Grant/Contract Title: UNCOVERING MECHANISMS FOR REPAIR AND PROTECTION IN COLD ENVIRONMENTS THROUGH STUDIES OF COLD ADAPTED ARCHAEA

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Introduction

Methanococcoides burtonii is a cold-adapted archaeon isolated from permanently cold (1-2ºC), methane saturated waters in Ace Lake, Antarctica. *M. burtonii* is a motile, flagellated microbe that uses methylated carbon compounds for growth (methylotrophy), such as methanol and trimethylamine. Although adapted to the cold, *M. burtonii* is capable of growth at much higher temperatures, with the highest (= optimal) growth rate occurring at 23ºC, and a maximum growth temperature of 28ºC. We have employed two separate but integrated approaches for investigating the basis of cold adaptation in *M. burtonii*: targeted protein studies, and global proteomic analysis. Our first approach focuses on the study of the chaperonin (Cpn60) complex, which is important for the correct folding of proteins inside the cell. Our second approach aims to determine how protein expression changes in the entire cell at different growth temperatures, using isotope labeling of proteins and liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS). Our research aims to provide fundamental knowledge about the molecular mechanisms of adaptation to growth at cold temperatures in archaea. This will enable us to identify molecular strategies that have evolved in order to cope with extreme cold, with the cell in a state of permanent cold stress. This is interesting in terms of both the limits of archaeal adaptation to low temperature extremes, and in considering whether archaeal mechanisms for cold adaptation may be extended to other systems.

Objective 1 is to define principles of protein folding at low temperature through studies conducted on the molecular chaperonin complex from *M. burtonii*. Our previous proteomic studies determined that one of the three molecular chaperonin (Cpn60) subunits is more abundant during growth at 4°C compared to 23°C. Consistent with this, cold shock studies in thermophilic archaea, and studies in bacteria by others indicate that proper protein folding is an important repair and protection mechanism for adapting to the cold. Recombinant studies of the three *M. burtonii* chaperonins, and studies of the native chaperonin complex from cells grown at 4°C and 23°C will enable the mechanism of low temperature protein folding to be assessed. Analysis of the native chaperonin complex composition will define the role of specific subunits in protein folding and thermal adaptation. This objective also seeks to determine the precise cellular abundance and regulation of expression of the 3 molecular chaperonins at the 7 different growth temperatures in *M. burtonii*. Western blot analysis will be used to determine protein

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14. ABSTRACT

Methanococcoides burtonii is a cold-adapted archaeon isolated from permanently cold (1-2?C), methane saturated waters in Ace Lake, Antarctica. M. burtonii is a motile, flagellated microbe that uses methylated carbon compounds for growth (methylotrophy), such as methanol and trimethylamine. Although adapted to the cold, M. burtonii is capable of growth at much higher temperatures, with the highest (= optimal) growth rate occurring at 23?C, and a maximum growth temperature of 28?C. We have employed two separate but integrated approaches for investigating the basis of cold adaptation in M. burtonii: targeted protein studies, and global proteomic analysis. Our first approach focuses on the study of the chaperonin (Cpn60) complex, which is important for the correct folding of proteins inside the cell. Our second approach aims to determine how protein expression changes in the entire cell at different growth temperatures, using isotope labeling of proteins and liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS). Our research aims to provide fundamental knowledge about the molecular mechanisms of adaptation to growth at cold temperatures in archaea. This will enable us to identify molecular strategies that have evolved in order to cope with extreme cold, with the cell in a state of permanent cold stress. This is interesting in terms of both the limits of archaeal adaptation to low temperature extremes, and in considering whether archaeal mechanisms for cold adaptation may be extended to other systems.

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abundance, and RT-PCR to determine RNA abundance. Protein modification (*e.g.* phosphorylation) will be determined by Fourier transform mass spectrometry, and studies will be initiated to identify the native protein targets of the archaeal chaperonin. Integrating knowledge of the abundance and protein state (modification and protein targets) with subunit composition and activity, will enable the role and regulation of the chaperonin complex to be accurately defined, and specifically related to the physiological state of the cell.

