STRESS RESPONSE BY ALTERNATIVE $\sigma$-FACTOR, RpoH, AND ANALYSIS OF POSTTRANSLATIONAL MODIFICATION OF THE HEAT SHOCK PROTEIN, DnaK, IN *Escherichia coli* 

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Bacteria have developed specialized responses that involve the expression of particular genes present in a given regulon. Sigma factors provide regulatory mechanisms to respond to stress by acting as transcriptional initiation factors. This work focuses on σ^{32} during oxidative stress in Escherichia coli. The differential response of key heat shock (HS) genes was investigated during HS and oxidative stress using qPCR techniques. While groEL and dnaJ experienced increases in transcriptional response to H_{2}O_{2} (10 mM), HS (42°C), and paraquat (50 mM) exposure, the abundance of dnaK over the co-chaperones was apparent. It was hypothesized that DnaK undergoes oxidative modification by reactive carbonyls at its Lys-rich C-terminus, accounting for the differential response during oxidative stress. A σ^{32}-mediated β-galactosidase reporter was devised to detect the activity of wild-type DnaK and DnaKV634X modified to lack the Lys-rich C-terminus. Under unstressed conditions and HS, σ^{32} was bound at the same rate in both strains. When subjected to H_{2}O_{2}, the WT DnaK strain produced significantly higher β-galactosidase than DnaKV634X (one-tailed Student’s t test p=0.000002, α=0.05) and approached the same level of output as the lacZ positive control. The β-galactosidase assay indicates that DnaK undergoes Lys modification in the WT strain, preventing the protein from binding σ^{32}, increasing the activity of σ^{32}, and resulting in higher β-galactosidase activity than the DnaKV634X strain. In the DnaKV634X strain DnaK continues to bind σ^{32} so that σ^{32} could
not promote the production of β-galactosidase. These findings demonstrate how DnaK is oxidatively modified, hindering the interaction with σ^{32} in manner distinct from HS.
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<td>ECF</td>
<td>Extracytoplasmic function</td>
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<td>Δttg</td>
<td><em>E. coli</em> cells lacking trigger factor</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<td>•OH</td>
<td>Hydroxyl radical</td>
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<td>PQ</td>
<td>Paraquat</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>HNE</td>
<td>4-hydroxynonenal</td>
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<td>HSP70</td>
<td>Heat shock protein 70</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>PMS-10</td>
<td>Photosynthetic medium</td>
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<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>GC/MS</td>
<td>Gas chromatography-mass spectrometry</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<td>ERCC</td>
<td>External RNA Control Consortium</td>
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GW  Gateway
SDM  Site-directed mutagenesis
S.O.C.  Super Optimal Broth (Catabolite repression)
LA  Luria broth agar
CHAPTER 1
INTRODUCTION

1.1 Bacterial Stress and Heat Shock Response

Microorganisms are constantly exposed to stress from the environment, including but not limited to temperature, UV exposure, changes in pH and exposure to harmful chemicals. Bacteria have developed complex responses to these stresses that involve the expression of a number of genes present in a given regulon. Sigma factors complex with RNA polymerases and direct them toward the specific promoter of the gene(s) to be expressed (Brooks 2007). The vegetative sigma factor, $\sigma^{70}$ directs RNA polymerase to bind the promoter consensus sequences -10 and -35 base pairs upstream from the transcriptional start site of a gene (Snyder 2007).

Extracytoplasmic function (ECF) or alternative sigma factors provide a regulatory mechanism to respond to these extracellular stressors by acting as transcriptional initiation factors. ECF sigma factors have binding regions that differ in structure from non-ECF factors such as $\sigma^{70}$ (Raivio 2001). Furthermore, ECF sigma factors regulate different promoters than those regulated by $\sigma^{70}$. Additionally, all ECF sigma factors are regulated by a common mechanism in which an inactive ECF sigma factor remains bound to an anti-sigma factor until an activating signal, sensed through the periplasm, causes induction of the sigma factor to the cytoplasm where it can associate with RNA polymerase to bind promoters of stress related genes (Raivio 2001). This work will focus on the role of the heat shock sigma factor, $\sigma^{32}$, during oxidative stress.

Heat shock is classically defined as the exposure of an organism to a higher than optimal temperature. The heat shock system of *Escherichia coli* (Figure 1.1) mediated by RpoH ($\sigma^{32}$) is
the best-studied stress response (Raivio 2001). Under standard cellular conditions $\sigma^{70}$, the “housekeeping” sigma factor plays a significant role in the transcription of $\sigma^{32}$. Four promoters have been shown to be involved in $rpoH$ transcription. The promoters P1, P4, and P5 are recognized by the $\sigma^{70}$-RNA polymerase complex. The $\sigma^{70}$-RNA polymerase complex transcribes most genes during the exponential bacterial growth phase. Under standard conditions, P1 and P4 mediate the transcription of $rpoH$. The P3 promoter is regulated by a $\sigma^{24}$-RNA polymerase complex, $\sigma^{24}$ characterized by its role in the maintenance of the cellular envelope. The $\sigma^{24}$-RNA polymerase complex is only active at higher temperatures (Yura 2000). $\sigma^{32}$ has been shown to regulate the expression of as many as 91 genes in *Escherichia coli* (Martinez-Salazar 2009). Among the 91 genes that $\sigma^{32}$ regulates, the best characterized are the heat shock protein (HSP) genes, including *dnaK*, *dnaJ*, and *grpE*. 
Figure 1.1. The bacterial heat shock system. Under unstressed conditions, promoters P1, P4, and P5 that regulate $\sigma^{32}$ transcription are modulated by $\sigma^{70}$. Promoter P3 is regulated by the $\sigma^{24}$ complex under envelope stress conditions. $\sigma^{32}$ goes on to regulate transcription of numerous genes by complexing with RNA polymerase to bind to the heat shock protein promoter regions (pHSP) to transcribe the genes for the heat shock proteins (HSP), DnaK, DnaJ, and GrpE that play a role in multiple stress responses.

1.2 Bacterial Heat Shock Protein, DnaK

DnaK belongs to the Hsp70 family of molecular chaperones found in the cytosol. Hsp70 proteins function by binding and releasing nascent or unfolded proteins in conjunction with ATP hydrolysis (Ulrich Hartl 2002). These proteins facilitate the assembly of protein complexes and are responsible for intracellular folding of proteins that are to be secreted or transported. Hsp70 proteins are also rapidly activated when a cell encounters environmental stress (Karlin
The heat shock protein, DnaK, is key to the regulation of $\sigma^{32}$. Under optimal cellular conditions, DnaK binds to and promotes the degradation of newly synthesized $\sigma^{32}$. DnaK binding destabilizes the sigma factor, leaving it susceptible to protease degradation (Chattopadhyay 2002). Under heat shock conditions, DnaK disassociates from $\sigma^{32}$. $\sigma^{32}$ then increases in stability and is free to recruit RNA polymerase to increase the transcription of heat shock genes (Straus 1987). The exact mechanism by which DnaK interacts with $\sigma^{32}$ under these conditions is not known (Chattopadhyay 2002). However, it is hypothesized that $\sigma^{32}$ interacts with the C-terminus of DnaK. Under oxidative stress conditions, the C-terminus is the putative target of oxidative modification, accounting for the variation in stress response between heat shock and oxidative stress.

