcriptomes were aligned to *P. fluorescens* Pf0-1, "experiments" were created within the program to allow for comparisons. Two experimental temperature conditions were compared at a time (six transcriptomes) to generate tables with expression fold changes. First, original expression data were normalized and transformed to log2 values. Normalized and transformed data were then used to perform statistical analysis of the "experiments". T-tests compared the average transformed expression data of the two conditions to give expression fold change, p-values, and corrected p-values accounting for the false discovery rate.

Table 2. Samples and Adapter Indexes

Sample	Index Name	Index Sequence	HiSeq Lane
TA25 A	A002	CGATGT	7
TA25 B	A004	TGACCA	7
TA25 C	A005	ACAGTG	7
S25 A	A006	GCCAAT	7
S25 B	A007	CAGATC	7
S25 C	A012	CTTGTA	7
TA4 A	A002	CGATGT	8
TA4 B	A014	AGTTCC	8
TA4 C	A015	ATGTCA	8
S4 A	A016	CCGTCC	8
S4 B	A018	GTCCGC	8
S4 C	A019	GTGAAA	8

CHAPTER IV: RESULTS

Pseudomonas fluorescens HK44 is a Psychrotroph

Pseudomonas fluorescens HK44 is a psychrotrophic bacterium capable of growing at temperatures ranging from 4˚C to 32˚C. Microplate assays were used to measure continuous growth of *P. fluorescens* HK44 in the Biotek Synergy 2 for temperatures ranging from 10˚C to 32˚C. However, the Synergy was limited in the temperature range that could be achieved due to the inability to cool internal temperatures below the ambient temperature and a loss of function when placed at temperatures below 10˚C. Growth rates and lag times are reported from growth microplate assays in Table 3. Growth rates do not appear to vary much based on temperature. There was no statistical difference in the growth rates calculated for cultures grown at 20˚C, 28˚C, and 30˚C, while growth rates were most affected by 10˚C and 32˚C (Table 4). In addition to temperature limitations, oxygen limitations were seen using the microplate assays; the optical densities of the cultures were lower than expected from previous batch culture experiments (Figure 2). In order to allow for ample oxygenation of cultures and to achieve low temperatures, *P. fluorescens* HK44 was grown at 4˚C and 25˚C in small batch cultures. Optical densities of the small batch cultures were higher than the optical densities in the microplate assays due to greater headspace allowing for cultures to be exposed to larger amounts of oxygen (Figure 3). No other growth curves were generated using the small batch culture method because the upper temperature limit had previously been determined with the microplate assays, and limitations in the laboratory did not allow for lower temperatures to be tested.

Table 3. Average Growth Rate and Lag Time for *P. fluorescens* HK44 Using Microplate Assays

Temperature	Average Growth Rate (OD/hour)	Lag Time
10˚C	0.0589	12 hours
15˚C	0.0571	7 hours
20˚C	0.0822	6 hours
28˚C	0.0781	4 hours
30˚C	0.0819	4 hours
32˚C	0.0698	5 hours

Table 4. Student Newman Keuls (SNK) Analysis of Variance of Microplate Assay Growth Rates. Growth rate means with the same SNK grouping are not statistically different from each other.

Temperature	Growth Rate Mean	SNK Grouping
10˚C	0.058733	A
15˚C	0.092200	B
20˚C	0.081700	B, C
28˚C	0.077733	B, C
30˚C	0.082333	B, C
32˚C	0.068833	A

Table 5. Cold Shock Protein and DEAD box Genes Identified in *P. fluorescens* HK44 Genome Using RAST and Corresponding *P. fluorescens* Pf0-1 Genome ID Calls.

Gene Annotation	Genome ID	Renamed
cspA	Pfl01_4395	*cspA* 1
cspA	Pfl01_1085	*cspA* 2
cspA	Pfl01_1201	*cspA* 3
cspC	Pfl01_4415	-
cspD	Pfl01_3592	-
cspG	Pfl01_1929	-
deaD	Pfl01_3987	-

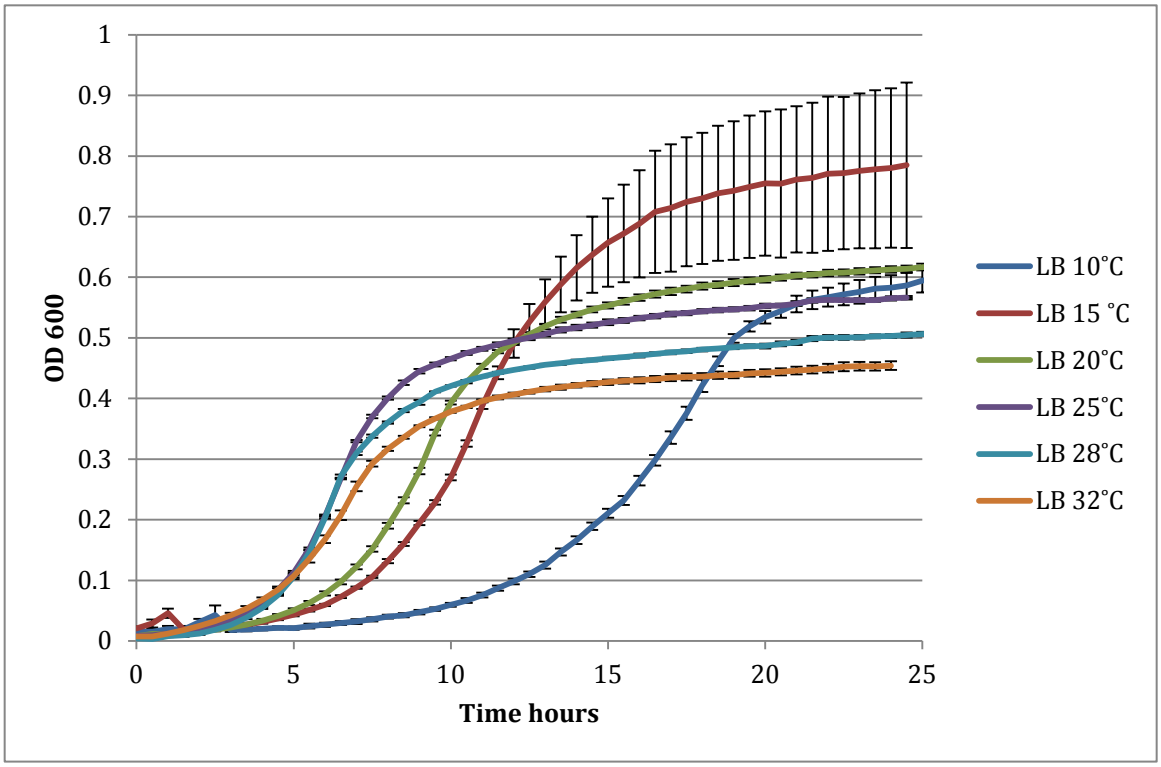

Figure 2. *Pseudomonas fluorescens* HK44 Growth Curves from Microplate Assays. Error bars represent the standard error from three technical replicate sample wells.

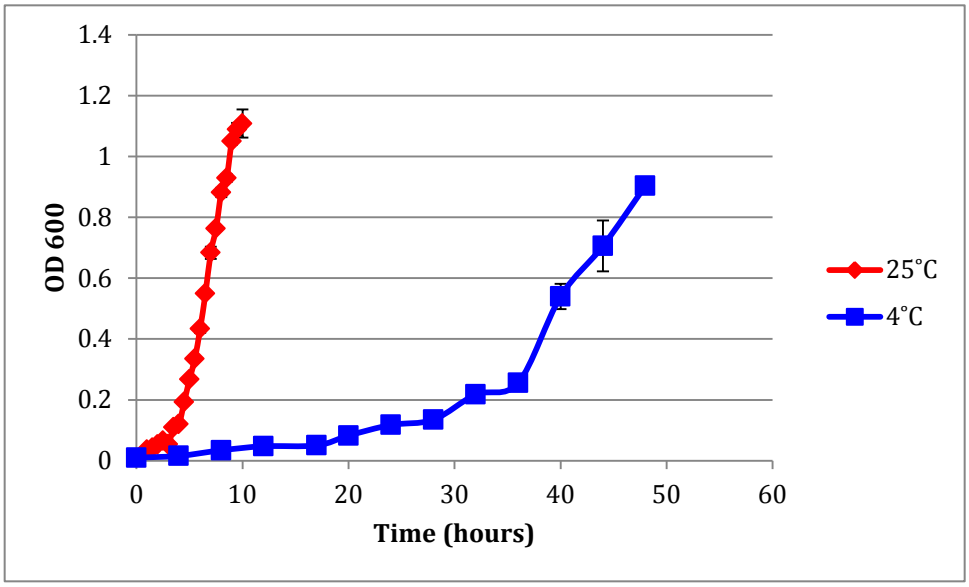

Figure 3. *Pseudomonas fluorescens* HK44 Growth Curves from Small Batch Cultures. Error bars indicate standard error from biological triplicates.

Bioinformatic Analysis

Half of Cold Shock Proteins in HK44 Genome are cspA

The *P. fluorescens* HK44 genome was initially evaluated for genes that are involved in cold shock using the online RAST server. Six cold shock protein genes and one DEAD box protein gene were identified through genome mining (Table 4). Unexpectedly, three copies of the major cold shock protein gene, *cspA*, were found within the HK44 genome, these copies were renamed *cspA* 1, *cspA* 2, and *cspA* 3 for this project (Table 5). Amino acid sequences from the three CspA proteins in HK44 were aligned with Clustal Omega against the only CspA from *E. coli* K12. The protein alignment showed regions of highly conserved amino acids, as well as divergent regions of the CspA proteins (Figure 4). Percent identities calculated indicate that CspA 3 of HK44 is most similar to CspA of *E. coli* K12, followed by CspA 2, and CspA 1, with calculated percent identities of 60.87%, 57.97%, and 49.28% respectively (Table 6). The percent identity matrix shown in Table 5 indicates that CspA 1 and CspA 3 of *P. fluorescens* HK44 are most similar, while CspA 2 and CspA 3 are least similar with percent identities of 79.71% and 55.07%, respectively.

Evaluating Cold Shock Proteins Found in Other Bacteria

The number of *cspA* gene copies found in *P. fluorescens* HK44 was unexpected, therefore, other *Pseudomonas* species were evaluated for the number of cold shock protein genes found within their genomes, as well as the number of *cspA* copies, using the RAST database. The average number of cold shock protein genes and the average copies of *cspA* are shown in Table 7. Three of the four strains of *Pseudomonas fluorescens* represented in the RAST database contained three copies of the *cspA* gene, where the fourth strain contained two copies. The number of *cspA* gene copies in *P. putida* genomes varied, with an average of 2.6 gene copies per

genome. Both *P. fluorescens* and *P. putida* contained an average of 6 total cold shock protein genes. On the other hand, *P. aeruginosa* strains had only one copy of *cspA*, but contained almost the same number of overall cold shock protein genes. Looking at other proteobacteria, *E. coli* species had the most overall cold shock protein genes, but only contained an average of one *cspA* gene. *Acinetobacter* and *Roseobacter* species had an average of 3.5 to 4.0 cold shock protein genes found in their genomes, respectively, where *Roseobacter* species had slightly greater copies of *cspA* genes found in the genome, 1.7 copies compared to 1.5. Similar to *Roseobacter* species, *Burkholderia* species had an average of four cold shock protein genes, but contained only one copy of *cspA*.

A maximum likelihood phylogenetic tree was generated in MEGA 5.2.2 to evaluate the cold shock proteins from *Pseudomonas* species and *E. coli* K12 (Figure 5). It is interesting that tree branching separates the CspA proteins from *Pseudomonas* genomes based on the presence of multiple *cspA* gene copies. All *Pseudomonas* species have at least one CspA protein that cluster together; this group contains the HK44 CspA protein copy that has been labeled CspA 2. *Pseudomonas fluorescens* and *Pseudomonas putida* genomes containing two or more *cspA* copies that cluster together on the opposite side of the phylogenetic tree, and contain CspA 3 from HK44. The final CspA cluster seen on the tree contains only *Pseudomonas fluorescens* strains that contain three copies of the *cspA* gene, and CspA 1 from HK44. The tree alignment shows that CspA 1 and CspA 3 from HK44 are most similar, while CspA 2 is more distantly related. These comparisons are in agreement with the protein alignment and calculated percent identities seen in Table 5 and Figure 4. Finally, the cold shock protein CspD is the most highly conserved protein because all of the *Pseudomonas* CspD proteins cluster with the *E. coli* K12 CspD.

Figure 4. CspA Protein Alignment from Clustal Omega. Symbols beneath sequences distinguish conserved vs. non-conserved regions of the proteins. * Indicates highly conserved region within protein, : indicates conserved region in protein. Blank spaces under the alignment show regions that differ between sequences.

Table 6. CspA Percent Identities Calculated in Clustal Omega. Cold shock protein sequences from *P. fluorescens* HK44 and *E. coli* were compared to distinguish similarities in amino acid sequences.

Gene	HK44 CspA 1	HK44 CspA 2	HK44 CspA 3	*E. coli* CspA
P. fluorescens HK44 CspA 1	100.00	56.52	79.71	57.97
P. fluorescens HK44 CspA 2	56.52	100.00	55.07	49.28
P. fluorescens HK44 CspA 3	79.71	55.07	100.00	60.87
E. coli K12 CspA	57.97	49.28	60.87	100.00

Table 7. Comparison of the Average Cold Shock Protein Genes and *cspA* Copies in Proteobacteria.

Organism	Average Cold Shock Protein Genes	Average Copies of *cspA*
Pseudomonas fluorescens	**6.0**	**2.8**
Pseudomonas putida	6.0	2.0
Pseudomonas aeruginosa	4.6	1.0
Burkholderia	4.0	1.0
Roseobacter	4.0	1.7
Acinetobacter	3.5	1.5
E. coli	7.2	1.0

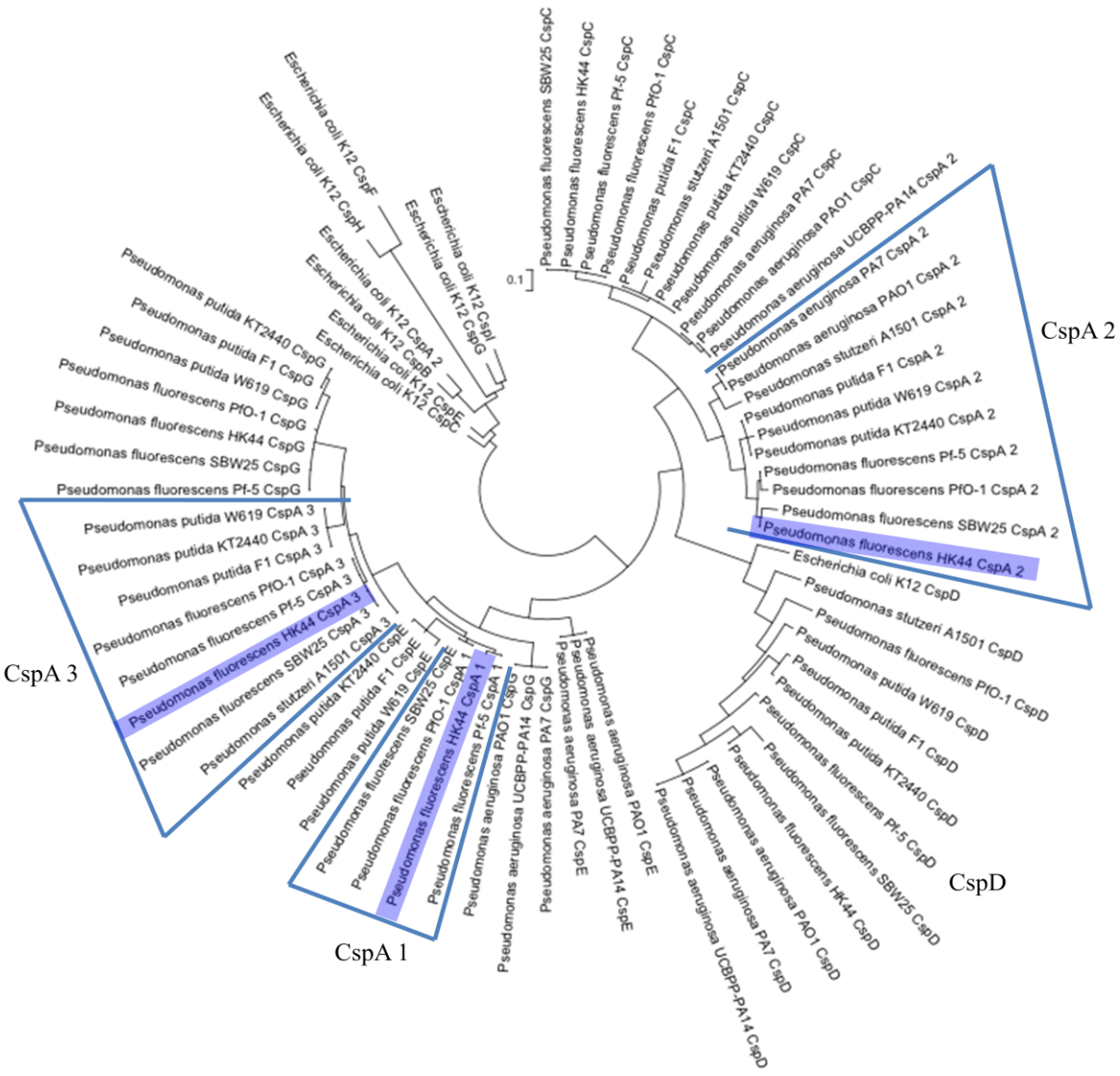

Figure 5. Maximum Likelihood Phlyogenetic Tree Generated in MEGA 5.2.2. *P. fluorescens* HK44 is highlighted in blue. The three CspA protein groups are outlined, highlighting the separation within the tree. The most highly conserved cold shock protein, CspD, is labeled.