Objective 2 is to determine how protein expression changes in the cell at incrementally different growth temperatures spanning from the lower (e.g. T_{min}, -2ºC) to the upper (e.g. T_{max}, 28ºC) growth temperature limits for *M. burtonii*. This approach expands on our previous work that compared growth at 4ºC and 23ºC to encompass a comparison of 7 growth temperatures (-2, 1, 4, 10, 16, 23 and 28°C). This will enable stress proteins and associated cellular processes to be carefully discriminated from those that are linked to growth at less stressful temperatures, and will provide a molecular means of defining truly optimal growth conditions. It will also specifically enable proteins and associated cellular processes to be identified that are most important for protection at low temperature extremes. Comparing protein levels across seven different growth conditions has not previously been examined for any biological system using proteomics and will provide a unique level of understanding about global gene expression and cellular adaptation in this cold adapted microorganism.

Objective 1: Progress

All three chaperonin genes from *M. burtonii* have been cloned and the gene products have been overproduced in *E. coli*. We tried out a few growth conditions and found the best expression in terms of solubility to be 16°C. We managed to get pure protein by purifying the His-tagged proteins with Ni-affinity and gel filtration columns. The stability, flexibility and hydrophobicity of the recombinant chaperonins have been assessed via heat- and urea- based Circular dichroism, Differential Scanning Calorimetry, Dynamic Fluorescence Quenching and ANS-Flourescence. Thus the biochemical characterization of the recombinant chaperonins has been completed. We had polyclonal antibodies made in rabbits against the chaperonin subunits after removing the tag. The antibodies turned out to be highly specific and show no cross-reactivity. We perform Western Blot analysis to detect the chaperonins in M. burtonii cells grown at different temperatures. The pure recombinant chaperonin subunits were used to set up big crystallization trials and the the manually improved crystals of one of the subunits are currently being analysed.

Objective 1: Accomplishments

The chaperonins could be recombinantly produced in *E. coli*. The proteins are soluble and give a gel filtration profile with one peak that corresponds to a molecular mass of 120 kDa. Therefore we assume that they form a dimer. All three recombinant chaperonins form just one band on both SDS-PAGE and native PAGE. There appear to be no

isoforms. We investigated the stability of the chaperonins and it turned out that they have different melting temperatures for the secondary and tertiary structure and different activation energies for the unfolding. One chaperonin is the least stable, one is the most stable and one is in between. Interestingly the melting temperatures (38 °C – 51°C) are far above the upper growth temperature of *M. burtonii* which is 28°C. We assessed the flexibility as well and the order didn't change. The most stable chaperonin is the most rigid one, the most labile chaperonin is the most flexible one. We looked for a reason for this difference and found out that the most labile and flexible chaperonin has more hydrophobic residues exposed than the two others and shows maximum hydrophobicity at low temperature whereas the other two chaperonins show maxium hydrophobicity at their corresponding melting temperatures.

Western Blot analysis of the chaperonins on *M. burtonii* cells grown at a temperature range from 1°C to 28°C showed that all three chaperonins can be detected at all temperatures. The most flexible chaperonin and the one that is in between the two others are produced in equal amounts at all temperatures whereas the most stable chaperonin is produced in higher amounts with higher temperature.

ATPase activity was measured for all three recombinant chaperonins in their dimeric state. The overall activity is very low with a peak between 37°C and 55°C for all chaperonins. We again see the same order. The most labile chaperonin is the least active, the most stable chaperonin is the most active.

The pure recombinant chaperonins were used to set up crystallization trials. And even here we see an order ranging from no crystals for the flexible chaperonin, crystals under only a few conditions for the chaperonin that is in between the two others, and an amazingly high crystal genesis for the most stable chaperonin. The crystal genesis is highly dependent on a high salt concentration and almost none of the PEG-containing condictions gave crystals even when combined with salt. The crystals could be manually improved in size and we were able to collect a data set at 2.9 Angstrom for one of them which is currently being processed.