In the DnaK signaling system, DnaK co-chaperones with DnaJ and the nucleotide exchange factor, GrpE. The DnaK protein is composed of an N-terminal ATPase domain and a C-terminal peptide-binding domain. The peptide-binding domain contains a beta-sandwich sub-domain with a peptide binding cleft and a latch-like segment that targets the hydrophobic residues of nascent or unfolded proteins. When bound, the $\alpha$-helical latch changes to an open conformation from the closed conformation by hydrolysis of bound ATP. The heat shock proteins DnaJ and GrpE regulate cycling between open and closed conformations of DnaK. The N-terminal J domain of DnaJ binds DnaK to accelerate hydrolysis of ATP while GrpE facilitates the release of ADP from DnaK. Binding of a second ATP molecule completes the reaction cycle by causing the DnaK complex with DnaJ and GrpE to disassociate (Figure 1.2).
Figure 1.2. DnaK binding cycle. DnaK (blue) is composed of an N-terminal ATPase domain (NBD) and a C-terminal peptide-binding domain (PBD). The peptide binding domain contains a peptide binding cleft (green) and a latch-like segment that targets the hydrophobic residues of unfolded proteins (red). The hydrolysis of ATP drives changed from open to closed conformation of the protein. The Co-chaperone DnaJ (purple) to accelerate hydrolysis of ATP, this closed the latch in the PBD. GrpE (yellow) facilitates the release of ADP and loads DnaK with a second ATP molecule. The refolded polypeptide and GrpE are released, completing the reaction cycle (Nakamura 2011; Qi 2013).

Like many chaperones, DnaK is constitutively expressed under standard cellular conditions. However during stress, such as heat shock mentioned above, levels of DnaK and other heat shock proteins increase (Ulrich Hartl 2002). Trigger factor, DnaK, and DnaJ share overlapping chaperone functions in “stabilizing nascent chains in a state competent for subsequent folding” and may share functional redundancy under stress conditions as well.
However, *E. coli* cells lacking trigger factor (Δttg) or DnaK exhibited no folding defects at 37°C but deletion of *dnaK* in a Δttg strain was lethal following heat shock (Deuerling 2003) suggesting the more central role of DnaK during heat shock.

1.3 σ^{32} and Oxidative Stress Response

In addition to mediating heat stress, σ^{32} has been shown to have a role in cellular response to a number of other stresses including oxidative stress, ethanol exposure, starvation, and exposure to toxic compounds (Kogoma 1992; Raivio 2001; Martinez-Salazar 2009). Despite the essential role of oxygen in the metabolism of many organisms, it is readily converted to harmful reactive oxygen species that can cause oxidative stress to the organism. Oxidative stress occurs in an organism when a compound oxidizes or reduces its molecular partner affecting signal transduction, gene expression, and cellular injury (Boelsterli 2009). Molecular oxygen is a biradical compound with two lone electrons that spin in the same direction. Molecular oxygen remains stable if these electrons maintain the same spin, however, should one of these electrons become excited, the compound will become singlet oxygen. This process occurs readily in photosynthetic systems like that found in the bacterium *Rhodobacter sphaeroides* (Dufour 2008). Singlet oxygen is particularly harmful because it can generate other reactive oxygen species (ROS), causing damage to cellular components including DNA, proteins, and lipids. Other harmful ROS are generated by the step-wise reduction of oxygen; these species include the superoxide anion, hydrogen peroxide (H_2O_2), and the hydroxyl radical (∙OH) (Boelsterli 2009). Electrons have been shown to leak at NADH dehydrogenase and ubiquinone sites in the bacterial respiratory chain leading to the production of the reactive oxygen species
H₂O₂ (Gonzalez-Flecha 1995; Ghouleh 2011). Bacteria can also experience oxidative stress in response to “environmental agents such as ionizing near-UV radiation,” or exposure to harmful xenobiotics such as the herbicide paraquat (Cabisco 2000). Paraquat (PQ) acts as a redox cycler; the oxidized form of PQ accepts electrons from the reduced form. The reduced PQ radical then reacts with oxygen to produce a superoxide radical and subsequent reactive oxygen species like H₂O₂ and \( \cdot \text{OH} \). These reactive oxygen species can induce protein degradation, DNA damage, and lipid peroxidation (Lascano 2012). \( \sigma^{32} \) has been shown to increase in the presence of reactive oxygen species like those mentioned above. When rpoH was deleted from E. coli heat shock mutants sodA and sodB, cells were sensitized to oxidative stress by paraquat. These results indicate that rpoH deletion inhibited the induction of the heat shock genes (Kogoma 1992).

1.4 Reactive Oxygen Species and Lipid Peroxidation

Harmful reactive oxygen species have been implicated in the cell damage related to aging, apoptosis and iron metabolism imbalance (Tamarit 1998). Oxidative stress, chiefly lipid peroxidation, has been increasingly implicated in the development of neurodegenerative diseases like Alzheimer’s disease (Reed 2008). Lipid peroxides have been shown to react with the amino acid residue lysine potentially disrupting protein function (Sayre 2006). The lipid aldehyde end products of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are as harmful as the peroxide products (Figure 1.3). At elevated levels, the lipid peroxidation by-product HNE has been shown to induce oxidative stress, impaired
protein function, cell death, and the disruption of cellular, organelle, and organ function in higher organisms (Romero 1998).

Figure 1.3. Oxidative modification of Lysine by the lipid aldehyde HNE. (A) Lysine modification of a lipid aldehyde such as HNE or MDA may occur by nucleophilic attack of the C3 carbon of the lipid aldehyde by the lysine residue leading to formation of the Michael adduct. (B) Lysine modification also results in the formation of Schiff bases through nucleophilic attach by the amino group of the lysine on the aldehyde carbon.

Heat shock protein 70 (hsp70) is the eukaryotic homolog of DnaK. The two proteins share 50% amino acid sequence identity including the presence of a conserved hypercharged sequence, proximal to the C-terminus and several other conserved regions rich in lysine residues (Figure 1.4) (Lindquist 1988; Karlin 1998). In the present work, the mixed charge
region located at the C-terminus of *E. coli* DnaK will be investigated (Figure 1.7). Reed et al., identified several proteins, including Hsp70 that were shown to be 4-hydroxynonenal (HNE)-modified in the brains of amnestic mild cognitive impairment (MCI) patients, further linking oxidative stress to Alzheimer’s disease (2008). DnaK and several other proteins were identified as having a higher carbonyl content when oxidized (Tamarit 1998). Of these preferentially carbonylated proteins, DnaK, enolase, and elongation factor G all possess multiple lysine residues, implicating Lys-modification as a key factor in oxidative stress signaling. Lipid aldehydes such as MDA have been shown to selectively modify single Lys residues and crosslink multiple lysine residues to form carbonyl adducts (Shao 2010). Posttranslational modification through the oxidation of proteins has been shown in a number of systems occurring at cysteine, tryptophan, methionine, and lysine residues (Houde 2006). Oxidative stress can affect signal transduction through redox sensitive compounds like the response regulator, in most cases by oxidation of the thiol residues present in these proteins. In *E. coli* the response regulator, OxyR is redox sensitive and acts as a transcription factor, activating antioxidant response (Storz 1992). The focus of the present work is on the role of C-terminal domain, particularly with respect to Lys modification, in DnaK regulation. It is postulated that DnaK is posttranslationally modified under oxidative stress conditions, altering its activity on RpoH.
Figure 1.4. ClustalW2 multiple sequence alignment of HSP70 proteins from prokaryotic and eukaryotic sources. Blocks of alignment are numbered and aligned residues shown in upper case letters. Prokaryotic HSP70 protein sequences are from completely sequenced bacterial and archaeal genomes. Eukaryotic sequences are from HSP70 A2 protein segments. Conserved hypercharged region and lysine residues are blocked.
In organisms containing polyunsaturated fatty acids, lipid peroxides could react to create modifications of DnaK under oxidative stress conditions. Lipid peroxidation is defined as the attack of polyunsaturated fatty acids in membranes by a reactive oxygen species such as hydrogen peroxide, the hydroxyl radical, or superoxide. Once attacked, the polyunsaturated fatty acids are converted to reactive oxygen products capable of undergoing a free radical chain reaction that results in either the cleavage of an RH bond or the abstraction of a hydrogen atom from the polyunsaturated fatty acid (Figure 1.5).