Transcriptomic Analysis

Twelve transcriptomes were generated in this experiment, comprising of biological triplicates of cultures grown under four temperature treatment regimes (Table A3). The average number of raw reads generated from the RNA-seq data per transcriptome ranged from 50,579,625 to 62,143,314 reads. CLC Genomics workbench software was used for quality trimming and downstream analysis. After the removal of low quality sequences and sequences less than 75 bp, the average number of reads for each experiment only decreased by an average of 4.23% (Table 8). The high quality sequences remaining after trimming were mapped to *Pseudomonas fluorescens* Pf0-1 in order to determine which genes were represented by the transcript sequences generated during sequencing (Table 8). The majority (80-90%) of raw reads were successfully trimmed and mapped to the *P. fluorescens* Pf0-1 genome. Principle component analysis of the 12 transcriptomes shows separation of each treatment temperature. Cold adapted (TA4) and cold shocked (S4) cells are the most separated, where the 25˚C Exponential (TA25) and 25˚C T30 minute (S25) treatments were overlapping (Figure 6).

mRNA Expression Analysis

Following trimming and sequence alignment, "experiments" were created in CLC Genomics Workbench, where T-tests were used to evaluate the change in gene expression between triplicate transcriptomes from two temperature treatments (Table 9). Analysis focused on transcripts that had significant p-values ($P < 0.05$) and a fold change $\geq |2|$, however, genes with fold changes $\geq|5|$ and $\geq|10|$ are listed in the appendix (Table A4) as well as whether the gene was up or down regulated (Table A5). Table 10 shows each "experiment" designed in CLC and the number of genes that had transcripts with significant p-values, with and without false

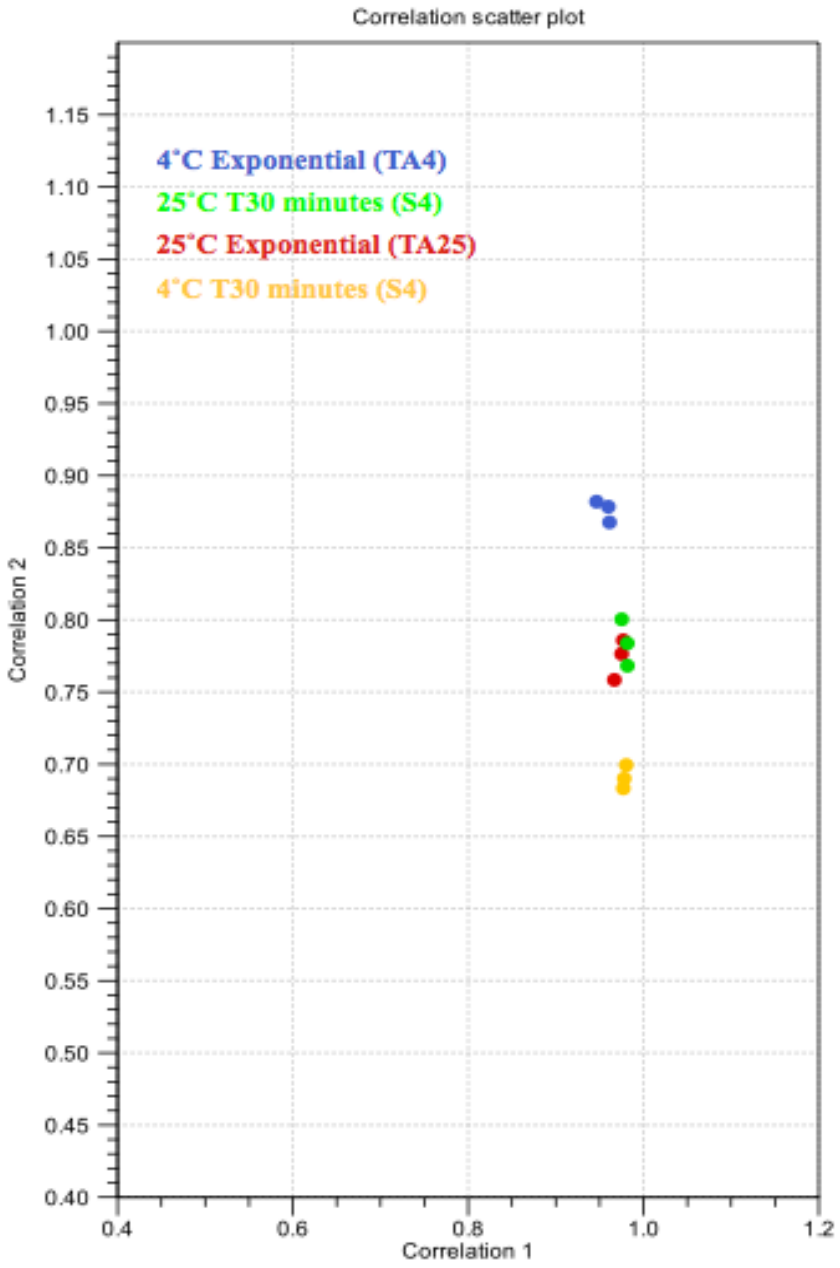

Figure 6. Principle Component Analysis of Transcriptomes. Blue dots represent cold adapted cells (4˚C Exponential TA4), yellow dots represent cold shocked cells (4˚C T30 minutes S4), green dots represent 25˚C shocked cells (25˚C T30 minutes S25), and red dots represent warm acclimated cells (25˚C Exponential TA25).

Table 8. Average Reads Processed for Each Experimental Condition.

Experimental Condition	Average Number of Reads	Average Reads After Trimming	Average Number of Mapped Reads
25˚C Exponential	53,392,097	51,460,285	47,691,285
4˚C Exponential	62,143,314	59,485,853	54,665,358
25˚C Shock	50,579,615	48,297,461	43,308,420
4˚C Shock	53,967,673	51,533,852	43,359,114

Table 9. Experiments Designed in CLC Genomics Workbench for Gene Expression Analysis. (See Figure 1 and Table 1 for TA and S)

Experiments	Transcriptome Condition 1	Transcriptome Condition 2
Control	S25	TA25
Cold Shock 1	S4	TA25
Cold Shock 2	S4	S25
Cold Adapted	TA4	TA25
Cold Adapted vs. Cold Shock	S4	TA4

Table 10. Genes with Significant Fold Change of mRNA Expression**.**

Experiments	Genome Total Genes	Genes with Transcripts	Significant Change in Transcript p-value <0.05	Significant Gene with Fold Change ≥ |2|	Significant Change in Transcript FDR Corrected p-value <0.05	FDR Corrected Significant Gene with Fold Change ≥ |2|
Control	5829	2955	293	80	0	0
Cold Shock 1	5829	3028	1620	569	475	207
Cold Shock 2	5829	3065	1933	724	0	0
Cold Adapted	5829	2754	1075	358	18	11
Cold Adapted vs. Cold Shock	5829	2807	2400	1001	1952	867

Table 11. Cold Shock Protein Gene Expression.

Cold Shock Gene	Control	Cold Shock 1	Cold Shock 2	Cold Adapted	Cold Adapted vs. Cold Shock
cspA 1	1.19†	1.28†	1.07	-1.90†	**9.07**†
cspA 2	4.65	**15.13**†	3.25	-1.65†	-1.08†
cspA 3	1.25†	1.48†	1.18	1.24†	1.06
cspD	1.01	1.19†	1.18†	1.07†	1.08
cspG	ND	ND	ND	ND	ND
cspC	-1.04	1.63	1.7	-1.13	2.13†

† $P < 0.05$

ND- Not Detected

discovery rate (FDR) correction. As expected, the control experiment contained the least number of genes with a significant fold change in expression, and once the FDR p-values were calculated, there were no genes differentially expressed between the two groups. The two cold shock experiments contain a similar number of genes that were differentially expressed; the cold shock 1 experiment identified 1620 genes, where the cold shock 2 experiment identified 1933 genes. Interestingly, after FDR p-values were calculated, no genes were differentially expressed in the cold shock 2 experiment, and genes differentially expressed in the cold shock 1 experiment had dropped from 1620 genes to 475 genes. The cold adapted experiment identified 1075 genes that had a significant fold change in expression; this number was reduced to 18 genes after FDR p-values were calculated (Table A7). The greatest number of differentially expressed genes was seen in the cold adapted vs. cold shock experiment, where 2400 genes were identified before FDR corrected p-values were calculated, and 1952 genes were identified after FDR corrected p-values were calculated.

Identifying Differential Gene Expression During Low Temperature Exposure

In order to determine which genes are involved in cell survivability at 4˚C, either during cold shock or adaptation, gene expression tables generated with CLC Genomics Workbench were mined to identify first the expression levels of the cold shock protein genes, followed by genes that were significantly expressed ≥ |2|-fold and surrounding cold shock protein genes. Expression data for the six cold shock protein genes found in the *P. fluorescens* HK44 genome is provided in Table 10. Significant expression of the *cspA* genes was seen under multiple experimental conditions; however, the fold change in expression was, for the most part small (< |2|-fold). Two *cspA* genes, *cspA* 1 and *cspA* 2, had fold changes greater than 2-fold in two separate experimental comparisons. The *cspA* 1 gene had a 9.07-fold greater expression in the

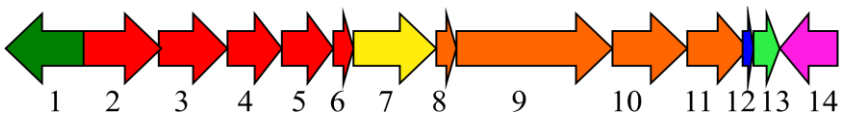

Figure 7. Gene Order Surrounding *P. fluorescens* HK44 *cspA* 1. Genes highlighted in the same color are part of the same operon. 1, arginine/ornithine antiporter; 2, arginine/ornithine antiporter; 3, arginine deiminase; 4, ornithine carbamoyltransferase; 5, carbamate kinase; 6, hypothetical protein; 7, transcriptional regulator TyrR; 8, glycine cleave system protein H; 9, glycine dehydrogenase; 10, L-serine ammonia-lyase; 11, glycine cleavage system T protein; 12, cspA 1; 13, hypothetical protein; 14, quinolinate synthetase.

Table 12. Gene Expression Values Surrounding *cspA* 1 in Cold Adapted vs. Cold Shock Experiment. (See Table 9) The gene numbers correspond to Figure 7.

Figure 7 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	arginine/ornithine antiporter	Pfl01_4384	ND	ND	ND
2	arginine/ornithine antiporter	Pfl01_4385	-25.69	1.12E-04	1.31E-02
3	arginine deiminase	Pfl01_4386	-7.60	2.81E-05	1.31E-02
4	ornithine carbamoyltransferase	Pfl01_4387	-14.35	1.59E-05	1.31E-02
5	carbamate kinase	Pfl01_4388	-6.46	2.39E-05	1.31E-02
6	hypothetical protein	Pfl01_4389	ND	ND	ND
7	transcriptional regulator TyrR	Pfl01_4390	4.04	1.20E-03	1.43E-02
8	glycine cleavage system protein H	Pfl01_4391	-6.43	1.72E-03	1.56E-02
9	glycine dehydrogenase	Pfl01_4392	2.40	4.56E-04	1.31E-02
10	L-serine ammonia-lyase	Pfl01_4393	-3.48	5.74E-03	2.52E-02
11	glycine cleavage system T protein	Pfl01_4394	4.71	7.68E-04	1.32E-02
12	**cspA 1**	**Pfl01_4395**	**9.07**	**8.32E-04**	**1.33E-02**
13	hypothetical protein	Pfl01_4396	-0.43	6.40E-03	2.67E-02
14	quinolinate synthetase	Pfl01_4397	2.10	1.20E-03	1.43E-02

ND- Not Detected

Table 13. Gene Expression Values Surrounding *cspA* 1 in Cold Adapted Experiment. (See Table 9) The gene numbers correspond to Figure 7.

Figure 7 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	arginine/ornithine antiporter	Pfl01_4384	ND	ND	ND
2	arginine/ornithine antiporter	Pfl01_4385	-18.87	1.64E-04	5.32E-02
3	arginine deiminase	Pfl01_4386	40.24	2.02E-04	5.53E-02
4	ornithine carbamoyltransferase	Pfl01_4387	-13.58	1.15E-04	5.16E-02
5	carbamate kinase	Pfl01_4388	-39.67	1.51E-04	5.28E-02
6	hypothetical protein	Pfl01_4389	ND	ND	ND
7	transcriptional regulator TyrR	Pfl01_4390	-2.08	3.20E-03	9.97E-02
8	glycine cleavage system protein H	Pfl01_4391	-2.22	2.05E-03	8.67E-02
9	glycine dehydrogenase	Pfl01_4392	-1.94	1.44E-02	1.47E-01
10	L-serine ammonia-lyase	Pfl01_4393	2.52	4.64E-01	9.95E-01
11	glycine cleavage system T protein	Pfl01_4394	-1.37	1.79E-02	1.59E-01
12	**cspA 1**	**Pfl01_4395**	**-1.90**	**1.73E-03**	**8.67E-02**
13	hypothetical protein	Pfl01_4396	-3.13	3.61E-02	2.26E-01
14	quinolinate synthetase	Pfl01_4397	-1.42	5.84E-03	1.10E-01

ND Not Detected

cold shocked cells than the cold adapted cells, where the *cspA* 2 gene had a 15.13-fold greater expression in cold shocked cells than cells adapted to 25˚C. Following the evaluation of gene expression of cold shock proteins, operons surrounding the two *cspA* genes resulting in a greater than 9-fold increase in expression were evaluated; expression data for the remainder of the *csp* and surrounding genes are found in the appendix (Tables A8-A33 and Figures A1-A4). An arginine deiminase operon is located upstream of the *cspA* 1 gene (Figure 7), consisting of four genes that degrade arginine. Gene expression analysis indicates that the operon is downregulated in cold shocked cells compared to cold adapted cells (Table 12). The arginine/ornithine antiporter is downregulated 25-fold and ornithine carbamoyltransferase is downregulated 14-fold, decreased expression of these two genes limits the export of ornithine from the cell, and limits the degradation of citrulline, respectively. This operon was also differentially expressed in cold adapted cells compared to 25˚C-adapted cells (Table 13). In the cold adapted experiment, arginine deiminase is upregulated 40 fold, allowing for the accumulation of citrulline, while the rest of the operon is down regulated, including the arginine/ornithine antiporter (-8 fold), which prevents the export of ornithine that may accumulate. Interestingly, of the 18 genes with FDR p-value < 0.05 in the cold adapted experiment (Table A7), three of the genes belong to the arginine deiminase operon found near *cspA* 1. This may indicate that these genes play a major role in cold adaptation.