Objective 2: Progress

For the second approach, we used global proteomic analysis to uncover those proteins that were differentially expressed in response to different growth temperatures. We initially focused on comparing two different growth temperatures for *M. burtonii* (4ºC versus 23ºC) before shifting to a comparison of seven temperatures (-2ºC, 1ºC, 4ºC, 10ºC, 16ºC, 23ºC, 28ºC), which encompasses the lower to upper growth limits of *M. burtonii*. We also examined the effect of different carbon sources (methanol versus trimethylamine) on protein expression at 4ºC and 23ºC.

For the two-way comparisons of 4ºC versus 23ºC (in either methanol medium or trimethylamine medium) and of methanol versus trimethylamine as carbon sources (at either 4ºC or 23ºC) we used the 4plex iTRAQ system (Applied Biosystems) for post-

incorporation labeling of the peptides. The availability of an 8plex iTRAQ system in the second half of 2008 allows us to assay all seven growth temperatures in the same liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS) run. We had initially intended to compare all seven temperatures using both N isotope metabolic labeling and 4plex post-incorporation labeling. However, cultures of *M. burtonii* grown at -2ºC require at least 2 years growth in order to produce sufficient biomass for harvesting and subsequent LC/LC-MS/MS. The 1ºC cultures usually require a minimum of 6 months. Therefore, in the intervening time we decided to focus on the 4ºC and 23ºC cultures, and carry out a 2-way temperature comparison for all three fractions (cell membrane, cytosolic, supernatant), using 4plex iTRAQ labeling. We also carried out a comparison of the effect of different media: 'MFM medium', a complex medium which has trimethylamine as the C substrate, versus 'M medium', a defined medium which has methanol as the C substrate. Our aim was to elucidate the effect of different substrates on the cellular processes of *M. burtonii*. M medium is a far less amenable medium for growth than MFM; M medium lacks yeast extract, and the C1 substrate methanol yields only one-third the energy of trimethylamine. These experiments have been **completed**, and revealed that many proteins that are differentially abundant according to temperature (4ºC v 23ºC) also show differential abundances according to media (methanol v trimethylamine), suggesting that they are not strictly temperature responsive proteins.

Further, the 4C v 23C and M v MFM comparisons allowed us to refine the methodology, especially with respect to the hydrophobic (cell membrane) fraction, which proved to be the most technically challenging. Development and optrimization of a method for preparation of the hydrophobic fraction took nearly a year. These proteins need to be altered to a soluble form prior to mass spectrometry, while remaining relatively chemically inert so as not to interfere with instrumentation. Treating the proteins with, for example, ionic detergents or agents that bind to the proteins (altering charge or local hydrophobicity) can interfere with LC separations, foul the detector, or interfere with peptide ionization. The method developed by Blonder et al. (2002), which utilizes the organic solvent methanol, combined with thermal denaturation and in-solvent digestion, proved to be the most successful, with some refinements.

After 2 years growth, several of the -2C cultures have now reached sufficient biomass for processing, which has coincided with the commercial availability of 8-plex iTRAQ reagents. This has allowed *M. burtonii* cells grown at all seven temperatures to be compared directly in the same MS/MS run. We have chosen 8plex iTRAQ post-incorporation labeling as our preferred labeling technique for the comparison of the seven *M. burtonii* growth temperatures. 8plex iTRAQ LC/LC-MS/MS investigation of all seven temperatures has now commenced, and is proceeding rapidly. **As of September 2009, these are now completed.**

Objective 2: Accomplishments - 4ºC versus 23ºC comparisons

Our proteomic analyses have identified many proteins that are implicated in archaeal cold adaptation. Many of these (e.g., stand-alone TRAM domain proteins, cadherin and dockerin domain proteins) represent classes of proteins whose function in archaea are unknown. **For the first time, we propose a role for these proteins in the cold-stress response of archaea**. Below, we provide a summary of the most interesting proteins identified so far in our two-way 4ºC versus 23ºC comparisons, resulting from no less than 72 runs of LC/LC-MS/MS. Each LC/LC-MS/MS run takes approximately 2-3 days, with subsequent data processing (including quantitation) also taking 2-3 days. Only those that proteins that are higher at 4ºC (and therefore putatively cold responsive) are given below. Proteins are designated by their Mbur locus tags.