![Initiation of lipid peroxidation injury. Lipid peroxidation in initiated with the generation of a hydroxyl radical by Fenton chemistry. This reaction is driven by electron transfer from compounds such an NADH. The hydroxyl radical abstracts a hydrogen atom from the lipid that can be found in the membranes of bacterial cells, generating a lipid radical. An isomerization event will yield a conjugated double bond that is oxidized to yield a highly reactive lipid peroxyl radical.](image-url)
Upon formation of the radical fatty acid, isomerization yields conjugated double bonds that are oxidized to yield lipid peroxides. Lipid peroxides are highly reactive and readily convert into more stable lipid aldehydes (Figure 1.6) (Lokireddy 2005). The reactive nature of lipid peroxides makes detection of these compounds difficult. Lipid peroxides are indirectly quantified by their more stable lipid aldehyde by-products, namely MDA and HNE. Lipid peroxides are routinely detected and quantified by the thiobarbituric acid reactive substances (TBARS) assay; a colorimetric assay in which the sample is heated at 100°C in an acid buffer for an hour, resulting in a pink chromogen that can be measured by absorbance at approximately 532 nm (Liu 1997).

![Figure 1.6. Propagation of lipid peroxides and fragmentation of lipid radicals to form reactive lipid aldehydes such as MDA and HNE. Lipid peroxides are propagated by the action of lipid radicals on nearby lipids. The lipidhydroperoxide intermediate generated during propagation undergoes a second Fenton reaction to yield a lipid alkoxyl radical. This radical is extremely unstable and readily degrades to form stable but still reactive products such as the lipid aldehydes MDA and HNE.](image-url)
The detection of lipid peroxides in bacteria is complicated by the fact that many bacteria simply lack the polyunsaturated fatty acids that generate lipid peroxides. In a review of the pathways of oxidative damage, Imlay posits that it is unclear if the lipid peroxidation chain reaction can occur in most bacteria because the propagation of lipid peroxides requires lipid peroxide radicals to remove electrons from nearby lipids, a reaction that is “kinetically efficient only when a bis-allylic methylene carbon, present only in polyunsaturated lipids, is the target” (Imlay 2003). The membranes of most bacteria contain only monounsaturated fatty lipids that have been shown to be insensitive to lipid peroxidation (Bielski 1983). However, the presence of reactive aldehyde species has been demonstrated in cells exposed to tert-butylhydroperoxide, hydrogen peroxide, potassium tellurite, and titanium oxide (Perez 2008). This suggests that even in organisms where polyunsaturated fatty acids are rare, such as *E. coli*, lipid peroxidation occurs and is physiologically relevant. In contrast, polyunsaturated fatty acids are known to exist in the extensive thylakoid membranes of photosynthetic bacteria. Photosynthetic systems found in these bacteria are also capable of synthesizing singlet oxygen, which can initiate the lipid peroxide chain reaction, making these organisms a viable alternative to the study of lipid peroxidation (Imlay 2003; Dufour 2008).

1.5 *Escherichia coli*, *Rhodopseudomonas palustris*, and Oxidative Stress

*Rhodopseudomonas palustris* is a Gram-negative purple non-sulfur bacterium belonging to the alpha-proteobacteria. *R. palustris* is capable of multiple modes of growth including photoautotrophic, photoheterotrophic, chemoheterotrophic, and chemoautotrophic growth. To grow phototrophically, *R. palustris* must use its extensive photosynthetic system located in
the thylakoid membranes of this organism. Given the presence of the thylakoid membranes shown to have polyunsaturated fatty acids in the genus *Rhodopseudomonas, R. palustris* is particularly suited to the study of lipid peroxidation (Wood 1965). Genetic manipulation of *R. palustris* is difficult in comparison to the bacterial model organism *E. coli*, and for this reason all cloning in this study was conducted using *E. coli*. The high similarity of the DnaK protein in these two organisms goes beyond the consensus sequence above to include multiple lysine residues in the C-terminal substrate-binding region of the protein (Figure 1.7). The sequence similarity between these two organisms allows for the study of functional similarity. Therefore, the mechanism of oxidative modification of DnaK should be conserved between these two bacterial species.

Figure 1.7. ClustalW2 protein sequence alignment of DnaK from *R. palustris* and *E. coli* showing last 32 residues of each protein including conserved C-terminal substrate-binding regions. Note the concentration of lysine residues (K) at the C-terminus.

1.6 Research Objectives

One of the primary goals of this project was to quantify the transcriptional responses of key regulatory components of the heat shock system such as *rpoH, dnaK, dnaJ,* and *groEL* under both heat shock and oxidative stress conditions in *E. coli*. The transcription of *rpoH* has been shown to increase under multiple stress conditions including heat shock and oxidative stress (Straus 1987; Raivio 2001; Chattopadhyay 2002; Martinez-Salazar 2009). Activation of the heat shock system is regulated by $\sigma^{32}$ under both of these stress conditions and $\sigma^{32}$ is regulated by
the heat shock protein, DnaK. In an unstressed system, DnaK binds to σ^{32} and marks it for degradation by the protease, FtsH. Under heat shock conditions σ^{32} increases in stability and abundance so that it is free to recruit RNA polymerase to increase the transcription of heat shock genes (Chattopadhyay 2002). Differences in active σ^{32} concentration could be attributed to changes in the activity of DnaK or its co-chaperone DnaJ. While DnaK and its co-chaperones bind and prevent the aggregation of unfolded proteins, the GroEL system provides a protective environment for refolding within its ring-like structure (Cardoso 2010). Transcription of both of these chaperone systems are under the control of σ^{32} and have been shown to be induced under heat shock and oxidative stress conditions (Kogoma 1992). However, there may be key differences in transcription of these genes that would point to differential regulation of this system under heat shock or oxidative stress.

Next, this project set out to determine the relationship between σ^{32} and its regulator, DnaK, during oxidative stress conditions by investigating the putative oxidative modification of the Lys-rich C-terminus of this protein. Under oxidative stress conditions, proteins rich in Lys-residues are preferentially modified (Lanouette 2014). DnaK contains a conserved Lys-rich C-terminus that is a potential target for oxidative modification. The posttranslational modification of DnaK would affect σ^{32} activities in a manner distinct from the canonical heat shock system.

Lastly, this work will inform the discussion of diseases linked to its homolog HSP70. The conserved chaperone functions of Hsp70 play a vital role in preventing the formation of protein aggregates and subsequent toxicity as a result of stress. Dysfunctional Hsp70 has been linked to degradation of neurons in Parkinson’s, Alzheimer’s, polyglutamine disease and amyotrophic
lateral sclerosis (Turturici 2011). Hsp70 is also involved in translocation of proteins that can affect signal transduction cascades including the apoptotic cascade (Turturici 2011). The effect of oxidative modification of DnaK is most evident in its association with σ^{32}. The response of the DnaK constructs in this work to oxidative stress could mirror the response of Hsp70 to ubiquitous oxidative stress in the complex mammalian system because the heat shock proteins have conserved function and sequences across species. Alternatively, there may be no change in σ^{32} levels due to modification of DnaK. This finding would refute the importance of DnaK’s C-terminal Lys-rich region in the interaction with σ^{32}. In this scenario, another region of DnaK may be important to interactions with σ^{32} or another protein in the DnaK complex such as DnaJ or GrpE may play a more central role in regulating stress response.