Genes positioned around *cspA* 2 were also investigated (Figure 8). Expression analysis of the surrounding genes from the cold shock 1 experiment, where *cspA* 2 was upregulated more than 15 fold, revealed no significant change in expression levels of a complete operon (Table 14). However, some genes that could be relevant to cold stress response were upregulated, including guanine-N(1)-methyltransferase and glycerol-3-phospate acyltransferase.

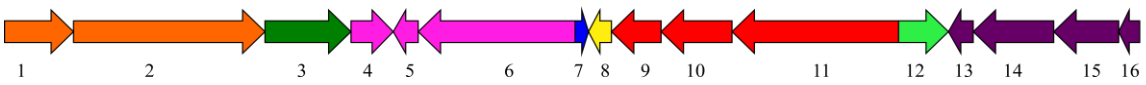

Figure 8. Gene Order Surrounding *P. fluorescens* HK44 *cspA* 2. Genes highlighted in the same color are part of the same operon. 1, secretion protein HlyD; 2, acriflavin resistance protein; 3, filamentation induced by cAMP protein Fic; 4, hypothetical protein; 5, putative lipoprotein; 6, glycerol-3-phosphate acyltransferase; 7, cspA 2; 8, hypothetical protein; 9, rRNA (guanine-N(1)-methyltransferase; 10, succinyl-diaminopimelate desuccinylase; 11, glycosyl transferase family protein; 12, UBA/THIF-type NAD/FAD binding domain-containing protein; 13, Fe-S metabolism associated SufE; 14, aromatic amino acid beta-eliminating lyase/threonine aldolase; 15, 2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase; 16, hypothetical protein.

Table 14. Gene Expression Values Surrounding *cspA* 2 in Cold Shock 1 Experiment. (See Table 9) The gene numbers correspond to Figure 8.

Figure 8 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	secretion protein HlyD	Pfl01_1079	-0.88	9.70E-01	9.99E-01
2	acriflavin resistance protein	Pfl01_1080	1.81	3.79E-02	1.52E-01
3	filamentation induced by cAMP protein Fic	Pfl01_1081	0.77	3.52E-01	7.86E-01
4	hypothetical protein	Pfl01_1082	0.26	2.28E-01	5.49E-01
5	putative lipoprotein	Pfl01_1083	ND	ND	ND
6	glycerol-3-phosphate acyltransferase	Pfl01_1084	2.29	1.37E-02	8.57E-02
7	**cspA 2**	**Pfl01_1085**	**15.13**	**1.22E-02**	**8.08E-02**
8	hypothetical protein	Pfl01_1086	3.72	2.44E-03	4.62E-02
9	rRNA (guanine-N(1)-)-methyltransferase	Pfl01_1087	11.79	7.40E-04	4.01E-02
10	succinyl-diaminopimelate desuccinylase	Pfl01_1088	1.52	2.65E-02	1.24E-01
11	glycosyl transferase family protein	Pfl01_1089	1.53	7.76E-03	6.75E-02
12	UBA/THIF-type NAD/FAD binding domain-containing protein	Pfl01_1090	2.56	6.60E-03	6.31E-02
13	Fe-S metabolism associated SufE	Pfl01_1091	0.02	1.74E-02	9.74E-02
14	aromatic amino acid beta-eliminating lyase/threonine aldolase	Pfl01_1092	4.89	1.04E-02	7.50E-02
15	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	Pfl01_1093	1.20	3.95E-02	1.56E-01
16	hypothetical protein	Pfl01_1094	1.79	6.48E-03	6.31E-02

ND- Not Detected

The expression of guanine-N(1)-methyltransferase was 11 fold greater in cold shocked cells than 25˚C-adapted cells, while a 2 fold increase was seen in the glycerol-3-phosphate acyltransferase gene.

Evaluating Expression Profiles Across Experiments

While the primary focus of this study has been the cold shock protein genes and genes immediately surrounding them, to better understand the whole cell response to cold shock and cold adaptation changes in the entire transcriptome must be examined. Each experiment designed in CLC Bio (Table 9) identified a group of genes that were differentially expressed in response to the temperature regimes evaluated. Within those differentially expressed genes, with a fold change $\geq|2|$, the majority of genes were unique to the temperature treatment regime (Figure 10). Cold shocked cells had 468 genes that were differentially expressed that were not identified in any other experiment, where cold adapted cells identified 243 unique genes, and control cells had only 37 genes identified. However, there were small groups of genes that were differentially expressed in two or more experiments. Transcriptomic analysis identified 86 genes that were differentially expressed in both cold shocked and cold adapted cells. Most of the genes (62 of 86) expressed in both cold adapted and cold shock experiments were oppositely regulated from one another (Table 15). For example, if a gene was upregulated during cold shock, it was downregulated during cold adaptation. Only 24 of the genes (Table A35) were under the same type of regulation in response to low temperatures.

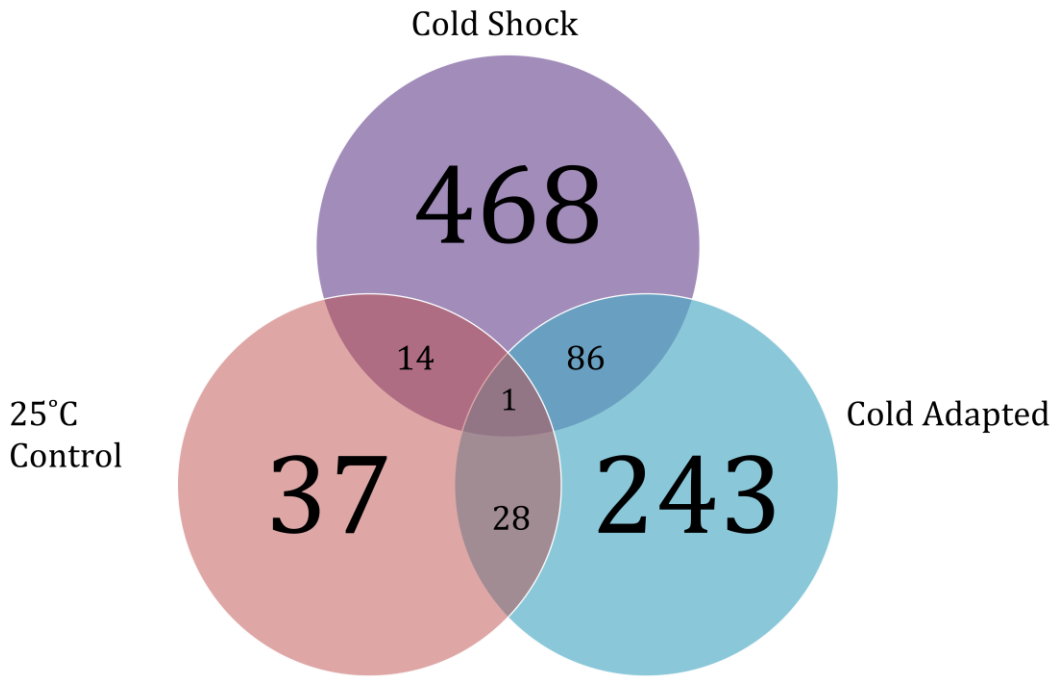

Figure 9. Quantification of Genes Representing Overlapping Transcripts Found in Each Experiment.

Table 15. Overlapping Differentially Expressed Genes from Cold Adapted and Cold Shock Experiments.

Gene Funciton	Gene Name	Cold Shock 1 Experiment Fold Change	Cold Adapted Experiment Fold Change
acetate permease	actP	2.2	2.5
peptide deformylase	def_2	3.8	-2.0
flagellar basal body P-ring protein	flgI	2.1	-2.5
flagellar rod assembly protein/muramidase FlgJ	flgJ	4.4	-11.1
glucokinase	glk	2.5	-2.3
apolipoprotein N-acyltransferase	lnt	2.3	-2.5
tetraacyldisaccharide 4'-kinase	lpxK	4.4	-7.6
Maf-like protein	maf_2	-2.7	-5.8
flagellar motor protein MotB	motB	5.9	-2.3
flagellar motor protein	motC	3.1	19.1
UDP-N-acetylenolpyruvoylglucosamine reductase	murB	6.1	-2.0
UDPdiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	murG	2.2	-2.1
ABC transporter-like protein	Pfl01_0038	3.4	-2.7
fusaric acid resistance protein	Pfl01_0171	3.9	5.5
surface antigen protein	Pfl01_0278	4.5	-2.1
lipoprotein	Pfl01_0355	9.4	-7.2
urocanate hydratase	Pfl01_0360	3.8	2.0
hypothetical protein	Pfl01_0437	-8.5	-2.0
glycosyl transferase family protein	Pfl01_0478	2.3	-2.9
hypothetical protein	Pfl01_0485	3.1	-2.1
3-deoxy-D-manno-octulosonic-acid transferase	Pfl01_0490	2.7	-37.6
(4Fe-4S)-binding protein	Pfl01_0517	3.9	2.5
branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	Pfl01_0590	2.6	-13.1
methylmalonate-semialdehyde dehydrogenase	Pfl01_0686	3.0	6.7
Phage integrase	Pfl01_0746	3.4	-9.2
maleylacetoacetate isomerase	Pfl01_0912	-2.8	3.5
phosphate-starvation-inducible E	Pfl01_0917	-3.7	-30.1
cyanate transport system protein	Pfl01_0978	2.6	4.0

Table 15. Continued

Gene Funciton	Gene Name	Cold Shock 1 Experiment Fold Change	Cold Adapted Experiment Fold Change
hypothetical protein	Pfl01_0979	4.3	-3.0
CheA Signal transduction histidine kinase (STHK)	Pfl01_1056	2.7	-5.1
hypothetical protein	Pfl01_1077	3.2	-10.9
hypothetical protein	Pfl01_1298	5.2	2.5
signal transduction protein	Pfl01_1356	-132.8	-3.4
anti sigma-E protein, RseA	Pfl01_1363	2.0	-3.4
cyclic nucleotide-binding protein	Pfl01_1398	2.7	-2.5
hypothetical protein	Pfl01_1425	3.5	-2.7
K+ transporter Trk	Pfl01_1479	3.0	-3.3
PAS/PAC sensor Signal transduction histidine kinase	Pfl01_1533	2.1	-3.0
cytochrome c biogenesis protein CcmA	Pfl01_1577	3.8	-3.8
threonine-phosphate decarboxylase	Pfl01_1645	6.8	-17.3
cobyric acid synthase	Pfl01_1646	3.6	-2.8
benzoate membrane transport protein	Pfl01_1792	2.1	-3.1
acyl-CoA dehydrogenase	Pfl01_1809	3.0	6.7
rhomboid-like protein	Pfl01_2197	4.1	-4.4
Short-chain dehydrogenase/reductase SDR	Pfl01_2526	5.2	4.9
NUDIX hydrolase	Pfl01_3237	-14.8	-2.1
glutaminase	Pfl01_3373	14.0	27.7
RND efflux system outer membrane lipoprotein NodT	Pfl01_3644	2.0	-5.2
hypothetical protein	Pfl01_3989	-3.7	-2.5
LysR family transcriptional regulator	Pfl01_3993	-3.0	6.0
hypothetical protein	Pfl01_4018	-3.5	5.1
DoxX	Pfl01_4019	5.3	-3.8
ABC transporter-like protein	Pfl01_4088	2.4	-9.4
periplasmic sensor Signal transduction histidine kinase	Pfl01_4239	2.1	756.5
periplasmic sensor Signal transduction histidine kinase	Pfl01_4244	2.4	3.7

Table 15. Continued

Gene Funciton	Gene Name	Cold Shock 1 Experiment Fold Change	Cold Adapted Experiment Fold Change
two component transcriptional regulator	Pfl01_4245	-8.6	-4.9
AraC family transcriptional regulator	Pfl01_4287	2.2	-2.3
gluconate transporter	Pfl01_4335	3.9	3.1
gluconate kinase	Pfl01_4336	-2.6	2.1
ribonuclease BN	Pfl01_4350	2.4	-2.5
citrate-proton symport	Pfl01_4459	-10.0	18.0
EmrB/QacA family drug resistance transporter	Pfl01_4479	6.4	2.2
DNA-(apurinic or apyrimidinic site) lyase / endonuclease III	Pfl01_4513	5.2	-6.2
5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	Pfl01_4545	-2.6	-6.6
molybdopterin biosynthesis protein MoeB	Pfl01_4746	-19.4	-3.4
Pas/Pac sensor-containing chemotaxis sensory transducer	Pfl01_4766	2.2	-2.5
cation efflux protein	Pfl01_4937	3.5	-6.3
hypothetical protein	Pfl01_4940	2.4	-2.2
peptidase U61, LD-carboxypeptidase A	Pfl01_4962	10.1	-7.2
rod shape-determining protein RodA	Pfl01_4969	2.9	-2.1
LrgB-like protein	Pfl01_4980	-4.0	18.5
Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	Pfl01_5155	56.5	5.3
cyclic nucleotide-binding protein (cNMP-bd) protein	Pfl01_5185	-4.7	-9.7
acyltransferase	Pfl01_5198	2.2	-40.9
intergral membrane protein	Pfl01_5250	2.8	4.4
hypothetical protein	Pfl01_5254	-3.3	-2.1
16S ribosomal RNA methyltransferase RsmE	Pfl01_5288	8.0	-10917.2
HylII	Pfl01_5292	4.6	-2.5
pilus retraction protein PilT	Pfl01_5318	2.2	-2.1
formamidopyrimidine-DNA glycosylase	Pfl01_5351	2.8	-2.3
hypothetical protein	Pfl01_5375	6.5	-2.8
hypothetical protein	Pfl01_5472	3.0	2.2

Table 15. Continued

Gene Funciton	Gene Name	Cold Shock 1 Experiment Fold Change	Cold Adapted Experiment Fold Change
lysine exporter protein LysE/YggA	Pfl01_5476	2.0	-3.2
Mg chelatase-like protein	Pfl01_5508	2.4	-2.8
lipoprotein	Pfl01_5553	4.1	-2.3
hypothetical protein	Pfl01_5679	10.3	-7.8

CHAPTER V: DISCUSSION

When this study initially started, the ability of *P. fluorescens* HK44 to grow at low temperatures (<10˚C) was unexpected. The organism was originally isolated from the soil rhizosphere and has previously been classified as a mesophile in online databases, such as the Genome OnLine Database (GOLD). Our study shows that *P. fluorescens* HK44 has an upper limit growth temperature of 32˚C, with an optimum growth temperature between 20˚C and 28˚C. The lower limitation of *P. fluorescens* HK44 was not determined due to limitations in the lab; however, it was proven that HK44 is capable of growing at 4˚C. Thus, the organism should now be classified as a psychrotroph. This finding also sheds a light on the misclassification of genomes published online. Generalizations should not be made regarding phenotypic traits until supporting evidence has been provided. The under representation of psychrotrophic and psychrophilic organisms in genomic databases may be a result of theses misclassifications.