TRAM domain proteins (Mbur_0304, Mbur_0604, Mbur_1445). TRAM domains are RNA-binding domains that have no catalytic function on their own, and are usually found on proteins that have other (catalytic) domains that alter RNA substrates (Anantharam et al., 2001). Proteins composed solely of a single TRAM domain ('stand-alone' TRAM proteins) are unique to archaea. The *M. burtonii* genome encodes three such stand-alone TRAM proteins, and all three showed higher abundances at 4ºC over 23ºC. All three also showed higher abundances in methanol medium over trimethylamine medium. These data suggest that these stand-alone TRAM proteins may be mobilized as part of the cell's general stress response, but are nevertheless important in cold adaptation. Their function is unknown; these small proteins (63-68 aa) may form a complex with other proteins, or bind to mRNA and serve to inhibit secondary structure formation (see above).

DEAD box helicases (Mbur_0245, Mbur_1950): These enzymes may be responsible for unwinding secondary structures in messenger RNA, and a role in cold adaptation in *M. burtonii* has been proposed (Lim et al., 2000). These secondary structures are more stable at low temperatures, and interfere with efficient transcription and translation (Jones et al., 1996). Thus, DEAD box helicases could aid growth and survival at cold temperatures by unwinding these secondary structures.

Winged helix domain protein (Mbur_1512): The function of this protein is unknown, but winged helix domains have been associated with transcriptional regulation (Gajiwala and Burley, 2000). Interestingly, although winged helix protein Mbur_1512 was higher at 4ºC over 23ºC, another winged helix domain protein (Mbur_0652) showed the opposite response (higher at 23ºC than 4ºC). This could indicate that individual winged helix proteins are involved in different stress responses in *M. burtonii*.

PPiases (peptidyl-prolyl *cis/trans* isomerases) (Mbur_1485, Mbur_2256) are intimately involved in protein folding, by catalyzing the *cis-trans* isomerization of proline imide bonds in polypeptides. Given that this is a rate-limiting step, it is unsurprising that these enzymes showed higher abundance at 4ºC.

ParA protein (Mbur_2141): ParA ATPases are a ubiquitous family of proteins associated with directed movement within cells of chromosomes, plasmids, proteins, and

protein complexes, and ensure that these macromolecules are efficiently partitioned during cell division (Motallebi-Veshareh et al., 1990; Thompson et al., 2006). For chromosome and plasmid partitioning, ParA proteins work in concert with ParB, which binds the DNA. For protein and protein complex positioning, the partner proteins are as yet unidentified (Thompson et al., 2006). No ParB protein was identified in our proteomics data, which indicates that ParA is performing a different but unknown function in *M. burtonii* in response to growth at cold conditions, such as by positioning certain proteins within the cytoplasm.

Proteolytic proteins, including proteasome alpha subunit (Mbur_1096), aminopeptidase (Mbur_0712), and a novel trypsin-like protease (Mbur_1349) may help "quality control", by removing damaged or unneeded proteins from the cell, and converting them to short peptides and amino acids. Such a function may be more important under cold conditions, if proteins are damaged by cold stress, or the action of protein chaperones is not always effective in ensuring properly folded proteins.