Microbial models are particularly useful in systems that are highly conserved due to the simplicity of the system, relatively short generation time, and ease of genetic manipulation. Photosynthetic \textit{Rhodobacter} species containing a bacterial respiratory chain have been successfully used as models for mitochondrial diseases linked to the point mutations in ND subunits encoded by mitochondrial DNA (Rea 2010). Like \textit{Rhodobacter}, \textit{E. coli} and \textit{R. palustris} could be used as a microbial model for oxidative stress because of putative lipid peroxide production.

It has been established that heat shock proteins protect the cell from a variety of stresses beyond heat shock. The link between human disease and the heat shock proteins is evident. Identification of lipid peroxide products in \textit{E. coli} and \textit{R. palustris} provides support for the development of these organisms as models for diseases linked to oxidative stress. Manipulation of an easily controlled microbial model such as \textit{E. coli} would allow for the study of
the heat shock system under a myriad of stresses that could elucidate the function of this system in higher organisms given the high genetic similarity. This model would not only aid in combating debilitating diseases but also provide information on the microbial response to multiple stresses that is proving crucial to the understanding and control of microbial ecosystems.
2.1 Bacterial Strains and Growth Conditions

*R. palustris* strain CGA009 for use in TBARS and quantitative real-time PCR experiments were grown anaerobically in the light at 30°C in PMS10 liquid media to exponential phase (OD$_{660}$=0.3). All chemicals for media making were obtained from Fisher Scientific. Per liter of PMS10, 25 mL of 0.5 M Na$_2$HPO$_4$, 0.5 mL of 0.5 M KH$_2$PO$_4$, 10 mL of 10% (NH$_4$)$_2$SO$_4$, 1 mL of trace metal solution, 1 mL of 0.1 M Na thiosulfate, 1 ml 2mg/mL p-aminobenzoic acid (filter sterilized), 10 mL 1.0 M sodium succinate, 1ml 20 mg/mL NiCl$_2$$\cdot$6H$_2$O, and 926 mL H$_2$O was added. The pH of the solution was adjusted to 6.8-7.0 with 5 N NaOH or 1 M HCl. Light was provided by a 40-W incandescent lamp kept 20 cm from the lamp. *R. palustris* culture tubes were inverted at least twice daily to prevent cells from settling. *R. palustris* culture tubes were also rotated around the light source to ensure equal exposure to the light, leading to equal growth. *E. coli* K12 and dnaK mutant strains were grown in 5 mL of Luria Broth (LB) at 37°C in an Innova® I-26 stackable incubator (New Brunswick Scientific) at 225 rpm. LB was made by combining 10 g Tryptone, 10 g NaCl, and 5 g yeast extract in 950 ml of H$_2$O. Once dissolved, the pH of the solution was adjusted to 6.8-7.0 with 5 N NaOH or 1 M HCl. For cloning experiments, *E. coli* mutants were grown on Luria Agar plates (LA) that were made according to the composition above with 8 g of agar added per liter. All media was sterilized by autoclaving for 15 minutes at 15 p.s.i. and 121°C on liquid cycle. For cloning experiments where antibiotic selection was utilized, LA was cooled to 55°C in a water bath. Once at 55°C, 50 µg/mL of kanamycin, 100 µg/mL of ampicillin, or 10 µg/mL of gentamycin was added prior to pouring.
into Petri dishes. Liquid media containing antibiotics was prepared by adding 50 µg/mL of kanamycin, 100 µg/mL of ampicillin, or 10 µg/mL of gentamycin to LB. All media containing antibiotics was covered with foil and kept at 4°C prior to use.

2.2 Quantitative Real-Time PCR of rpoH and Heat Shock Genes dnaK, dnaJ, and groeEL

For RNA extraction cells were subjected to stress treatment (oxidative stress, heat shock, or no treatment) followed by centrifugation to pellet the exposed cells. Oxidative stress was achieved by exposing cells to either 50 mM paraquat (Sigma-Aldrich) for 30 minutes or 10 mM H2O2 for 30 minutes. Heat shock was simulated by incubating cells in a 42°C water bath. Samples were taken from each treatment condition at 10 minute intervals. The supernatant was then removed and the pellet was allowed to air dry. Samples were dried by inverting the tube containing the pellet onto an absorbent paper towel, usually overnight. The samples were then stored at -20°C until RNA extraction using the RNeasy® Plus Universal Mini kit (Qiagen®) could be performed. In order to disrupt the cells for RNA extraction, the MP Biomedicals™ FastPrep® 24 Instrument was used to achieve mechanical disruption. 700 µL of Buffer RLT from the Qiagen® RNeasy kit was added to the dried pellets and the tubes were vortexed to redistribute the samples. Samples were then transferred to MP Biomedicals™ lysing matrix B tubes and subjected to bead-beating at 6.5 m/s for 1 minute. The lysed samples were then centrifuged for 10 seconds at 17,000 x g. The supernatant was carefully transferred to a new microcentrifuge tube so as not to disturb the pelleted beads. An equal volume of 70% ethanol was then added to the extracted supernatant (~220 µL). The entire volume of this solution was then transferred to the RNeasy Mini spin column for total RNA extraction of the bacterial
lysate. The column was then centrifuged for 15 seconds at 8,000 x g. RNase-free DNase treatment was included at this point to digest DNA from the samples (Qiagen®). Buffer RW1 was added to the spin column and centrifuged for 15 s at 8,000 x g. DNase treatment was performed by addition of a 10:70 µL DNase I to Buffer RDD solution to the RNeasy® spin column. Following a 15 minute incubation at room temperature, an additional 350 µL of buffer RW1 was added to the sample and incubated an additional 5 minutes before centrifugation at 8,000 x g for 15 seconds. At this point, the RNeasy Mini column was placed into a new 2 mL collection tube and 500 µL of Buffer RPE was applied to the column. The column was then washed by centrifugation at 8,000 x g for 15 seconds. The wash step was then repeated the RNeasy column was then transferred to and a new 1.5 mL microcentrifuge for collection. 50 µL of RNase-free water was added to the column. RNA was eluted by centrifugation of the column at 8,000 x g for 1 minute. This step was repeated with a second collection tube and additional 50 µL of RNase-free water in order to isolate the highest concentration of RNA possible. cDNA was synthesized from extracted mRNA using the iScript™ cDNA synthesis kit (Bio-Rad) to serve as template for qPCR analysis. Quality and concentration of mRNA was confirmed prior to cDNA synthesis, RNA concentrations ~300 ng/µL were typical and deemed acceptable for this analysis. Suboptimal samples were included in the analysis but subjected to additional incubation time during the reverse transcription step. For cDNA synthesis, the following reaction was prepared:

- Total RNA: 3 µL
- Random primers (3 µg/µL): 1 µL
- RNAsase-free dd H₂O: 3 µL
Once prepared, the solution was heated to 70°C for 10 minutes then placed on ice while the following components were added:

5x reverse transcription buffer: 2.5 µL
reverse transcriptase: 0.5 µL

The reaction, totaling 10 µL in volume was then incubated at room temperature for 10 minutes, followed by incubation at 42°C for 2 hours. The reaction was terminated by heating the samples to 65°C for 7 minutes. Once complete, the samples were diluted with 5x RNase-free ddH₂O and stored at -80°C for later use. cDNA were checked using the NanoDrop-1000 spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA).