Significance of *cspA* Gene Copies

Subsequent to determining that *Pseudomonas fluorescens* HK44 is a psychrotroph, genomic mining was used to identify genes that may be representative of the psychrotrophic phenotype seen. Unexpectedly, during HK44 genomic analysis, three copies of the *cspA* gene were identified. Looking at Figure 5, the group labeled as CspA 2 contains *Pseudomonas* species that are known to grow at both psychrotrophic and mesophilic temperatures. *Pseudomonas aeruginosa* strains can be an opportunistic pathogen in humans, and grow in temperatures ranging from 25˚C to 42˚C [73]. The dynamic temperature growth ranges seen in organisms within this cluster suggest that *cspA* 2 may be the major cold shock protein found in all *Pseudomonas* species without regard to temperature classification. A group of *Pseudomonas* CspA proteins was identified in Figure 5 that cluster together without a CspA protein from *P.*

aeruginosa. This group, labeled CspA 3, contains *Pseudomonas* species that have a diverse temperature range, but exclude *P. aeruginosa* species that are unable to grow at low temperatures. Additionally, all of the species found in this cluster contain at least two copies of the *cspA* gene. This suggests that *Pseudomonas* species containing genes homologous to *cspA* 3 have at least two copies of the *cspA* genes and are capable of growing at temperatures below 25˚C. The smallest group of CspA proteins clustering on the phylogenetic tree belong to the protein group named CspA 1. This group contains all *Pseudomonas fluorescens* species known to have three copies of *cspA* genes. Thus, this gene copy may weigh heaviest in determining an organisms ability to survive low temperatures and suggests that CspA 1 is representative of true psychrotrophic *Pseudomonas* species. These data indicate that multiple copies of the *cspA* gene may be indicative of temperature ranges organisms can proliferate.

Genes Located Near *cspA* Genes and Possible Roles in Cold Acclimation

The major cold shock protein gene, *cspA*, is known to be an RNA chaperone in *E. coli*, allowing for efficient transcription and translation at low temperatures. This being known, it is important to identify genes surrounding cold shock protein genes that may be transcriptionally regulated by Csps, thereby allowing a cell to adapt to low temperatures. This project narrowed its focus to the *cspA* genes due to the unexpected high copy number identified in the *P. fluorescens* HK44 genome. Beginning with the cold shock protein gene that weighs heaviest on determining an organisms psychrotrophic phenotype, genes surrounding *cspA* 1 were identified (Figure 7). Genomic investigation identified an arginine deiminase pathway located upstream of *cspA* 1. In this pathway, arginine is converted to citrulline, then citrulline is converted to ornithine and carbamoyl phosphate, and finally carbamoyl phosphate is converted to carbon dioxide that can

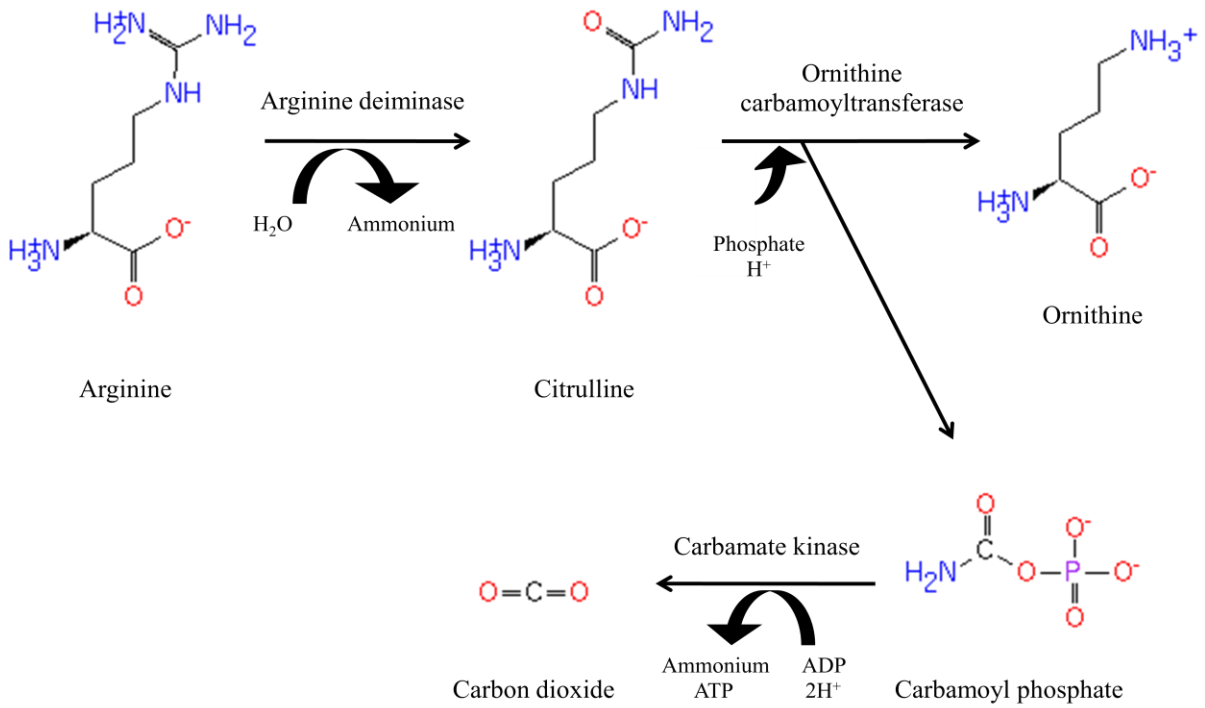

Figure 10. Arginine Deiminase Pathway.

be used downstream in cellular processes (Figure 10). While several papers have examined the expression of this pathway in lactic acid bacteria, acid is usually the environmental factor implied to regulate the expression of this pathway [74, 75]. Under low pH conditions, the pathway is upregulated causing an accumulation of ammonium in efforts to increase the pH. To my knowledge there are no references in the literature that associates the regulation of the arginine deiminase pathway with cold acclimation. However, some studies evaluating metabolites have shown increased concentrations of citrulline and ornithine in response to low temperatures. In an early study evaluating the ability of winter rape plants to survive at 5˚C, amino acid analysis found an increase in citrulline concentration in plants at 5˚C than in control plants [76]. In a more recent metabolomics study, Singh et al. [77] showed that *Listeria monocytogenes* cultures grown at 8˚C had 5.3 fold greater concentration of ornithine than cultures grown at 37˚C. They suggest an increase in ornithine is seen because it is a precursor to polyamine biosynthesis, which is associated with nucleic acid and protein biosynthesis and structure, as well as cell growth and differentiation. Polyamines also accumulate in plants exposed to low temperatures, and are required for these plants to adapt to low temperatures [78]. Other studies show that the accumulation of ornithine can be used in proline biosynthesis [79]. The accumulation of proline can be utilized in plants and microbes as a cryoprotectant [80-82].

Two genes surrounding *cspA* 2 in HK44 were identified as having the potential to be involved in cold acclimation. The first gene, encoding glycerol-3-phosphate acyltransferase, is located just upstream of the *cspA* 2 gene, which is involved in phospholipid biosynthesis. Altering the phospholipids found within the cell membrane can alter membrane fluidity. For example, an increase in unsaturated fatty acids allows for greater membrane fluidity, which is necessary for an organism exposed to low temperatures. Sui et al.[83] evaluated the role of

glycerol-3-phosphate acyltransferase in tomatos stressed by low temperatures. They found that over expression of this gene allowed for the otherwise cold sensitive tomato to grow at low temperatures. The second gene found near *cspA* 2 that may be involved in cold acclimation is guanine-N(1)-methyltransferase. A study by Das et al.[84] indicated that this gene methylates 23S rRNA at the hairpin 35 position, and deletion of the gene causes a decrease in growth rate in *E. coli.* However, in a more recent study, an *E. coli* knockout strain lacking the guanine-N(1)-methyltransferase gene initially saw a decrease in growth rate, but later reverted to the wild type growth rate after the mutant strain underwent serial passages [85]. Therefore, Lui et al. [85] suggested that methylation of the 23S rRNA is a secondary function of this gene; furthermore, they suggest that the primary function of the gene product is ribosomal related, and probably serves as an RNA chaperone aiding, in ribosomal assembly

Transcriptomic Data

Transcriptomic data was first evaluated to determine the expression levels of the cold shock protein genes. Early expression studies of the *cspA* gene from *E. coli* show increased expression when *E. coli* was shifted from 37˚C to 10˚C [43]. Therefore, a large fold increase in the expression levels of *cspA* 1, *cspA* 2, and *cspA* 3 in the two cold shock experiments performed with *P. fluorescens* HK44 was expected. However, after performing statistical analysis and setting a minimum fold change threshold of 2-fold, only one *cspA* gene, *cspA* 2, showed significant fold change in expression (~15-fold) in the cold shock 1 experiment. Although there was a large fold increase in *cspA* 2 expression, the majority of surrounding genes saw less than a 2-fold change in expression (Table 14). However, the two genes predicted to be involved in cold acclimation were upregulated. The glycerol-3-phosphate acyltransferase gene was upregulated 2.29-fold in cold shocked cells, where guanine-N(1)-methyltransferase was upregulated 11.79-

fold. The small increase in expression levels of glycerol-3-phosphate acyltransferase may indicate that HK44 modifies the membrane phospholipids produced during cold shock. Membrane modification in response to temperature stress has been seen in other *Pseudomonas* species, and would not be an unexpected result of this study. The large fold change seen from the methyltransferase gene may support Lui et al. [85] and their proposed RNA chaperone function. Inducing a gene that aids with ribosomal assembly under low temperature stress would assist transcription and translation activity that can be reduced from low temperatures.

While *cspA* 1 did not become induced during the cold shock experiments, a high fold change in expression was seen when cold shocked cells were compared to cold adapted cells (Table 12). It was expected that the *cspA* 1 gene to be expressed at higher levels in the cold shocked cells than in cold adapted cells. Goldstein et al. [43] described the expression of *E. coli cspA* during a cold shock from 37˚C to 10˚C, where *cspA* was significantly induced within 30 minutes of the temperature downshift and returned to baseline expression levels within 1.5 hours of the temperature downshift. Similar to the Goldstein experiment, cold shocked cells were harvested 30 minutes after the temperature downshift. However, the cold adapted cells were harvested after 50 hours of growth at 4˚C. This would be more than sufficient time for the cold shock protein gene to have peaked in expression and return to low levels. This may explain the higher expression levels of *cspA* 1 in cold shocked cells than cold adapted cells. This experiment also identified a significant change in expression of the arginine deiminase pathway (Table 11). While there are large fold changes seen by comparing cold adapted and cold shocked cells, this does not tell us which temperature treatment effects the pathway more. A better understanding of how this pathway is utilized can be gained by looking at the expression data from the cold adapted experiment and the cold shock experiments,

The arginine deiminase pathway was more affected in cells that have long-term exposure to 4˚C (cold adapted cells). Expression data from the cold adapted experiment showed large fold changes of the arginine deiminase pathway in cold adapted cells compared to 25˚C-adapted cell (Table 13). The antiporter that removes ornithine from the cell in exchange for arginine is down regulated ~8-fold and arginine deiminase, the gene responsible for converting arginine to citrulline is upregulated ~40-fold. There is a decrease in expression levels of the gene responsible for converting citrulline to ornithine (13-fold), however with such a high fold change in citrulline production ornithine could still be produced. The accumulation of ornithine can be used to produce proline, a metabolite that can act as a cryoprotectant [82, 86].

Expression levels of *cspA* 3 identified in transcriptomic analysis were the most unexpected, in that no experimental comparison had a significant fold change greater than |2|. This was surprising because the CspA 3 protein is the most similar of the cold shock proteins in *P. fluorescens* HK44 to CspA of *E. coli.* Furthermore, if *cspA* 3 expression acts atypically of the *E. coli cspA,* it would expected to act similarly to *cspA* 1 of HK44 because their proteins are highly similar (Table 5). The expression levels of genes surrounding *cspA* 3 were also low, including a DEAD-box helicase gene, which aids in the unwinding of nucleic acids for transcription and translation.

Limiting our study to cold shock protein genes and genes immediately surrounding them eliminates a large portion of the genes that are differentially expressed in response to low temperature acclimation. Evaluating transcripts that are differentially expressed in one or more experiments can give a better picture of how *P. fluorescens* HK44 adapts to low temperature. The majority of genes differentially expressed in cold shock and cold adaptation experiments were specific to the temperature treatment regime they represented (Figure 9). The genes that are

unique to each treatment provide a better understanding of how HK44 acclimates to cold shock and long-term cold exposure. There were 468 genes with a greater than |2|-fold change during cold shock, suggesting that HK44 must make large changes to its expression profile to acclimate during a rapid downshift in temperatures. Cold adapted cells identified 243 genes specific to long-term low temperature exposure. Fewer genes were identified in the cold adapted experiment most likely because cells were already adjusted to low temperatures and had reached an exponential rate of growth. Perhaps more interesting, are the 86 genes that were found in both cold shock and cold adaptation experiments. By evaluating these genes, a core group of genes that are affected by low temperature could be identified.

Finally, comparing data from this experiment to previous experiments evaluating gene expression can validate these findings. The RNA-seq study carried out by Frank et al. [4] identified 2337 genes differentially expressed in *P. putida* after adapting to 10˚C for 2 hours. This is far more than the number of genes identified during cold adaptation of *P. fluorescens* HK44 (243), but differences may be due to the expression analysis. Frank et al. [4] used an RPKM comparison method published by Mortazavi et al [88], where this study calculated significance using student T- tests, which generated p-values. Spaniol et al [41] used a more stringent statistical analysisto determine 831 genes were differentially expressed during cold shock in *M. catarrhalis;* however, the data included genes with a fold change ≥|1.5|. This study only included genes with a ≥|2| fold change in expression and may account for the differences in the number of genes differentially expressed in *P. fluorescens* HK44 during cold shock (569). The number of *P. fluorescens* HK44 genes differentially expressed was much less than the previous RNA-seq studies mentioned. However, the number of genes identified in this study is much larger than the 26 to 28 genes predicted to be induced during cold shock in *E. coli* by

Table 16. Original Genes Predicted to be Induced During Cold Shock and Expression values of HK44.

Gene Name	Description/Function	Fold Change During Cold Shock 1 in *P. fluorescens* HK44
aceE	Pyruvate dehydrogenase, decarboxylase	1.09
aceF	Pyruvate dehydrogenase, dihydrolipoaminde acetyltransferase	1.15
cspA	Cold inducible RNA chaperone, antiterminator, transcriptional enhancer	1.28† (*cspA* 1) 15.13† (*cspA* 2) 1.48† (*cspA* 3)
cspB	Cold shock inducible RNA chaperone	NA
cspE	RNA chaperone, transcriptional antitermination	NA
cspG	Cold inducible cold shock protein	1.18†
cspI	Cold inducible cold shock protein	NA
deaD	ATP-dependent RNA helicase	1.67†
dnaA	DNA binding and replication initiator, global transcription regulator	1.41†
gyrA	DNA gyrase, subunit A, DNA binding/cleaving/regoining subunit of gyrase	1.18†
hns	Nucleoid protein, transcriptional repressor, repressor supercoiling	0
hscA	DnaK-like chaperone	1.65†
hscB	DnaJ-like co-chaperone for HscA	3.14†
hupB	Nucleoid protein, DNA supercoiling	
infA	Protein chain initiation factor IF1, translation initiation	2.23†
infB	Protein chain initiation factor IF2, translation initiation, fMet-tRNA binding, protein chaperone	1.38†
infC	Protein chain initiation factor IF3, translation initiation, stimulates mRNA translation	1.28†
lpxP	Lipid A synthesis, cold- inducible	NA
nusA	Transcription termination/antitermination/elongation L factor	1.42†
otsA	Trehalose phosphate synthase, cold and heat induced	NA
otsB	Trehalose phosphate phosphatase, cold and heat induced	NA
pnp	Polynucleotide phosphorylase, 3'- 5' exoribonuclease, component of RNA degradosome	1.24†

† Significant p-value <0.05

Table 16. Continued

Gene Name	Description/Function	Fold Change During Cold Shock 1 in *P. fluorescens* HK44
rnr	Ribonucleotide reductase, 3'– 5' exonuclease	NA
rbfA	Ribosome binding factor for 16S rRNA processing	2.22†
recA	General recombination and DNA repair, induction of SOS response	1.29†
tig	Protein folding chaperone, multiple stress protein	1.10
ves	Cold and stress inducible	NA
yfiA	Protein Y associated with 30S ribosomal subunit, inhibits translation	NA

† Significant p-value <0.05

Gualerzi et al [87] and Barria et al [7]; Table 16 identifies these genes and the expression change determined in this experiment. The majority of the genes predicted by Gualerzi and Barria are present in this data, but lack a |2| fold or more change in expression. This may indicate that these genes are required for a mesophilic organism such as *E. coli*, but pyschrotrophic organisms may regulate other genes during cold shock.