Cohesin and dockerin proteins (Mbur_0728, Mbur_0729): This pair of secreted proteins (but no domains for anchoring the protein to the cell) exhibited higher abundance at 4ºC over 23ºC, but were unaffected by the presence of methanol, suggesting that they may be specific to cold adaptation. Mbur_0728 (1126 aa) contains multiple domains, including an an immunoglobulin-like domain, a large (but weak) agglutinin domain, a cohesin domain at its C-terminus. Mbur_0729 (226 aa) has a single central cohesin domain. Cohesin and dockerin domains serve as complementary binding modules, and are found in many Euryarchaeota genomes, but their function in archaea is unknown (Bayer et al., 1999; Peer et al., 2008). In bacteria proteins containing dockerin and cohesin domains are used to build cell-surface protein complexes (e.g., cellulosomes) (Shoham et al., 1999). Mbur_0729 may function in intercellular interactions, such as promoting cell aggregation at cold temperatures. One possible scenario is that the agglutinin domain of Mbur_0728 binds to the glycan moieties of S-layer glycoproteins, and the protein promote cell aggregation through interactions between complementary dockerin and cohesin domains of different cells. Mbur_0729 may regulate cohesin-dockerin interactions through competitive inhibition by binding the dockerin domains of Mbur_0728.

Cell-surface proteins including serine-rich adhesin (Mbur_0060), a cadherin-like protein (Mbur_0314), and two DUF1608 (DUF="domain of unknown function") proteins (Mbur_0288, Mbur_1690). These have transmembrane domains, indicating that they are anchored to the membrane. Mbur_0060 and Mbur_0314 two contain domains associated with intercellular interactions, suggesting such a role in *M. burtonii*.

Glycine betaine solute-binding protein (Mbur_0503): Given the ability of *M. burtonii* to grow is dependent upon the stability of enzymes at low temperatures, this may be facilitated by the intracellular accumulation of osmolytes such as glycine betaine, which serve as cryoprotectants. Mbur_0503 is one part of a three-component ABC transport system, which also includes an ATPase and a permease (both detected in our proteomics data, but not differentially abundant in response to different temperatures).

Hypothetical proteins. These have no identifiable domains or signatures that allow us to infer what their respective functions might be. These include Mbur_0209 (116 aa), Mbur_0513 (340 aa), Mbur_0886 (208 aa), and Mbur_2063 (391 aa).

Objective 2: Accomplishments – 7x temperature comparisons (-2ºC, 1ºC, 4ºC, 10ºC, 16ºC, 23ºC, 28ºC)

Results are preliminary, but the availability of the 8-plex iTRAQ labeling system has facilitated this phase of this project. We began with the supernatant fraction (the fraction enriched for cell surface and secreted proteins), and in the experiments carried out so far, several cell surface and secreted proteins show differential abundance across all seven temperatures tested. Six proteins discussed above in the context of the 4ºC versus 23ºC comparisons (Mbur_0060, Mbur_0268, Mbur_0728, Mbur_0729, Mbur_0314, Mbur_1690) tend to show high abundance for 4ºC and 10ºC growth temperatures, with the lowest abundances recorded for the lower- and uppermost growth temperatures (-2ºC and 28ºC). However, two (Mbur_0060, Mbur_0268) peaked at 1ºC, which is close to the natural growth temperature of *M. burtonii* at the bottom of Ace Lake.

The above may indicate a combination of two factors: (a) these proteins are not necessary for growth at the higher temperatures (concordant with these being cold-adapted proteins); and (b) certain parts of *M. burtonii* biosynthetic metabolism being repressed at very cold temperatures (-2ºC), and is consistent with a scenario in which a larger proportion of cellular metabolism is dedicated to fulfilling the cell's maintenance energy demand at this extreme (Goodchild et al., 2005).

The winged helix protein Mbur_1512 was recovered in the supernatant fraction, despite being a cytosolic protein, probably as a result of cell lysis during growth. This protein also showed the highest abundance at 1ºC. It also showed a high abundance at -2ºC, which suggests an important role for this nucleic acid-binding protein.

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