Primers for qPCR of rpoH, dnaK, dnaJ, and groEL were designed using Invitrogen’s Oligoperfect™ Designer under the PCR: Detection menu (http://tools.lifetechnologies.com/content.cfm?pageid=9716). Primers were designed to be between 17-28 bp long, have 50-60% GC content, and melting temperature (Tm) between 55-80 °C. Primers were ordered at 25 mM scale with standard desalting purification.

cDNA for rpoH and the heat shock genes dnaK, dnaJ, and groEL were quantified using the fluorochrome iQ™SYBR® green Supermix. (Bio-Rad) Amplification of each gene was performed using the MyiQ™ single-color Real-Time PCR Detection System. The 2-step protocol was set up was follows:

Cycle 1: (1x)
Step 1: 95°C for 3 minutes
Cycle 2: (40x)
Step 1: 95°C for 10 seconds
Step 2: The annealing step ~60°C has been optimized for each gene’s primer set. The experiments performed on R. palustris rpoH and gapDH had an optimal temperature of 60.5°C.
while the experiments performed on *E. coli rpoH, dnaK, dnaJ, groEL, and gapA* had an optimal temperature of 60.2°C. Each temperature was held for 30 seconds. Data collection and RT analysis enabled

**Cycle 3:** (81x)

**Step 1:** 55°C-95°C for 30 seconds

Melt curve data collection and analysis enabled

The Livak method was used to analyze the relative changes in gene expression (Livak 2001; Bio-Rad 2011).

### 2.3 Whole Transcriptome Sequencing: RNAseq

Approximately 200-500 ng of total RNA was extracted from cells treated with 50 mM paraquat, 42°C, and a no treatment control using the RNeasy® Plus Universal Midi kit without the RNAProtect (Qiagen®) step as it has been shown to have downstream effects. RNA was quantified and quality was assessed with Experion™ Automated Electrophoresis Station (Bio-Rad Laboratories). RNA extracts were then depleted of ribosomal RNA using the RiboMinus™ Bacteria Transcriptome Isolation Kit (LifeTechnologies). ERCC RNA Spike-In Mix (Ambion®) was added to aid in mapping of transcripts following sequencing. The rRNA depleted RNA samples were then converted to cDNA using the Ion Total RNA-Seq Kit (LifeTechnologies). The cDNA samples were then subjected to size selection using Agencourt® AMPure® XP (Beckman Coulter) magnetic beads to target fragments 200-300 bp long. Next, the whole genome library was constructed (LifeTechnologies 2011), followed by preparation of template positive Ion Sphere particles (ISPs) using the Ion OneTouch™ ES system (LifeTechnologies 2012). Finally, the Ion PGM™ system using 316 chips (Ion Torrent™, LifeTechnologies) was used to sequence templates that were clonally amplified on ISPs (LifeTechnologies 2012). Once sequences were
obtained, the reads were mapped to the reference genome to reveal the transcriptional structure and any variation between treatment types.

2.4 Reporter Studies

Reporter studies were carried out by constructing plasmids containing lacZ under the control of a σ^{32}-dependent heat shock protein promoter that were then transformed into DnaK-deficient mutant strain of E. coli. Beta-galactosidase activity was monitored by a kinetic plate assay as a measure of σ^{32} activity in each E. coli strain.

2.4.1 Construction of σ^{32}-Dependent Reporter

E. coli mutant strains were obtained from The Coli Genetic Stock Center at Yale University. LacZ reporter plasmids were constructed with the MultiSite Gateway® Pro system (Invitrogen) to contain the lacZ fragment under the control of an σ^{32}-dependent heat shock promoter, p_{groE} (Cowing 1985; Wang 2003). A lacZ plasmid with no DnaK complement was used as a control. DnaK complement plasmids were generated using the Gateway® Pro System (Invitrogen 2006). See Figure 2.3 for cloning workflow. To generate P_{groE} fragments compatible with the Gateway® Pro System, P_{groE} PCR products flanked by specific attB sites were generated. Primers were designed for P_{groE} flanked by attB1 and attB5r sites (Table 2) that could be combined with the entry vector plasmid pDONR P1-P5r (Figure 2.1). The PCR reaction was performed with illustra™ puREtaq Ready-To-Go PCR Beads where 1 µL of each primer, 2 µL of genomic DNA, and 21 µL of RNase-free diH₂O was added to each PCR tube, a DNA
negative control was included. The cycling conditions were: 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds and a temperature gradient of 55°C to 69°C, with a final extension of 72°C for 5 minutes. The PCR product was purified using the LONZA FlashGel™ System, where products approximately 118 base pairs long were recovered. The recovered product was quantified on the NanoDrop1000, where 150 ng of product was considered acceptable for use in the BP reaction of the Gateway® system. The BP reaction was prepared by the addition of 2 µL of attB PCR product, 1 µL of pDONR P1-P5r (150 ng/µL), and 5 µL of 1X TE Buffer, pH 8.0, and 2 µL of BP Clonase™ II enzyme mix. The mix was incubated at 25°C for an hour. At this point, 1 µL of 2 µg/µL Proteinase K solution was added and incubated at 37°C for 10 minutes to stop the BP reaction. 2 µL of the BP reaction was then added to a vial of One Shot® Mach1™ T1R chemically competent E. coli (LifeTechnologies) for transformation. The vial containing the reaction was placed on ice for 30 minutes followed by heat-shock in a 42°C water bath for 30 seconds without shaking. Immediately following heat shock, the vial was placed on ice for an additional 2 minutes. 250 µL of room temperature S.O.C. media was then aseptically added to the vial. The vial was capped tightly and incubated horizontally at 37°C for an hour with shaking (225 rpm). Following incubation, 20 µL and 100 µL were plated on separate LA plates containing 50 µg / mL of kanamycin to select for transformants. Plates were incubated overnight at 37°C. Putative transformant colonies were grown in LB containing kanamycin (50 µg / mL) from which a plasmid prep was performed using the 5Prime FastPlasmid™ Mini Kit. Plasmid DNA was quantified using the NanoDrop1000 and verified by electrophoresis on a 1% agarose gel at 80V for 30 minutes. Plasmids were sent to Eurofins MWG Operon for sequencing
using the M13 forward and M13 reverse primers that flank the inserted region in the pDONR P1-P5r plasmid (Figure 2.1).

To create the lacZ reporter under the control of the P\textsubscript{groE} promoter both attB-flanked fragments were inserted into the Gateway\textsuperscript{®} pETDEST42 destination vector (Invitrogen 2010) using the LR reaction. 2 µL of 150 ng plasmid DNA from both pDONR\textsuperscript{TM} 221 P1-P5r containing P\textsubscript{groE} described above and a lab strain of pDONR\textsuperscript{TM} 221 P5-P2 containing lacZ were added along with 2 µL of MultiSite Gateway\textsuperscript{®} LR recombination reaction to a vial of One Shot\textsuperscript{®} Mach1\textsuperscript{TM} chemically competent E. coli (LifeTechnologies). The vial was then incubated on ice for 30 minutes followed by heat shock for 30 seconds in a 42°C water bath without shaking. The vial was immediately transferred to ice for an additional 2 minutes. Next, 250 µL of room temperature S.O.C. media was added to the vial. The vial was capped tight and placed horizontally in a 37°C incubator for 1 hour with shaking (225 rpm). 50 µL and 100 µL of the LR reaction mixed was plated on LA plates containing 100 µg/mL of ampicillin to select for pETDEST42: P\textsubscript{groE} + lacZ transformants. A plasmid prep was performed on the pETDEST42: P\textsubscript{groE} + lacZ clones and transformation was verified by electrophoresis on a 1% agarose gel at 80V for 30 minutes.
Figure 2.1. Map of pDONR™ 221 P1-P5r. pDONR™221 P1-P5r contains the kanamycin resistance gene useful for transformant selection. The attP1 and attP5r facilitate insertion of the PCR amplified gene of interest P_{groE}. The M13 forward and M13 reverse priming sites allow for detection of the desired insert by Sanger sequencing. Adapted from “MultiSite Gateway® Pro: using Gateway® Technology to simultaneously clone multiple DNA fragments,” by Invitrogen™, 2006, p. 55. Copyright 2006 by Invitrogen™.
Figure 2.2. Map of pETDEST42. pETDEST42 contains the ampicillin resistance gene for selection of transformed clones. The attR1 and attR2 sites allow for insertion of fragments flanked by the appropriate att sites. Adapted from “pET-DEST42 Gateway® Vector: A destination vector for high-level, inducible expression in E. coli,” by Invitrogen™, 2010, p. 9. Copyright 2010 by Invitrogen™.
Figure 2.3. Construction of *dnaK* complement mutants. The 2-site Gateway® system (Invitrogen) was used to construct a reporter plasmid containing a $\sigma^{32}$-dependent promoter, $p_{\text{groE}}$, fused to lacZ and cloned into the pDONR entry vector before the final pETDEST destination vector. The *dnaK* complement plasmids were generated by first cloning the insert into a pCR®2.1 by TOPO® TA Cloning® (Invitrogen). This plasmid was used as a template for Gateway® cloning into the pDONR entry vector. At this point site-directed mutagenesis (SDM) was performed to generate the C-terminally modified *dnaKV634X*. Both *dnaK* and *dnaKV634X* constructs were transferred to the pBBRDEST destination before being transformed into a $\Delta\text{dnaK} \ E. \ coli$ host.
2.4.2 Construction of DnaK Strains and Site-Directed Mutagenesis