Hypothesis Evaluation

Growth assays preformed with *P. fluorescens* HK44 failed to reject our first hypothesis, H_1: *P. fluorescens* HK44 is a psychrotroph. Therefore, HK44 should no longer be classified as a mesophile, and should be recognized as a psychrotroph with a growth range between 4˚C and 32˚C. Our second hypothesis was also not rejected. There are unique genes in the *P. fluorescens* HK44 genome that are responsible for psychrotrophic phenotypes. Multiple copies of the *cspA* gene appear to be unique to the psychrotrophic Pseudomonads found in the RAST database.

Based on the analysis of the transcriptomic expression data, H_3: The *cspA* and surrounding genes will be differentially expressed during low temperature conditions, is rejected. Expression data did not conclude that all *cspA* genes were significantly expressed during low temperature conditions in all experiments. Only *cspA* 1 and *cspA* 2 were differentially expressed during two different experiments. Furthurmore, the expression levels of genes surrounding the cold shock proteins genes did not always correspond to the expression levels of the cold shock protein genes. A large increase in expression of a cold shock protein gene did not guarantee that there was a large increase in expression of surrounding genes. For example, when *cspA* 2 was shown to be upregulated 15 fold in the cold shock 1 experiment, the majority of surrounding genes did not have changes in expression greater than |2|-fold. Likewise, downregulation of the cold shock protein genes did not mean that surrounding genes were downregulated; for example,

cspA 1 was downregulated 1.9-fold, while there was a 40-fold increase in expression of the arginine deiminase gene.

Our last hypothesis, H_4: Genes involved in the production of cryoprotectants and/or membrane modification will be expressed in response to cold shock and cold adaptation, cannot be accepted or rejected based on the data generated in this study. However, it is anticipated that it will be accepted following the completion of supplementary experiments. The genes upregulated surrounding *cspA* genes have been suggested to be involved with production cryoprotectants and membrane modifications. However, until metabolite and fatty acid membrane analyses are completed, it can only be hypothesized the gene expression found in this study correlates to the production of cryoprotectants and changes in the phospholipid membrane.

Limitations and Future Directions

These data provide insight to how *Pseudomonas fluorescens* HK44 adapts to low temperature and reclassifies and organism's temperature growth range. However, some limitations should be noted. One of the limitations of this study was the ability to grow cells at low temperatures. The absolute lowest temperature at which *P. fluorescens* HK44 can grow was unable to be determined because the lab does not have equipment that allows for growth rate evaluation below 4˚C. Growth chambers capable of cooling below 4˚C should be used to further define the growth parameters the *P. fluorescens* HK44 can withstand. Another limitation of this study was not having a complete *P. fluorescens* HK44 reference genome for transcriptomic data alignment. Even though *P. fluorescens* Pf0-1 is the closest *Pseudomonas fluorescens* strain to HK44 with respect to percent identity, there are still genomic differences between them. Differences in the genome may have caused transcripts to be eliminated from analysis that are relevant to temperature stress. When these data were generated the HK44 genome was in 181

contigs, however, now that the number of contigs have been significantly reduced, re-aligning the raw sequencing data to the HK44 genome may further provide insights to how the organism adapts to low temperatures.

Future experiments should be carried out to support the genomic and transcriptomic data generated during this project. This experiment was designed to contain triplicate transcriptomes for each temperature treatment in efforts to generate reproducible and reliable data. Validation of the expression data can be achieved by running quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays. Widely accepted as a reliable method for quantifying gene expression levels, qRT-PCR is capable of detecting fold changes as low as 2-fold, which is sufficient for detecting the levels of expression found during transcriptomic analysis. My gene expression data suggests that ornithine accumulates in the cell. A metabolomic study would quantify the concentrations of metabolites in the cell and clarify if ornithine accumulated during long-term exposure to 4˚C. Furthermore, this data could determine if the concentration of ornithine correlated to proline concentration to discern whether or not ornithine was accumulating to be used in the production of proline for use as a cryoprotectant. Quantifying metabolites throughout the growth stages may indicate if a metabolite must accumulate before exponential growth can be achieved, this would explain the long lag times seen at low temperatures. Finally, fatty acid membrane analysis at each temperature condition would provide insight to membrane modifications that occur rapidly in response to cold shock, or over a long period of low temperature acclimation.

Data generated during this project provides evidence of a well adapted organism, capable of withstanding cold temperature stress. Further analysis of HK44 to include aspects for determining reporter function at low temperatures would be a novel future direction. Exposing

the psychrotrophic HK44 strain to varying naphthalene concentrations at low temperatures while evaluating naphthalene degradation and bioluminescent output would provide data to further validate *P. fluorescens* HK44 as a bioremediation tool. It is unknown whether or not the pUTK21 plasmid can operate under low temperature conditions, which is the determining factor for HK44 functioning as a real-time bioreporter.

Conclusions

This study provided several findings regarding *Pseudomonas fluorescens* HK44. First, it has been shown through growth kinetics studies, that *P. fluorescens* HK44 is a psychrotrophic bacterium. Redefining the temperature classification of the organism may encourage future studies to broaden temperatures conditions while assessing HK44 as a bioremediator. Genomic mining revealed multiple copies of *cspA* are found within *P. fluorescens* HK44 and other *Pseudomonas fluorescens* strains. Based on what has been reported on *Pseudomonas* growth temperatures, it is suggested that the number of *cspA* copies present in the genome can be an indicator of psychrotrophic growth. Furthermore, it is possible that protein sequences my be able to be used as an indication of functional gene activity at low temperatures. Finally, evidence was presented that *P. fluorescens* HK44 responds to cold shock and long-term cold exposure by regulating genes involved in cryoprotectant production and membrane modification. These findings further our understanding of cold acclimation of *P. fluorescens* HK44 and show that genomics and transcriptomics are effective tools for evaluating cold acclimation.

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APPENDIX

Table A1. RNA Extraction Comparison. Hot phenol extractions utilizing mechanical lysis produce the greatest concentration of high-quality total RNA.

Extraction Method	Mechanical Lysis vs No Mechanical Lysis	µg Total RNA per Sample 25˚C	µg Total RNA per Sample 4˚C	Average µg/mL Cells Extracted Total RNA 25˚C	Average µg/mL Cells Extracted Total RNA 4˚C
Hot Phenol Extraction	Bead Beating	168.456 †¥	203.570†¥	16.847	20.357
	No Bead Beating	38.680†	9.260†¥	3.868	0.926
MP Bio Fast RNA Extraction (Soil)	Bead Beating	11.435†	13.035†	3.812	4.345
	No Bead Beating	2.215†	2.090	0.738	0.697
QIAGEN RNeasy	Bead Beating	0.598†	0.296	1.993	1.021
	No Bead Beating	4.714†	2.724†	15.713	9.393

† 260/280 greater than 1.80

¥ 260/230 greater than 1.80

Table A2. Growth Time and Optical Density of *P. fluorescens* HK44 Cultures Harvested for RNA Extractions. RNA collected at these time points was used to generate transcriptomic libraries.

Growth Phase	25ºC	4ºC
Lag	2.5 hr OD 0.0970	24 hr OD 0.0911
Exponential	6.5 hr OD 0.5947	50 hr OD 0.7560
Stationary	10 hr OD 1.1107	58 hr OD 1.0613
Cold Shock	6.5 hr OD .6027	---

Table A3. Raw Data for Each Transcriptome. Between 80% and 90% of raw reads remained after quality trimming and transcript mapping to the *P. fluorescen* Pf0-1 genome.

Experiment	Number of Reads	Number of Reads After Trimming	Number of Mapped Reads
TA25 A	23,398,104	22,487,867	20,748,091
TA25 B	73,096,304	70,703,494	65,732,221
TA25 C	63,681,884	61,189,493	56,593,543
S25 A	72,595,398	69,820,495	63,902,342
S25 B	54,530,646	52,717,107	48,968,935
S25 C	59,303,898	55,919,957	51,124,797
TA4 A	50,655,720	48,649,741	43,553,263
TA4 B	54,344,704	51,578,972	46,321,658
TA4 C	46,738,420	44,663,671	40,050,338
S4 A	45,281,414	43,221,990	36,735,530
S4 B	54,619,670	52,201,583	44,882,800
S4 C	62,001,936	59,177,984	48,459,011

Table A4. Total Genes with Significant Differentially Expressed Genes.

Experiments	Genome Total Genes	Genes with Transcripts	Significant Change in Transcript p-value <0.05	Significant Gene with Fold Change > |2|	Significant Gene with Fold Change > |5|	Significant Gene with Fold Change > |10|
Control	5829	2955	293	80	23	16
Cold Shock 1	5829	3028	1620	569	179	92
Cold Shock 2	5829	3065	1933	724	221	103
Cold Adapted	5829	2754	1075	358	112	53
Cold Adapted vs. Cold Shock	5829	2807	2400	1001	361	181

Table A5. Differentially Expressed Genes Classified as Up or Down- Regulated

Experiments Compared	Significant Genes Up-regulated >|2| Fold	Significant Genes Down-regulated >|2| Fold	Significant Genes Up-regulated >|5| Fold	Significant Genes Down-regulated >|5| Fold	Significant Genes Up-regulated >|10| Fold	Significant Genes Down-regulated >|10| Fold
Control	27	53	11	12	10	6
Cold Shock 1	429	140	106	73	48	44
Cold Shock 2	535	189	134	87	70	46
Cold Adapted	94	264	46	66	26	27
Cold Adapted vs Cold Shock	688	313	209	152	93	88
Cold Adapted	94	264	46	66	26	27

Table A6. Fold Changes in Expression of DEAD-box Genes.

P. fluorescens HK44	*P. fluorescens* Pf0-1 DEAD/DEAH Gene	Control	Cold Shock 1	Cold Shock 2	Cold Adapted	Cold Adapted vs. Cold Shock
HisP	Pfl01_1205	ND	ND	ND	ND	ND
SrmB	Pfl01_1437	-1.01	1.40†	1.42†	1.04	1.35†
DinG	Pfl01_2690	0.65	-0.12†	-0.18	-0.89	-0.10†
DeaD	Pfl01_3987	1.21	1.67†	1.38†	1.11	1.50†
RNA Helicase	Pfl01_4920	-1.18	1.59†	1.88†	-1.35	2.45†
Helicase	Pfl01_5032	0.67	-1.01	-1.5	-0.46†	-0.21†
RhlE	Pfl01_5282	1.09	1.47†	1.35†	1.1	1.33†

† Significant p-value (<0.05)

Table A7. Significant Genes During Cold Adapted Experiment After FDR P-value Corrections

Gene Funciton	Gene Name	Fold Change	P-value	FDR P-value
carbamate kinase	Pfl01_4388	-39.67	1.51E-04	5.28E-02
arginine/ornithine antiporter	Pfl01_4385	-18.87	1.64E-04	5.32E-02
ornithine carbamoyltransferase	Pfl01_4387	-13.58	1.15E-04	5.16E-02
DNA-(apurinic or apyrimidinic site) lyase / endonuclease III	Pfl01_4513	-6.19	1.72E-06	1.00E-02
chaperone clpB	Pfl01_4834	-2.60	1.54E-04	5.28E-02
hypothetical protein	Pfl01_1013	0.11	1.27E-04	5.27E-02
cbb3-type cytochrome c oxidase subunit I	Pfl01_2512	0.29	4.23E-06	1.23E-02
cytochrome c oxidase subunit II	Pfl01_0079	0.67	4.19E-05	4.56E-02
PTS fructose IIC component	Pfl01_0795	0.90	1.38E-04	5.28E-02
D-lactate dehydrogenase	Pfl01_4223	0.90	6.26E-05	4.56E-02
hypothetical protein	Pfl01_1791	1.17	1.14E-04	5.16E-02
phosphoenolpyruvate--protein phosphotransferase	Pfl01_0793	1.59	1.09E-04	5.16E-02
FAD dependent oxidoreductase	Pfl01_4841	2.32	5.78E-05	4.56E-02
PfkB	Pfl01_0794	2.59	9.05E-05	5.16E-02
cytochrome c oxidase cbb3-type subunit III	Pfl01_1824	3.17	4.47E-05	4.56E-02
two component, sigma-54 specific, Fis family transcriptional regulator	Pfl01_1534	3.78	7.14E-05	4.63E-02
endoribonuclease L-PSP	Pfl01_5525	8.68	4.41E-05	4.56E-02
peptidase U32	Pfl01_3718	13.10	6.16E-05	4.56E-02

Table A8. Gene Expression Values Surrounding *cspA* 1 in Cold Shock 1 Experiment. (See Table 9) The gene numbers correspond to Figure 7.

Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	arginine/ornithine antiporter	Pfl01_4384	ND	ND	ND
2	arginine/ornithine antiporter	Pfl01_4385	-1.0932	0.1706	0.4341
3	arginine deiminase	Pfl01_4386	1.0490	0.4967	0.9993
4	ornithine carbamoyltransferase	Pfl01_4387	1.0365	0.5864	0.9993
5	carbamate kinase	Pfl01_4388	1.0579	0.4109	0.8933
6	hypothetical protein	Pfl01_4389	ND	ND	ND
7	transcriptional regulator TyrR	Pfl01_4390	1.4745	0.0340	0.1430
8	glycine cleavage system protein H	Pfl01_4391	1.2281	0.2084	0.5093
9	glycine dehydrogenase	Pfl01_4392	1.3396	0.0328	0.1407
10	L-serine ammonia-lyase	Pfl01_4393	-11.8694	0.0011	0.0425
11	glycine cleavage system T protein	Pfl01_4394	2.4699	0.0007	0.0400
12	**cspA 1**	**Pfl01_4395**	**1.2798**	**0.0021**	**0.0443**
13	hypothetical protein	Pfl01_4396	-3.4825	0.1881	0.4695
14	quinolinate synthetase	Pfl01_4397	1.2780	0.0324	0.1397

ND Not Detected

Table A9. Gene Expression Values Surrounding *cspA* 1 in Cold Shock 2 Experiment. (See Table 9) The gene numbers correspond to Figure 7.

Figure 7 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	arginine/ornithine antiporter	Pfl01_4384	ND	ND	ND
2	arginine/ornithine antiporter	Pfl01_4385	1.1018	0.1984	0.4230
3	arginine deiminase	Pfl01_4386	1.1820	0.0181	0.0941
4	ornithine carbamoyltransferase	Pfl01_4387	1.2265	0.0073	0.0793
5	carbamate kinase	Pfl01_4388	1.2900	0.0041	0.0773
6	hypothetical protein	Pfl01_4389	ND	ND	ND
7	transcriptional regulator TyrR	Pfl01_4390	1.7625	0.0205	0.0994
8	glycine cleavage system protein H	Pfl01_4391	0.0000	0.0000	0.0000
9	glycine dehydrogenase	Pfl01_4392	1.8529	0.0021	0.0773
10	L-serine ammonia-lyase	Pfl01_4393	-12.2590	0.0007	0.0757
11	glycine cleavage system T protein	Pfl01_4394	2.7550	0.0056	0.0782
12	**cspA 1**	**Pfl01_4395**	**1.0729**	**0.1963**	**0.4197**
13	hypothetical protein	Pfl01_4396	-1.0839	0.0342	0.1219
14	quinolinate synthetase	Pfl01_4397	1.4179	0.0315	0.1171

ND Not Detected

Table A10. Gene Expression Values Surrounding *cspA* 1 in Control Experiment. (See Table 9)

The gene numbers correspond to Figure 7.