A WT dnaK-complemented mutant was generated using the Gateway® Pro system (Invitrogen). First, dnaK fragments flanked by attB1 and attB2 sites were amplified, see Table 2 for primers. PCR products were purified using the LONZA FlashGel™ System, where products approximately 2000 base pairs long were recovered. The recovered product was quantified on the NanoDrop1000, where 150 ng of product was considered acceptable for use in the BP reaction. The products were inserted into pDONR™221 and used to transform chemically competent One shot® Mach1™ E. coli cells (LifeTechnologies) as described in section 2.4.1. Transformants were selected on LA kanamycin (50 µg/mL) plates. Plasmid DNA was extracted from transformed cells using the 5Prime FastPlasmid™ Mini Kit. Inserts were confirmed by restriction digest with the enzymes Sph1 and BstB1, followed by electrophoresis on 1% agarose gel at 80V for 30 minutes. Plasmid DNA was also sequenced by Eurofins MWG Operon using the M13 and M13 reverse primers. Primers (Table 2) for introduction of a premature stop at the first Lys residue of DnaK’s C-terminal region (Figure 2.4) via site-directed mutagenesis (SDM) were designed using the QuickChange® Primer Design tool (Agilent Technologies). Site-directed mutagenesis was performed according to the protocol (Stratagene 2006) on the pDONR™221: dnaK plasmid DNA and then transferred to Gateway® compatible pBBRDEST42 destination vector (Figure 2.5) (Khalsa-Moyers 2010). The LR reaction was used to insert wild type dnaK or dnaKV634X into the pBBRDEST42 terminal vector and used to transform a chemically competent dnaK knockout E. coli strain.
Chemically competent dnaK knockout E. coli cells were made by growing an overnight 5 mL culture of the cells in LB containing kanamycin (50 µg/mL) at 37°C with shaking. The overnight culture was then centrifuged at 8,000 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended by vortex in 5 mL of ice-cold 30 mM CaCl₂. The cells were then distributed into three 1.5 mL microcentrifuge tubes. The tubes were centrifuged for 30 seconds at maximum speed. The supernatant was discarded and the pellets were re-suspended in 0.5 mL ice-cold CaCl₂ by gently flicking the tubes. Each tube was then aliquoted into single use 50 µL microcentrifuge tubes. To transform the chemically competent ΔdnaK E. coli cells, 1 µL of pBBRDEST-42:dnak (Figure 2.6) or pBBRDEST-42:dnakV634X was added. The cells were incubated for 30 minutes on ice, followed by heat shock at 42°C for 30 seconds. The cells were chilled on ice for 5 minutes and 500 µL of S.O.C was added to the cells. The cells were then recovered by incubating at 37°C for 1 hour with shaking (225 rpm). 50 µL and 100 µL of cells were then plated on LA plates containing gentamycin (10 µg/mL).
Figure 2.5. Map of pBBRDEST-42 vector. pBBRDEST-42 contains the gentamycin resistance gene for selection of transformants. The vector also includes Gateway compatible $attR1$ and $attR2$ sites for transfer of desired fragments by the LR reaction. Adapted from “Use of proteomics tools to investigate protein expression in *Asospirillum brasiliense*,” by Khalsa-Moyers, G. K., 2010, PhD diss., University of Tennessee p. 202. Copyright 2010 by University of Tennessee, Knoxville.

In order to create the $\Delta dnaK$:pBBRDEST42:$dnaK$ (Figure 2.6) with pETDEST42:$pgroE/lacZ$ (Figure 2.7) and $\Delta dnaK$:pBBRDEST42:$dnaKV634X$ with pETDEST42:$pgroE/lacZ$ strains, the tri-parental mating technique was employed. 5 mL of *E. coli* $\Delta dnaK$:pBBRDEST42:$dnaK$ or *E. coli* $\Delta dnaK$:pBBRDEST42:$dnaKV634X$, Mach1TM T1R *E. coli*:pET-DEST42: $pgroE/lacZ$, and *E. coli*:pRK2013 (a helper plasmid strain) were grown overnight at 37°C with shaking (225 rpm) in
LB containing gentamycin (10 µg/mL), ampicillin (100 µg/mL), and kanamycin (50 µg/mL) respectively. 2 mL of each culture was centrifuged at 8,000 rpm for 5 minutes. The supernatant was decanted. The pellets were washed with 1 mL of 10% glycerol three times and finally re-suspended in 1 mL of 10% glycerol. Each strain was combined into a single tube. The combined mixture was spot plated on non-selective LA. The plates were grown overnight at 37°C. The overnight growth was swabbed a suspension was made from this growth in 2 mL of 10% glycerol. The suspension was plated on LA plates containing gentamycin (10 µg/mL) and ampicillin (100 µg/mL) to select for transformed colonies.

Figure 2.6. Map of pBBRDEST-42: dnaK plasmids. pBBRDEST-42: dnaK plasmid is complemented with wild type dnaK or dnaKV634X. The plasmid also contains the gentamycin resistance gene for selection of transformants.
Figure 2.7. Map of pETDEST42:$_{p\text{groE/lacZ}}$. pETDEST42:$_{p\text{groE/lacZ}}$ contains the $\text{lacZ}$ reporter under the control of the $\text{groE}$ promoter consensus sequence. The plasmid also contains an ampicillin resistance gene for selection of transformed clones.

The $\text{dnaK}$ knock-out complemented with the wild-type $\text{dnaK}$, and the modified $\text{dnaK}$ strains were then exposed to heat shock, oxidative stress and no treatment conditions; induction of the $\text{lacZ}$ reporter were quantified using a plate reader as described in 2.4.3.
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<td>$\Delta dnaK$:pBBRDEST42:$dnaK^+$$p_{groE}/lacZ$, Amp', Gm'</td>
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<td>pRK2013</td>
<td>Helper plasmid for mobilization of plasmids, Km'</td>
<td>Lab strain</td>
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2.4.3 Plate Reader Assay

The dnaK-deficient mutant and dnaK-complemented strains were grown to the exponential phase of growth (OD$_{600}$=0.3). Cells were then exposed to heat shock, oxidative stress by H$_2$O$_2$, and no treatment for 10 minutes (Table 1). X-gal (40 µg/mL) was added to each sample (MP Biomedicals). Cells were then transferred to 96-well plates in triplicate and absorbance measurements at both $A_{635}$ (beta-galactosidase) and $A_{600}$ (growth) measurements were read over a 3-hour assay at 15-minute intervals with a Synergy®2 plate reader (BioTek). Correlation of beta-galactosidase activity by absorbance levels with growth allowed for quantification of the DnaK-σ$^{32}$ interaction in vivo.