Figure 7 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	arginine/ornithine antiporter	Pfl01_4384	ND	ND	ND
2	arginine/ornithine antiporter	Pfl01_4385	-1.2044	0.0411	0.9991
3	arginine deiminase	Pfl01_4386	-1.1268	0.1838	0.9991
4	ornithine carbamoyltransferase	Pfl01_4387	-1.1833	0.0718	0.9991
5	carbamate kinase	Pfl01_4388	-1.2195	0.0663	0.9991
6	hypothetical protein	Pfl01_4389	ND	ND	ND
7	transcriptional regulator TyrR	Pfl01_4390	-1.1954	0.2992	0.9991
8	glycine cleavage system protein H	Pfl01_4391	-19.6711	0.0064	0.8780
9	glycine dehydrogenase	Pfl01_4392	-1.3831	0.0574	0.9991
10	L-serine ammonia-lyase	Pfl01_4393	0.9682	0.9830	0.9991
11	glycine cleavage system T protein	Pfl01_4394	-1.1154	0.6938	0.9991
12	**cspA 1**	**Pfl01_4395**	**1.1929**	**0.0142**	**0.9171**
13	hypothetical protein	Pfl01_4396	-0.3112	0.3330	0.9991
14	quinolinate synthetase	Pfl01_4397	-1.1095	0.3724	0.9991

ND Not Detected

Table A11. Gene Expression Values Surrounding *cspA* 2 in Cold Adapted Experiment. (See Table 9) The gene numbers correspond to Figure 8.

Figure 8 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	secretion protein HlyD	Pfl01_1079	2.0517	0.0840	0.3695
2	acriflavin resistance protein	Pfl01_1080	31.7924	0.0329	0.2155
3	filamentation induced by cAMP protein Fic	Pfl01_1081	-0.6058	0.2437	0.7584
4	hypothetical protein	Pfl01_1082	1.0856	0.9167	0.9946
5	putative lipoprotein	Pfl01_1083	ND	ND	ND
6	glycerol-3-phosphate acyltransferase	Pfl01_1084	1.0797	0.7862	0.9946
7	**cspA 2**	**Pfl01_1085**	**-1.6457**	**0.0184**	**0.1620**
8	hypothetical protein	Pfl01_1086	1.1910	0.0204	0.1685
9	rRNA (guanine-N(1)-)-methyltransferase	Pfl01_1087	-1.1368	0.5662	0.9946
10	succinyl-diaminopimelate desuccinylase	Pfl01_1088	-1.1543	0.3504	0.9839
11	glycosyl transferase family protein	Pfl01_1089	-1.2578	0.1869	0.6261
12	UBA/THIF-type NAD/FAD binding domain-containing protein	Pfl01_1090	-1.1321	0.5154	0.9946
13	Fe-S metabolism associated SufE	Pfl01_1091	0.6306	0.5456	0.9946
14	aromatic amino acid beta-eliminating lyase/threonine aldolase	Pfl01_1092	1.1561	0.7088	0.9946
15	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	Pfl01_1093	-1.1100	0.1007	0.4186
16	hypothetical protein	Pfl01_1094	1.1090	0.1220	0.4721

ND Not Detected

Table A12. Gene Expression Values Surrounding *cspA* 2 in Cold Shock 2 Experiment. (See Table 9) The gene numbers correspond to Figure 8.

Figure 8 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	secretion protein HlyD	Pfl01_1079	0.1840	0.2165	0.4552
2	acriflavin resistance protein	Pfl01_1080	2.0300	0.0292	0.1121
3	filamentation induced by cAMP protein Fic	Pfl01_1081	ND	ND	ND
4	hypothetical protein	Pfl01_1082	0.1655	0.1609	0.3552
5	putative lipoprotein	Pfl01_1083	ND	ND	ND
6	glycerol-3-phosphate acyltransferase	Pfl01_1084	2.2676	0.0135	0.0877
7	**cspA 2**	**Pfl01_1085**	**3.2515**	**0.0582**	**0.1661**
8	hypothetical protein	Pfl01_1086	2.5073	0.0163	0.0896
9	rRNA (guanine-N(1)-)-methyltransferase	Pfl01_1087	4.2481	0.0152	0.0884
10	succinyl-diaminopimelate desuccinylase	Pfl01_1088	1.6343	0.0221	0.1018
11	glycosyl transferase family protein	Pfl01_1089	1.6220	0.0088	0.0803
12	UBA/THIF-type NAD/FAD binding domain-containing protein	Pfl01_1090	3.2253	0.0073	0.0793
13	Fe-S metabolism associated SufE	Pfl01_1091	0.0145	0.1040	0.2502
14	aromatic amino acid beta-eliminating lyase/threonine aldolase	Pfl01_1092	2.7300	0.0125	0.0851
15	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	Pfl01_1093	1.2673	0.0286	0.1116

ND Not Detected

Table A13. Gene Expression Values Surrounding *cspA* 2 in Control Experiment. (See Table 9)

The gene numbers correspond to Figure 8.

Figure 8 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	secretion protein HlyD	Pfl01_1079	-0.1615	0.3444	0.9991
2	acriflavin resistance protein	Pfl01_1080	-1.1221	0.6479	0.9991
3	filamentation induced by cAMP protein Fic	Pfl01_1081	ND	ND	ND
4	hypothetical protein	Pfl01_1082	-0.6367	0.3930	0.9991
5	putative lipoprotein	Pfl01_1083	ND	ND	ND
6	glycerol-3-phosphate acyltransferase	Pfl01_1084	1.0111	0.9640	0.9991
7	**cspA 2**	**Pfl01_1085**	**4.6544**	**0.4000**	**0.9991**
8	hypothetical protein	Pfl01_1086	1.4826	0.3080	0.9991
9	rRNA (guanine-N(1)-)-methyltransferase	Pfl01_1087	2.7748	0.4021	0.9991
10	succinyl-diaminopimelate desuccinylase	Pfl01_1088	-1.0731	0.6228	0.9991
11	glycosyl transferase family protein	Pfl01_1089	-1.0612	0.6145	0.9991
12	UBA/THIF-type NAD/FAD binding domain-containing protein	Pfl01_1090	-1.2605	0.4119	0.9991
13	Fe-S metabolism associated SufE	Pfl01_1091	-0.9341	0.8890	0.9991
14	aromatic amino acid beta-eliminating lyase/threonine aldolase	Pfl01_1092	1.7913	0.4541	0.9991
15	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	Pfl01_1093	-1.0537	0.4863	0.9991
16	hypothetical protein	Pfl01_1094	1.0904	0.5941	0.9991

ND Not Detected

Table A14. Gene Expression Values Surrounding *cspA* 2 in Cold Adapted vs Cold Shock Experiment. (See Table 9) The gene numbers correspond to Figure 8.

Figure 8 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	secretion protein HlyD	Pfl01_1079	0.0922	0.0108	0.0368
2	acriflavin resistance protein	Pfl01_1080	5.7714	0.0041	0.0214
3	filamentation induced by cAMP protein Fic	Pfl01_1081	0.5120	0.0940	0.2147
4	hypothetical protein	Pfl01_1082	0.2598	0.2117	0.4631
5	putative lipoprotein	Pfl01_1083	ND	ND	ND
6	glycerol-3-phosphate acyltransferase	Pfl01_1084	2.1816	0.0109	0.0369
7	**cspA 2**	**Pfl01_1085**	**-1.0839**	**0.0024**	**0.0174**
8	hypothetical protein	Pfl01_1086	2.1426	0.0081	0.0306
9	rRNA (guanine-N(1)-)-methyltransferase	Pfl01_1087	7252.9694	0.0020	0.0164
10	succinyl-diaminopimelate desuccinylase	Pfl01_1088	1.8687	0.0150	0.0463
11	glycosyl transferase family protein	Pfl01_1089	1.8985	0.0032	0.0193
12	UBA/THIF-type NAD/FAD binding domain-containing protein	Pfl01_1090	3.5554	0.0108	0.0368
13	Fe-S metabolism associated SufE	Pfl01_1091	0.0130	0.0153	0.0468
14	aromatic amino acid beta-eliminating lyase/threonine aldolase	Pfl01_1092	3.7167	0.0019	0.0162
15	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	Pfl01_1093	1.3731	0.0048	0.0230
16	hypothetical protein	Pfl01_1094	1.4402	0.0094	0.0338

ND Not Detected

Figure A1. Gene Order Surrounding *P. fluorescens* HK44 *cspA* 3. Genes highlighted in the same color are part of the same operon. 1, putative lipoprotein; 2, prolyl-tRNA synthetase; 3, AmpG permease; 4, methylated-DNA-(protein)-cysteine S-methyltransferase; 5, hypothetical protein; 6, cspA 3; 7, deoxycytidine triphosphate deaminase; 8, hypothetical protein
9, metallophosphoesterase; 10, DEAD/DEAH box helicase-like protein; 11, ABC transporter-like protein; 12, succinylglutamate desuccinylase/aspartoacylase; 13, amino acid ABC transporter permease; 14, amino acid ABC transporter permease

Table A15. Gene Expression Values Surrounding *cspA* 3 in Cold Adapted Experiment. (See Table 9) The gene numbers correspond to Figure A1.

Figure A1 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	putative lipoprotein	Pfl01_1196	-1.1379	0.3066	0.8897
2	prolyl-tRNA synthetase	Pfl01_1197	-1.0914	0.2532	0.7779
3	AmpG permease	Pfl01_1198	-1.5976	0.2320	0.7299
4	methylated-DNA-(protein)-cysteine S-methyltransferase	Pfl01_1199	-0.8019	0.0515	0.2760
5	hypothetical protein	Pfl01_1200	ND	ND	ND
6	cspA 3	Pfl01_1201	**1.2402**	**0.0043**	**0.1029**
7	deoxycytidine triphosphate deaminase	Pfl01_1202	-1.3279	0.0122	0.1393
8	hypothetical protein	Pfl01_1203	-6.1609	0.4259	0.9946
9	metallophosphoesterase	Pfl01_1204	ND	ND	ND
10	DEAD/DEAH box helicase-like protein	Pfl01_1205	ND	ND	ND
11	ABC transporter-like protein	Pfl01_1206	1.0389	0.7609	0.9946
12	succinylglutamate desuccinylase/aspartoacylase	Pfl01_1207	-1.1616	0.4318	0.9946
13	amino acid ABC transporter permease	Pfl01_1208	1.0759	0.7883	0.9946
14	amino acid ABC transporter permease	Pfl01_1209	-1.1150	0.6041	0.9946

ND Not Detected

Table A16. Gene Expression Values Surrounding *cspA* 3 in Cold Shock 1 Experiment. (See Table 9) The gene numbers correspond to Figure A1.

Figure A1 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	putative lipoprotein	Pfl01_1196	1.5700	0.0110	0.0763
2	prolyl-tRNA synthetase	Pfl01_1197	1.1487	0.1362	0.3636
3	AmpG permease	Pfl01_1198	2.2735	0.0188	0.1014
4	methylated-DNA-(protein)-cysteine S-methyltransferase	Pfl01_1199	0.1299	0.0023	0.0455
5	hypothetical protein	Pfl01_1200	ND	ND	ND
6	cspA 3	Pfl01_1201	**1.4771**	**0.0095**	**0.0726**
7	deoxycytidine triphosphate deaminase	Pfl01_1202	1.3020	0.0656	0.2182
8	hypothetical protein	Pfl01_1203	0.4520	0.0322	0.1391
9	metallophosphoesterase	Pfl01_1204	ND	ND	ND
10	DEAD/DEAH box helicase-like protein	Pfl01_1205	ND	ND	ND
11	ABC transporter-like protein	Pfl01_1206	1.9036	0.0191	0.1020
12	succinylglutamate desuccinylase/aspartoacylase	Pfl01_1207	1.9312	0.0419	0.1615
13	amino acid ABC transporter permease	Pfl01_1208	2.4165	0.0898	0.2690
14	amino acid ABC transporter permease	Pfl01_1209	6.4215	0.0107	0.0754

ND Not Detected

Table A17. Gene Expression Values Surrounding *cspA* 3 in Cold Shock 2 Experiment. (See Table 9) The gene numbers correspond to Figure A1.

Figure A1 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	putative lipoprotein	Pfl01_1196	1.5141	0.0239	0.1044
2	prolyl-tRNA synthetase	Pfl01_1197	1.2066	0.0971	0.2369
3	AmpG permease	Pfl01_1198	2.4471	0.0189	0.0959
4	methylated-DNA-(protein)-cysteine S-methyltransferase	Pfl01_1199	0.1598	0.0120	0.0841
5	hypothetical protein	Pfl01_1200	ND	ND	ND
6	cspA 3	Pfl01_1201	**1.1811**	**0.1066**	**0.2545**
7	deoxycytidine triphosphate deaminase	Pfl01_1202	1.4639	0.0239	0.1044
8	hypothetical protein	Pfl01_1203	0.3870	0.0002	0.0649
9	metallophosphoesterase	Pfl01_1204	ND	ND	ND
10	DEAD/DEAH box helicase-like protein	Pfl01_1205	ND	ND	ND
11	ABC transporter-like protein	Pfl01_1206	1.8782	0.0229	0.1035
12	succinylglutamate desuccinylase/aspartoacylase	Pfl01_1207	2.1880	0.0237	0.1041
13	amino acid ABC transporter permease	Pfl01_1208	5.3103	0.0050	0.0782
14	amino acid ABC transporter permease	Pfl01_1209	-5.8336	0.0080	0.0799

ND Not Detected

Table A18. Gene Expression Values Surrounding *cspA* 3 in Control Experiment. (See Table 9)

The gene numbers correspond to Figure A1.

Figure A1 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	putative lipoprotein	Pfl01_1196	1.0369	0.7268	0.9991
2	prolyl-tRNA synthetase	Pfl01_1197	-1.0504	0.5104	0.9991
3	AmpG permease	Pfl01_1198	-1.0764	0.8377	0.9991
4	methylated-DNA-(protein)-cysteine S-methyltransferase	Pfl01_1199	0.8129	0.2280	0.9991
5	hypothetical protein	Pfl01_1200	ND	ND	ND
6	cspA 3	Pfl01_1201	**1.2506**	**0.0192**	**0.9745**
7	deoxycytidine triphosphate deaminase	Pfl01_1202	-1.1244	0.3341	0.9991
8	hypothetical protein	Pfl01_1203	-0.8561	0.3841	0.9991
9	metallophosphoesterase	Pfl01_1204	ND	ND	ND
10	DEAD/DEAH box helicase-like protein	Pfl01_1205	ND	ND	ND
11	ABC transporter-like protein	Pfl01_1206	1.0135	0.9468	0.9991
12	succinylglutamate desuccinylase/aspartoacylase	Pfl01_1207	-1.1329	0.6893	0.9991
13	amino acid ABC transporter permease	Pfl01_1208	-2.1976	0.4317	0.9991
14	amino acid ABC transporter permease	Pfl01_1209	0.9085	0.2187	0.9991

ND Not Detected

Table A19. Gene Expression Values Surrounding *cspA* 3 in Cold Adapted vs Cold Shock Experiment. (See Table 9) The gene numbers correspond to Figure A1.