2.5 Detection of Lipid Peroxides

These experiments characterized the type of reactive oxygen species that modifies DnaK in an organism capable of lipid peroxidation because the eukaryotic homologue HSP70 is a target for lipid peroxide products (Reed 2008). TBARS assay kit (Cayman Chemical) was used to determine if exposure to the reactive oxygen generating compounds hydrogen peroxide or paraquat would induce lipid peroxides in *E. coli* and *R. palustris*, respectively. Lipid peroxide products are highly unstable and readily degrade to lipid aldehyde by-products such as MDA. MDA concentration was estimated by comparison of a color reagent to an MDA standard curve. Cells were exposed to paraquat concentrations of 1, 10, 50, and 100 mM. A no treatment control was also included. For the *E. coli* assays, cells were exposed to 1, 10, 50, and 100 mM of H$_2$O$_2$. 

35
2.5.1 TBARS Detection of Putative Lipid Aldehyde Products in *E. coli* and *R. palustris*

1 mL of cells in PMS-10 (*R. palustris*) or LB (*E. coli*) was collected and sonicated 3 X for 5-second intervals at 40 V on ice. 100 µL of SDS were then added to 100 µL of the sample (Sigma-Aldrich). 4 mL of the color reagent was then added and samples were boiled (~100°C) for an hour. Following boiling, samples were placed on ice then centrifuged for 10 minutes at 1,600 x g at 4°C. 150 µL of sample were then loaded onto a 96-well plate and absorbance at 530-540 nm was read using a Synergy™ 2 plate reader (BioTek). MDA concentration in the samples was estimated by comparing absorbance readings to an MDA standard curve.

2.5.2 GC/MS Detection of Lipid Aldehyde Products in *R. palustris*

GC/MS provided an alternative method for the detection of lipid aldehydes as evidence of lipid peroxidation induced by the herbicide paraquat. All chemicals were obtained from Sigma-Aldrich for this set of experiments. *R. palustris* cells were exposed to 50 mM paraquat for 30 minutes and collected by centrifugation at 5000 rpm for 20 minutes and dried to yield a dry weight of ~15mg. The dried cell pellets were washed in 25 mL of cold water and centrifuged at 4000 rpm for 45 minutes at 4°C. The pellets were subjected to Folch extraction during which the cells were homogenized with a 15 mL chloroform/methanol (2:1) solution. The mixture was then agitated for 15 minutes in an orbital shaker at room temperature. The homogenate was centrifuged again to recover the liquid phase. The solvent was washed with 3.75 mL of water then vortexed to re-suspend. The mixture was centrifuged at 2000 rpm. The upper layer was removed and the lower layer was evaporated under a steady nitrogen stream (Bohin 2005). The samples were suspended in 100 µL of dimethylformamide (DMF). 5 µL of
internal standard was added at this point. For standards, 100 uL of each solution was used for derivatization. 200 µL of 0.05 M aqueous O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride was added to each sample or standard followed by agitation for 30 minutes at room temperature. 0.5 mL of methanol and 4 mL of hexane was then added followed by vortexing for 1 minute. 140 uL of sulphuric acid was then added dropwise to the solution followed by 1 minute of vortexing. Samples and standards were then centrifuged at 3000 rpm for 5 minutes. The upper phase was extracted and dried over sodium sulphate followed by blowdown under a steady N₂ stream. 50 µl of N., O-bis (trimethylsilyl) trifluoroacetamide in 1% trimethylchlorosilane and 50 µL of pyridine were added to samples extracts, HNE, and deuterated HNE standards and reacted at 80°C for 2 hours (Rauli 1998). Samples were injected in splitless mode to GC coupled with a mass selective detector. Absorbed volatiles were desorbed to a column at an initial temperature of 40°C that was held for 2 minutes, followed by an increase to 100°C at 10°C/min and a further increase to 240°C at 10°C/min held for 1 minute. Lipid aldehydes were detected by the selected ion monitoring focusing on the ions 250 and 181 for malondialdehyde and 352 and 242 for 4-hydroxynonenal. Ions used for deuterated 4-hydroxynonenal were 355 and 245.
Table 2. List of primers used for qPCR amplification and cloning. No change in font format is the portion of the sequence homologous to the gene of interest. For complete gene sequences see APPENDIX A. For oligonucleotide calculation results see APPENDIX B. For the Gateway cloning primers, the bold portion includes *att* sequences.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Description</th>
<th>Primer Sequence</th>
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<tbody>
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<td>RPAL rpoH FWD</td>
<td>Forward qPCR primer for <em>rpoH</em> in <em>R. palustris</em></td>
<td>CAAGGCGTGCATTCAAGAGT</td>
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<tr>
<td>RPAL rpoH REV</td>
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<td>rpoH EC2F</td>
<td>Forward qPCR primer for <em>rpoH</em> in <em>E. coli</em></td>
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<tr>
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<td>gapA EC1R</td>
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<td>Sample</td>
<td>Primer Description</td>
<td>Primer Sequence</td>
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<tr>
<td>groE attB1</td>
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<td>groE attB5r</td>
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CHAPTER 3