Figure A1 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	putative lipoprotein	Pfl01_1196	1.9551	0.0117	0.0388
2	prolyl-tRNA synthetase	Pfl01_1197	1.2694	0.0398	0.0998
3	AmpG permease	Pfl01_1198	4.6698	0.0056	0.0249
4	methylated-DNA-(protein)-cysteine S-methyltransferase	Pfl01_1199	0.1996	0.0263	0.0708
5	hypothetical protein	Pfl01_1200	ND	ND	ND
6	cspA 3	Pfl01_1201	**1.0634**	**0.4643**	**0.9669**
7	deoxycytidine triphosphate deaminase	Pfl01_1202	2.2161	0.0017	0.0155
8	hypothetical protein	Pfl01_1203	0.3591	0.0250	0.0683
9	metallophosphoesterase	Pfl01_1204	ND	ND	ND
10	DEAD/DEAH box helicase-like protein	Pfl01_1205	ND	ND	ND
11	ABC transporter-like protein	Pfl01_1206	1.8012	0.0385	0.0969
12	succinylglutamate desuccinylase/aspartoacylase	Pfl01_1207	2.5496	0.0097	0.0342
13	amino acid ABC transporter permease	Pfl01_1208	2.0737	0.0292	0.0770
14	amino acid ABC transporter permease	Pfl01_1209	27.7380	0.0071	0.0284

ND Not Detected

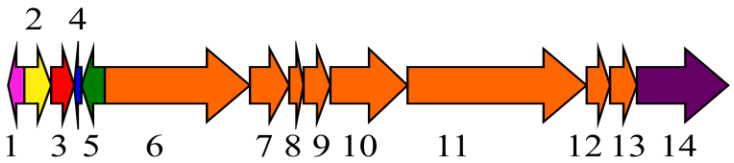

Figure A2. Gene Order Surrounding *P. fluorescens* HK44 *cspG*. Genes highlighted in the same color are part of the same operon. 1, hypothetical protein; 2, hypothetical protein; 3, hypothetical protein; 4, cspG; 5, hypothetical protein; 6, threonyl-tRNA synthetase; 7, translation initiation factor IF-3; 8, 50S ribosomal protein L35; 9, 50S ribosomal protein L20; 10, phenylalanyl-tRNA synthetase subunit alpha; 11, phenylalanyl-tRNA synthetase subunit beta; 12, integration host factor subunit alpha; 13, MerR family transcriptional regulator; 14, DNA helicase-like protein

Table A20. Gene Expression Values Surrounding *cspG* in Cold Adapted Experiment. (See Table 9) The gene numbers correspond to Figure A2.

Figure A2 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	hypothetical protein	Pfl01_1926	ND	ND	ND
2	hypothetical protein	Pfl01_1927	ND	ND	ND
3	hypothetical protein	Pfl01_1928	ND	ND	ND
4	cspG	Pfl01_1929	**1.0731**	**0.0256**	**0.1866**
5	hypothetical protein	Pfl01_1930	ND	ND	ND
6	threonyl-tRNA synthetase	Pfl01_1931	-1.0058	0.9088	0.9946
7	translation initiation factor IF-3	Pfl01_1932	1.0518	0.0653	0.3173
8	50S ribosomal protein L35	Pfl01_1933	1.0046	0.9376	0.9946
9	50S ribosomal protein L20	Pfl01_1934	1.0200	0.2272	0.7183
10	phenylalanyl-tRNA synthetase subunit alpha	Pfl01_1935	-1.0737	0.4888	0.9946
11	phenylalanyl-tRNA synthetase subunit beta	Pfl01_1936	-1.0717	0.4912	0.9946
12	integration host factor subunit alpha	Pfl01_1937	-1.1066	0.0131	0.1433
13	MerR family transcriptional regulator	Pfl01_1938	-1.1147	0.0491	0.2675
14	DNA helicase-like protein	Pfl01_1939	ND	ND	ND

ND Not Detected

Table A21. Gene Expression Values Surrounding *cspG* in Cold Shock 1 Experiment. (See Table 9) The gene numbers correspond to Figure A2.

Figure A2 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	hypothetical protein	Pfl01_1926	ND	ND	ND
2	hypothetical protein	Pfl01_1927	ND	ND	ND
3	hypothetical protein	Pfl01_1928	ND	ND	ND
4	cspG	Pfl01_1929	**1.1888**	**0.0004**	**0.0400**
5	hypothetical protein	Pfl01_1930	ND	ND	ND
6	threonyl-tRNA synthetase	Pfl01_1931	1.3879	0.0009	0.0404
7	translation initiation factor IF-3	Pfl01_1932	1.2754	0.0001	0.0400
8	50S ribosomal protein L35	Pfl01_1933	1.3965	0.0166	0.0951
9	50S ribosomal protein L20	Pfl01_1934	1.2997	0.0031	0.0487
10	phenylalanyl-tRNA synthetase subunit alpha	Pfl01_1935	1.2517	0.0644	0.2159
11	phenylalanyl-tRNA synthetase subunit beta	Pfl01_1936	1.2623	0.0375	0.1516
12	integration host factor subunit alpha	Pfl01_1937	1.2032	0.0106	0.0754
13	MerR family transcriptional regulator	Pfl01_1938	1.5515	0.0024	0.0462
14	DNA helicase-like protein	Pfl01_1939	ND	ND	ND

ND Not Detected

Table A22. Gene Expression Values Surrounding *cspG* in Cold Shock 2 Experiment. (See Table 9) The gene numbers correspond to Figure A2.

Figure A2 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	hypothetical protein	Pfl01_1926	ND	ND	ND
2	hypothetical protein	Pfl01_1927	ND	ND	ND
3	hypothetical protein	Pfl01_1928	ND	ND	ND
4	cspG	Pfl01_1929	**1.1817**	**0.0005**	**0.0704**
5	hypothetical protein	Pfl01_1930	ND	ND	ND
6	threonyl-tRNA synthetase	Pfl01_1931	1.2929	0.0034	0.0773
7	translation initiation factor IF-3	Pfl01_1932	1.1197	0.0141	0.0877
8	50S ribosomal protein L35	Pfl01_1933	1.0954	0.1894	0.4069
9	50S ribosomal protein L20	Pfl01_1934	1.0985	0.1600	0.3537
10	phenylalanyl-tRNA synthetase subunit alpha	Pfl01_1935	1.2616	0.0583	0.1661
11	phenylalanyl-tRNA synthetase subunit beta	Pfl01_1936	1.2735	0.0471	0.1450
12	integration host factor subunit alpha	Pfl01_1937	1.3034	0.0077	0.0797
13	MerR family transcriptional regulator	Pfl01_1938	1.7659	0.0085	0.0803
14	DNA helicase-like protein	Pfl01_1939	ND	ND	ND

ND Not Detected

Table A23. Gene Expression Values Surrounding *cspG* in Control Experiment. (See Table 9)

The gene numbers correspond to Figure A2.

Figure A2 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	hypothetical protein	Pfl01_1926	ND	ND	ND
2	hypothetical protein	Pfl01_1927	ND	ND	ND
3	hypothetical protein	Pfl01_1928	ND	ND	ND
4	cspG	Pfl01_1929	**1.0061**	**0.8107**	**0.9991**
5	hypothetical protein	Pfl01_1930	ND	ND	ND
6	threonyl-tRNA synthetase	Pfl01_1931	1.0735	0.1770	0.9991
7	translation initiation factor IF-3	Pfl01_1932	1.1391	0.0092	0.8780
8	50S ribosomal protein L35	Pfl01_1933	1.2749	0.0437	0.9991
9	50S ribosomal protein L20	Pfl01_1934	1.1831	0.0245	0.9745
10	phenylalanyl-tRNA synthetase subunit alpha	Pfl01_1935	-1.0080	0.9278	0.9991
11	phenylalanyl-tRNA synthetase subunit beta	Pfl01_1936	-1.0088	0.9215	0.9991
12	integration host factor subunit alpha	Pfl01_1937	-1.0833	0.1307	0.9991
13	MerR family transcriptional regulator	Pfl01_1938	-1.1382	0.3821	0.9991
14	DNA helicase-like protein	Pfl01_1939	ND	ND	ND

ND Not Detected

Table A24. Gene Expression Values Surrounding *cspG* in Cold Adapted vs Cold Shock Experiment. (See Table 9) The gene numbers correspond to Figure A2.

Figure A2 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	hypothetical protein	Pfl01_1926	ND	ND	ND
2	hypothetical protein	Pfl01_1927	ND	ND	ND
3	hypothetical protein	Pfl01_1928	ND	ND	ND
4	cspG	Pfl01_1929	**1.0747**	**0.0811**	**0.1876**
5	hypothetical protein	Pfl01_1930	ND	ND	ND
6	threonyl-tRNA synthetase	Pfl01_1931	1.4025	0.0008	0.0132
7	translation initiation factor IF-3	Pfl01_1932	1.2012	0.0008	0.0132
8	50S ribosomal protein L35	Pfl01_1933	1.3939	0.0088	0.0322
9	50S ribosomal protein L20	Pfl01_1934	1.2699	0.0039	0.0211
10	phenylalanyl-tRNA synthetase subunit alpha	Pfl01_1935	1.3775	0.0368	0.0935
11	phenylalanyl-tRNA synthetase subunit beta	Pfl01_1936	1.3586	0.0170	0.0508
12	integration host factor subunit alpha	Pfl01_1937	1.4440	0.0020	0.0164
13	MerR family transcriptional regulator	Pfl01_1938	2.0027	0.0010	0.0135
14	DNA helicase-like protein	Pfl01_1939	ND	ND	ND

ND Not Detected

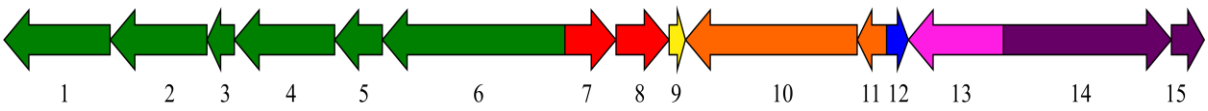

Figure A3. Gene Order Surrounding *P. fluorescens* HK44 *cspD*. Genes highlighted in the same color are part of the same operon. 1, precorrin-2 dehydrogenase / uroporphyrinogen-III C-methyltransferase; 2, seryl-tRNA synthetase; 3, camphor resistance protein CrcB; 4, recombination factor protein RarA; 5, outer-membrane lipoprotein carrier protein; 6, DNA translocase FtsK; 7, leucyl/phenylalanyl-tRNA--protein transferase; 8, arginyl-tRNA-protein transferase; 9, translation initiation factor IF-1; 10, ATP-dependent Clp protease ATP-binding subunit ClpA; 11, ATP-dependent Clp protease adaptor protein ClpS; 12, cspD; 13, isocitrate dehydrogenase (NADP); 14, isocitrate dehydrogenase (NADP+); 15, NUDIX hydrolase

Table A25. Gene Expression Values Surrounding *cspD* in the Cold Adapted Experiment. (See Table 9) The gene numbers correspond to Figure A3.

Figure A3 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	precorrin-2 dehydrogenase / uroporphyrinogen-III C-methyltransferase	Pfl01_3581	-1.2528	0.1017	0.4209
2	seryl-tRNA synthetase	Pfl01_3582	-1.1567	0.0594	0.3005
3	camphor resistance protein CrcB	Pfl01_3583	-1.3072	0.0064	0.1117
4	recombination factor protein RarA	Pfl01_3584	-2.1824	0.0994	0.4149
5	outer-membrane lipoprotein carrier protein	Pfl01_3585	-1.0644	0.4326	0.9946
6	DNA translocase FtsK	Pfl01_3586	-1.1584	0.0988	0.4139
7	leucyl/phenylalanyl-tRNA--protein transferase	Pfl01_3587	0.6073	0.5005	0.9946
8	arginyl-tRNA-protein transferase	Pfl01_3588	-0.2897	0.1650	0.5825
9	translation initiation factor IF-1	Pfl01_3589	1.2002	0.0202	0.1675
10	ATP-dependent Clp protease ATP-binding subunit ClpA	Pfl01_3590	-1.3013	0.0089	0.1231
11	ATP-dependent Clp protease adaptor protein ClpS	Pfl01_3591	-1.3340	0.0058	0.1094
12	cspD	Pfl01_3592	**ND**	**ND**	**ND**
13	isocitrate dehydrogenase (NADP)	Pfl01_3593	-1.0827	0.4015	0.9946
14	isocitrate dehydrogenase (NADP+)	Pfl01_3594	1.0877	0.1499	0.5425
15	NUDIX hydrolase	Pfl01_3595	1.7801	0.6131	0.9946

ND Not Detected

Table A26. Gene Expression Values Surrounding *cspD* in Cold Shock 1 Experiment. (See Table 9) The gene numbers correspond to Figure A3.

Figure A3 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	precorrin-2 dehydrogenase / uroporphyrinogen-III C-methyltransferase	Pfl01_3581	1.8609	0.0025	0.0462
2	seryl-tRNA synthetase	Pfl01_3582	1.1888	0.0241	0.1176
3	camphor resistance protein CrcB	Pfl01_3583	1.8328	0.0097	0.0735
4	recombination factor protein RarA	Pfl01_3584	3.3184	0.0041	0.0531
5	outer-membrane lipoprotein carrier protein	Pfl01_3585	2.4982	0.0013	0.0425
6	DNA translocase FtsK	Pfl01_3586	1.4823	0.0038	0.0513
7	leucyl/phenylalanyl-tRNA--protein transferase	Pfl01_3587	0.4554	0.0518	0.1848
8	arginyl-tRNA-protein transferase	Pfl01_3588	0.3311	0.0884	0.2657
9	translation initiation factor IF-1	Pfl01_3589	2.2286	0.0005	0.0400
10	ATP-dependent Clp protease ATP-binding subunit ClpA	Pfl01_3590	1.0730	0.2245	0.5422
11	ATP-dependent Clp protease adaptor protein ClpS	Pfl01_3591	1.0005	0.9965	0.9993
12	cspD	Pfl01_3592	**ND**	**ND**	**ND**
13	isocitrate dehydrogenase (NADP)	Pfl01_3593	-1.0097	0.8500	0.9993
14	isocitrate dehydrogenase (NADP+)	Pfl01_3594	1.0099	0.9150	0.9993
15	NUDIX hydrolase	Pfl01_3595	0.0415	0.0456	0.1705

ND Not Detected

Table A27. Gene Expression Values Surrounding *cspD* in Cold Shock 2 Experiment. (See Table 9) The gene numbers correspond to Figure A3.

Figure A3 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	precorrin-2 dehydrogenase / uroporphyrinogen-III C-methyltransferase	Pfl01_3581	1.6734	0.0121	0.0841
2	seryl-tRNA synthetase	Pfl01_3582	1.2256	0.0293	0.1123
3	camphor resistance protein CrcB	Pfl01_3583	2.5637	0.0140	0.0877
4	recombination factor protein RarA	Pfl01_3584	2.4141	0.0279	0.1106
5	outer-membrane lipoprotein carrier protein	Pfl01_3585	1.9365	0.0071	0.0793
6	DNA translocase FtsK	Pfl01_3586	1.5013	0.0108	0.0810
7	leucyl/phenylalanyl-tRNA--protein transferase	Pfl01_3587	0.4264	0.0306	0.1153
8	arginyl-tRNA-protein transferase	Pfl01_3588	0.2780	0.0485	0.1476
9	translation initiation factor IF-1	Pfl01_3589	1.8709	0.0018	0.0773
10	ATP-dependent Clp protease ATP-binding subunit ClpA	Pfl01_3590	1.4549	0.0074	0.0793
11	ATP-dependent Clp protease adaptor protein ClpS	Pfl01_3591	2.2909	0.0102	0.0803
12	cspD	Pfl01_3592	**ND**	**ND**	**ND**
13	isocitrate dehydrogenase (NADP)	Pfl01_3593	1.3172	0.0161	0.0896
14	isocitrate dehydrogenase (NADP+)	Pfl01_3594	1.1394	0.2258	0.4729
15	NUDIX hydrolase	Pfl01_3595	0.0259	0.0273	0.1098

ND Not Detected

Table A28. Gene Expression Values Surrounding *cspD* in Control Experiment. (See Table 9)

The gene numbers correspond to Figure A3.

Figure A3 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	precorrin-2 dehydrogenase / uroporphyrinogen-III C-methyltransferase	Pfl01_3581	1.1120	0.4978	0.9991
2	seryl-tRNA synthetase	Pfl01_3582	-1.0310	0.6955	0.9991
3	camphor resistance protein CrcB	Pfl01_3583	-1.3988	0.2334	0.9991
4	recombination factor protein RarA	Pfl01_3584	1.3746	0.5775	0.9991
5	outer-membrane lipoprotein carrier protein	Pfl01_3585	1.2901	0.1208	0.9991
6	DNA translocase FtsK	Pfl01_3586	-1.0128	0.8996	0.9991
7	leucyl/phenylalanyl-tRNA--protein transferase	Pfl01_3587	-0.9365	0.7153	0.9991
8	arginyl-tRNA-protein transferase	Pfl01_3588	-0.8394	0.5628	0.9991
9	translation initiation factor IF-1	Pfl01_3589	1.1912	0.1700	0.9991
10	ATP-dependent Clp protease ATP-binding subunit ClpA	Pfl01_3590	-1.3559	0.0178	0.9745
11	ATP-dependent Clp protease adaptor protein ClpS	Pfl01_3591	-2.2897	0.0043	0.8780
12	cspD	Pfl01_3592	**ND**	**ND**	**ND**
13	isocitrate dehydrogenase (NADP)	Pfl01_3593	-1.3300	0.0229	0.9745
14	isocitrate dehydrogenase (NADP+)	Pfl01_3594	-1.1282	0.1193	0.9991
15	NUDIX hydrolase	Pfl01_3595	-0.6244	0.2433	0.9991

ND Not Detected

Table A29. Gene Expression Values Surrounding *cspD* in Cold Adapted vs. Cold Shock Experiment. (See Table 9) The gene numbers correspond to Figure A3.

Figure A3 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	precorrin-2 dehydrogenase / uroporphyrinogen-III C-methyltransferase	Pfl01_3581	2.5450	0.0008	0.0132
2	seryl-tRNA synthetase	Pfl01_3582	1.4207	0.0007	0.0132
3	camphor resistance protein CrcB	Pfl01_3583	6.8902	0.0016	0.0153
4	recombination factor protein RarA	Pfl01_3584	804.2263	0.0004	0.0131
5	outer-membrane lipoprotein carrier protein	Pfl01_3585	2.9876	0.0018	0.0158
6	DNA translocase FtsK	Pfl01_3586	1.7205	0.0009	0.0134
7	leucyl/phenylalanyl-tRNA--protein transferase	Pfl01_3587	0.5241	0.1143	0.2581
8	arginyl-tRNA-protein transferase	Pfl01_3588	0.2240	0.0087	0.0321
9	translation initiation factor IF-1	Pfl01_3589	1.4570	0.0111	0.0375
10	ATP-dependent Clp protease ATP-binding subunit ClpA	Pfl01_3590	1.3995	0.0017	0.0157
11	ATP-dependent Clp protease adaptor protein ClpS	Pfl01_3591	1.8481	0.0112	0.0377
12	cspD	Pfl01_3592	**ND**	**ND**	**ND**
13	isocitrate dehydrogenase (NADP)	Pfl01_3593	1.0906	0.4050	0.8619
14	isocitrate dehydrogenase (NADP+)	Pfl01_3594	-1.0719	0.3980	0.8477
15	NUDIX hydrolase	Pfl01_3595	0.0475	0.0333	0.0859

ND Not Detected

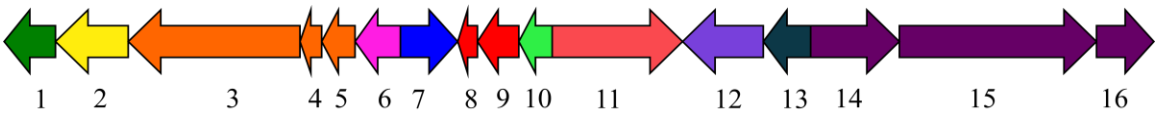

Figure A4. Gene Order Surrounding *P. fluorescens* HK44 *cspC*. Genes highlighted in the same color are part of the same operon. 1, Holliday junction resolvase; 2, hypothetical protein; 3, aspartyl-tRNA synthetase; 4, hypothetical protein; 5, hypothetical protein; 6, Ferritin and Dps; 7, cspC; 8, hypothetical protein; 9, histidine triad (HIT) protein; 10, hypothetical protein; 11, outer membrane porin; 12, mechanosensitive ion channel MscS; 13, putative nucleotide-binding protein; 14, 2-dehydropantoate 2-reductase; 15, multi-sensor Signal transduction histidine kinase; 16, ATP:cob(I)alamin adenosyltransferase

Table A30. Gene Expression Values Surrounding *cspC* in Cold Adapted Experiment. (See Table 9) The gene numbers correspond to Figure A4.

Figure A4 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	Holliday junction resolvase	Pfl01_4409	-1.0021	0.0014	0.0147
2	hypothetical protein	Pfl01_4410	22.0330	0.0036	0.0204
3	aspartyl-tRNA synthetase	Pfl01_4411	1.4201	0.0031	0.0192
4	hypothetical protein	Pfl01_4412	3.4911	0.0045	0.0224
5	hypothetical protein	Pfl01_4413	ND	ND	ND
6	Ferritin and Dps	Pfl01_4414	ND	ND	ND
7	cspC	Pfl01_4415	**2.1327**	**0.0227**	**0.0634**
8	hypothetical protein	Pfl01_4416	0.0000	#NUM!	#NUM!
9	histidine triad (HIT) protein	Pfl01_4417	0.0978	0.0675	0.1592
10	hypothetical protein	Pfl01_4418	ND	ND	ND
11	outer membrane porin	Pfl01_4419	2.4642	0.2125	0.4644
12	mechanosensitive ion channel MscS	Pfl01_4420	2.0900	0.0001	0.0131
13	putative nucleotide-binding protein	Pfl01_4421	1.7015	0.0031	0.0192
14	2-dehydropantoate 2-reductase	Pfl01_4422	ND	ND	ND
15	multi-sensor Signal transduction histidine kinase	Pfl01_4423	-3.8508	0.0050	0.0234
16	ATP:cob(I)alamin adenosyltransferase	Pfl01_4424	0.0932	0.0026	0.0178

ND Not Detected

Table A31. Gene Expression Values Surrounding *cspC* in Cold Shock 1 Experiment. (See Table 9) The gene numbers correspond to Figure A4.

Figure A4 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	Holliday junction resolvase	Pfl01_4409	-0.8219	0.0357	0.1472
2	hypothetical protein	Pfl01_4410	2.0210	0.0049	0.0564
3	aspartyl-tRNA synthetase	Pfl01_4411	1.1987	0.0356	0.1468
4	hypothetical protein	Pfl01_4412	2.0125	0.0074	0.0662
5	hypothetical protein	Pfl01_4413	ND	ND	ND
6	Ferritin and Dps	Pfl01_4414	ND	ND	ND
7	cspC	Pfl01_4415	**1.6310**	**0.0722**	**0.2325**
8	hypothetical protein	Pfl01_4416	0.6018	0.1249	0.3413
9	histidine triad (HIT) protein	Pfl01_4417	0.3095	0.4265	0.9211
10	hypothetical protein	Pfl01_4418	ND	ND	ND
11	outer membrane porin	Pfl01_4419	1.0243	0.9493	0.9993
12	mechanosensitive ion channel MscS	Pfl01_4420	1.7420	0.0003	0.0400
13	putative nucleotide-binding protein	Pfl01_4421	1.2605	0.0367	0.1495
14	2-dehydropantoate 2-reductase	Pfl01_4422	-0.8685	0.6727	0.9993
15	multi-sensor Signal transduction histidine kinase	Pfl01_4423	3.4761	0.0443	0.1673
16	ATP:cob(I)alamin adenosyltransferase	Pfl01_4424	0.2517	0.0554	0.1942

ND Not Detected

Table A32. Gene Expression Values Surrounding *cspC* in Cold Shock 2 Experiment. (See Table 9) The gene numbers correspond to Figure A4.

Figure A4 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	Holliday junction resolvase	Pfl01_4409	1.2719	0.0333	0.1198
2	hypothetical protein	Pfl01_4410	1.8408	0.0243	0.1049
3	aspartyl-tRNA synthetase	Pfl01_4411	-3.1292	0.0110	0.0819
4	hypothetical protein	Pfl01_4412	2.1959	0.0064	0.0793
5	hypothetical protein	Pfl01_4413	ND	ND	ND
6	Ferritin and Dps	Pfl01_4414	ND	ND	ND
7	cspC	Pfl01_4415	**1.7036**	**0.0659**	**0.1797**
8	hypothetical protein	Pfl01_4416	0.5538	0.1076	0.2562
9	histidine triad (HIT) protein	Pfl01_4417	0.0734	0.0458	0.1430
10	hypothetical protein	Pfl01_4418	ND	ND	ND
11	outer membrane porin	Pfl01_4419	4.0523	0.0945	0.2323
12	mechanosensitive ion channel MscS	Pfl01_4420	1.5929	0.0061	0.0790
13	putative nucleotide-binding protein	Pfl01_4421	1.3844	0.0316	0.1171
14	2-dehydropantoate 2-reductase	Pfl01_4422	0.8839	0.7267	0.9986
15	multi-sensor Signal transduction histidine kinase	Pfl01_4423	7.0335	0.0145	0.0881
16	ATP:cob(I)alamin adenosyltransferase	Pfl01_4424	0.1759	0.0163	0.0896

ND Not Detected

Table A33. Gene Expression Values Surrounding *cspC* in Control Experiment. (See Table 9)

The gene numbers correspond to Figure A4.

Figure A4 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	Holliday junction resolvase	Pfl01_4409	0.2626	0.3023	0.9991
2	hypothetical protein	Pfl01_4410	1.0979	0.6326	0.9991
3	aspartyl-tRNA synthetase	Pfl01_4411	-1.0611	0.4440	0.9991
4	hypothetical protein	Pfl01_4412	-1.0911	0.4180	0.9991
5	hypothetical protein	Pfl01_4413	ND	ND	ND
6	Ferritin and Dps	Pfl01_4414	ND	ND	ND
7	cspC	Pfl01_4415	**-1.0445**	**0.8036**	**0.9991**
8	hypothetical protein	Pfl01_4416	-0.9201	0.7883	0.9991
9	histidine triad (HIT) protein	Pfl01_4417	-0.2373	0.0948	0.9991
10	hypothetical protein	Pfl01_4418	ND	ND	ND
11	outer membrane porin	Pfl01_4419	-3.9560	0.0343	0.9991
12	mechanosensitive ion channel MscS	Pfl01_4420	1.0936	0.4787	0.9991
13	putative nucleotide-binding protein	Pfl01_4421	-1.0983	0.3391	0.9991
14	2-dehydropantoate 2-reductase	Pfl01_4422	-0.7677	0.2753	0.9991
15	multi-sensor Signal transduction histidine kinase	Pfl01_4423	-2.0234	0.5154	0.9991
16	ATP:cob(I)alamin adenosyltransferase	Pfl01_4424	-0.6991	0.2050	0.9991

ND Not Detected

Table A34. Gene Expression Values Surrounding *cspC* in Cold Adapted vs. Cold Shock Experiment. (See Table 9) The gene numbers correspond to Figure A4.

Figure A4 Gene Number	Gene Function	Gene Name	Fold Change	P-value	FDR P-value
1	Holliday junction resolvase	Pfl01_4409	-1.0021	0.0014	0.0147
2	hypothetical protein	Pfl01_4410	22.0330	0.0036	0.0204
3	aspartyl-tRNA synthetase	Pfl01_4411	1.4201	0.0031	0.0192
4	hypothetical protein	Pfl01_4412	3.4911	0.0045	0.0224
5	hypothetical protein	Pfl01_4413	ND	ND	ND
6	Ferritin and Dps	Pfl01_4414	ND	ND	ND
7	cspC	Pfl01_4415	**2.1327**	**0.0227**	**0.0634**
8	hypothetical protein	Pfl01_4416	ND	ND	ND
9	histidine triad (HIT) protein	Pfl01_4417	0.0978	0.0675	0.1592
10	hypothetical protein	Pfl01_4418	ND	ND	ND
11	outer membrane porin	Pfl01_4419	2.4642	0.2125	0.4644
12	mechanosensitive ion channel MscS	Pfl01_4420	2.0900	0.0001	0.0131
13	putative nucleotide-binding protein	Pfl01_4421	1.7015	0.0031	0.0192
14	2-dehydropantoate 2-reductase	Pfl01_4422	ND	ND	ND
15	multi-sensor Signal transduction histidine kinase	Pfl01_4423	-3.8508	0.0050	0.0234
16	ATP:cob(I)alamin adenosyltransferase	Pfl01_4424	0.0932	0.0026	0.0178

ND Not Detected

Table A35. Genes Overlapping from Cold Adapted and Cold Shock Transcripts. The genes represented in this table are only the genes that are either up or down regulated in both experiments. The majority of overlapping genes were affected oppositely based on temperature treatment (ie. Upregulated in cold shock and downregulated in cold adaptation, or vice versa).

Gene Function	Feature ID	Cold Shock 1 Experiment Fold Change	Cold Adapted Experiment Fold Change
acetate permease	actP	2.2	2.5
flagellar motor protein	motC	3.1	19.1
fusaric acid resistance protein	Pfl01_0171	3.9	5.5
urocanate hydratase	Pfl01_0360	3.8	2.0
(4Fe-4S)-binding protein	Pfl01_0517	3.9	2.5
[acylating]	Pfl01_0686	3.0	6.7
phosphate-starvation-inducible E	Pfl01_0917	-3.7	-30.1
cyanate transport system protein	Pfl01_0978	2.6	4.0
hypothetical protein	Pfl01_1298	5.2	2.5
acyl-CoA dehydrogenase	Pfl01_1809	3.0	6.7
Short-chain dehydrogenase/reductase SDR	Pfl01_2526	5.2	4.9
glutaminase	Pfl01_3373	14.0	27.7
hypothetical protein	Pfl01_3989	-3.7	-2.5
periplasmic sensor Signal transduction histidine kinase	Pfl01_4239	2.1	756.5
periplasmic sensor Signal transduction histidine kinase	Pfl01_4244	2.4	3.7
two component transcriptional regulator	Pfl01_4245	-8.6	-4.9
gluconate transporter	Pfl01_4335	3.9	3.1
5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	Pfl01_4545	-2.6	-6.6
molybdopterin biosynthesis protein MoeB	Pfl01_4746	-19.4	-3.4
Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	Pfl01_5155	56.5	5.3
cyclic nucleotide-binding protein (cNMP-bd) protein	Pfl01_5185	-4.7	-9.7
intergral membrane protein	Pfl01_5250	2.8	4.4
hypothetical protein	Pfl01_5254	-3.3	-2.1
hypothetical protein	Pfl01_5472	3.0	2.2

VITA

Abby Smartt was born June 30, 1987 in San Antonio, Texas. She is the second child of Dr. William and Mary Ellen Smartt. After her father retired from the military, her family moved to Knoxville, Tennessee, where Abby attended West High School. After graduating in 2005, Abby enrolled at the University of Tennessee and obtained a Bachelor of Science in Biological Sciences with a concentration in Microbiology in 2009. Following graduation, Abby worked as a research technician for Dr. Gary Sayler and Dr. Alice Layton until entering graduate school in the fall of 2011. Abby is expected to graduate with her Master's in Microbiology in August 2014.