RESULTS


To explore the differential expression of *rpoH* under stress conditions, quantitative real-time PCR (qPCR) analysis of *rpoH* mRNA transcript levels was performed following heat shock and oxidative stress compared to an unstressed system. Oxidative stress was achieved by exposure of cells to the concentration of H$_2$O$_2$ shown to be maximally effective in production of lipid aldehyde products from the TBARS experiment (Figure 3.2), 10 mM, for 30 minutes. Heat shock of cells was performed by placing the cells in a 42°C water bath for 30 minutes. The superoxide generator paraquat (50 mM) was also used to induce oxidative stress by exposure for 30 minutes (Figure 3.1). Samples were extracted at 10-minute intervals to determine the time course of stress response.
Figure 3.1. Detection of MDA or similar reactive substances in *R. palustris* by TBARS assay. A standard curve was generated with 0-10 μM MDA standards gave the equation $y=0.0017x-0.0004$, $R^2=0.99159$. Experimental MDA/TBARS content in *R. palustris* cells when exposed to 1, 10, 50, and 100 mM PQ was determined by using the best fit equation. Experiment performed in duplicate. Error bars represent standard deviation.
Figure 3.2. Detection of MDA or similar reactive substances in \textit{E. coli} by TBARS assay. A standard curve was generated with 0-50 μM MDA standards gave the equation $y=0.0014x-0.0023$, $R^2=0.99798$. Experimental MDA/TBARS content in \textit{E. coli} cells when exposed to 1, 10, 50, and 100 mM hydrogen peroxide. Experiment performed in duplicate. Error bars represent standard deviation.
qPCR studies (Figure 3.3) of the H₂O₂-treated E. coli cells (Figure 3.5) have shown that rpoH expression in E. coli increases from 1X to 6.08X between 0 and 10 minutes of exposure, rpoH levels decrease to 1.89 at 20 minutes and then increase to 10.00X at 30 minutes of exposure. Under heat shock conditions (Figure 3.4), rpoH expression in E. coli shifts from 1X, 2.20X, 1.08X, and 3.36X at 0, 10, 20, and 30 minutes. RpoH levels for cells treated with H₂O₂ (Figure 3.5) were significantly higher than rpoH levels following heat shock at 10, 20, and 30 minutes (one-tailed Student’s t test, p=0.00009, p=0.000002, and p=0.0005 for each time point respectively, α=0.05). When exposed to 50 mM PQ (Figure 3.6), rpoH expression was observed at 1X, 1.93X, 2.52X, and 7.73X at each consecutive time point. RpoH levels for PQ treated cells were significantly greater than rpoH levels for heat shocked cells at 20, and 30 minutes (one-tailed Student’s t test, p=0.0049 and p=0.0026 respectively, α=0.05). RpoH levels for cells treated with H₂O₂ were significantly greater than PQ at 10 and 30 minutes (one-tailed Student’s t test, p=0.046 and p=0.05 respectively, α=0.05).
Figure 3.3. Relative transcription levels of *rpoH* during 10 mM H$_2$O$_2$, 50 mM PQ, and heat shock (42°C) exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcription levels were normalized relative to the housekeeping gene *gapA* at each time point. *RpoH* levels for cells treated with H$_2$O$_2$ were significantly higher than *rpoH* levels following heat shock at 10, 20, and 30 minutes (one-tailed Student’s t test, $p=0.00009$, $p=0.000002$, and $p=0.0005$ for each time point respectively, $\alpha=0.05$). *RpoH* levels for PQ treated cells were significantly greater than *rpoH* levels for heat shocked cells at 20, and 30 minutes (one-tailed Student’s t test, $p=0.0049$ and $p=0.0026$ respectively, $\alpha=0.05$). *RpoH* levels for cells treated with H$_2$O$_2$ were significantly greater than PQ at 10 and 30 minutes (one-tailed Student’s t test, $p=0.046$ and $p=0.05$ respectively, $\alpha=0.05$). Error bars represent standard error.
Figure 3.4. Relative transcription levels of rpoH during heat shock at 42°C for 0, 10, 20, and 30 minutes in E. coli cells. RpoH transcript levels shift from 1X, 2.20X, 1.08X, and 3.36X at 0, 10, 20, and 30 minutes of heat shock. RpoH levels for cells treated with H2O2 were significantly higher than rpoH levels following heat shock at 10, 20, and 30 minutes (one-tailed Student’s t test, p=0.00009, p=0.000002, and p=0.0005 for each time point respectively, α=0.05). RpoH levels for PQ treated cells were significantly greater than rpoH levels for heat shocked cells at 10, and 30 minutes (one-tailed Student’s t test, p=0.0049 and p=0.05 respectively, α=0.05). Error bars represent standard error.
Figure 3.5. Relative transcription levels of *rpoH* during 10 mM H$_2$O$_2$ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcription levels were normalized relative to the housekeeping gene *gapA* at each time point. Transcript levels decrease to 1.89X at 20 minutes, and then increase to 10X at 30 minutes under H$_2$O$_2$ exposure. *RpoH* levels for cells treated with H$_2$O$_2$ were significantly higher than *rpoH* levels following heat shock at 10, 20, and 30 minutes (one-tailed Student’s t test, p=0.00009, p=0.00002, and p=0.0005 for each time point respectively, α=0.05). *RpoH* levels for cells treated with H$_2$O$_2$ were significantly greater than PQ at 10 and 30 minutes (one-tailed Student’s t test, p=0.046 and p=0.05 respectively, α=0.05). Error bars represent standard error.
Figure 3.6. Relative transcription levels of *rpoH* during 50 mM PQ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcription levels were normalized relative to the housekeeping gene *gapA* at each time point. *RpoH* transcript levels were observed at 1X, 1.93X, 2.25X, and 7.73X at 0, 10, 20, and 30 minutes. *RpoH* levels for PQ treated were significantly higher than *rpoH* levels following heat shock at 10, 20, and 30 minutes (one-tailed Student’s *t* test, *p*=0.00009, *p*=0.000002, and *p*=0.0005 for each time point respectively, *α*=0.05). *RpoH* levels for cells treated with H$_2$O$_2$ were significantly greater than PQ at 10 and 30 minutes (one-tailed Student’s *t* test, *p*=0.046 and *p*=0.05 respectively, *α*=0.05). Error bars represent standard error.
qPCR studies of paraquat-treated *R. palustris* cells have shown that *rpoH* expression increased over 30 minute exposure to 50 mM paraquat with fold changes greater than 10.7X at 10 minutes, 14.8X at 20 minutes and 8.1X at 30 minutes compared to *rpoH* expression under control conditions (Figure 3.7). Under heat shock conditions *rpoH* transcript levels showed fold changes of 9.4X, 1.9X, and 3.3X relative to the control at 10, 20, and 30 minutes, respectively (Figure 3.8). Treatment of cells with paraquat resulted in significantly higher levels of *rpoH* mRNA than in heat-shocked cells at 20 and 30 minutes (one-tailed Student’s t test, p=0.0002 and p=0.006 respectively, α=0.05).
Figure 3.7. Relative transcription levels of rpoH during 50 mM paraquat exposure for 10, 20, and 30 minutes in R. palustris cells. The transcript levels reported were normalized relative to the housekeeping gene gapDH at each time point. RpoH transcript levels were observed at 10.7X, 14.8X, and 8.1X at 10, 20, and 30 minutes. RpoH levels for cells treated with paraquat were significantly higher than gapDH at each time point (One-tailed Student’s t test, p=0.02, p=0.002, and p=0.007 respectively, α=0.05). Error bars represent standard error.
Figure 3.8. Relative transcription levels of *rpoH* during heat shock at 42°C for 10, 20, and 30 minutes in *R. palustris* cells. *RpoH* transcript levels were observed at 9.4X, 1.9X, and 3.3X at 10, 20, and 30 minutes of heat shock. *RpoH* levels for cells treated with heat shock were significantly higher than *gapDH* at each time point (One-tailed Student's t test, *p*=0.002, *p*=0.02, and *p*=0.04 respectively, α=0.05). Error bars represent standard error.

qPCR studies of the H$_2$O$_2$-treated *E. coli* cells have shown that $dnaK$ expression in *E. coli* increases to 1.53X, 2.00X, and 2.44X after 10, 20, and 30 minutes of exposure (Figure 3.9, Figure 3.11). At 10 minutes $dnaK$ transcription levels under H$_2$O$_2$ stress were significantly greater than when exposed to heat shock conditions (one-tailed Student’s t test, $p=0.0001$, $\alpha=0.05$). Under heat shock conditions (Figure 3.10), $dnaK$ expression in *E. coli* shifts from 1X, 0.76X, 1.91X, and 3.39X at 0, 10, 20, and 30 minutes. When exposed to 50 mM PQ, $dnaK$ expression was observed at 1X, 0.85X, 2.28X, and 1.95X at each consecutive time point (Figure 3.12). H$_2$O$_2$ treated $rpoH$ levels were significantly higher than PQ treated levels at 10 minutes (one-tailed Student’s t test, $p=0.0008$, $\alpha=0.05$).
Figure 3.9. Relative transcription levels of dnaK during 10 mM H$_2$O$_2$, 50 mM PQ, and heat shock exposure for 0, 10, 20, and 30 minutes in E. coli cells. The transcript levels were normalized relative to the housekeeping gene gapA at each point. At 10 minutes H$_2$O$_2$ exposure dnaK transcription levels were significantly greater than when exposed to heat shock conditions (one-tailed Student’s t test, p=0.0001, $\alpha=0.05$). H$_2$O$_2$ treated dnaK levels were significantly higher than PQ treated levels at 10 minutes (one-tailed Student’s t test, p=0.0008, $\alpha=0.05$). Error bars represent standard error.
Figure 3.10. Relative transcription levels of *dnaK* during heat shock at 42°C for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *DnaK* transcript levels shift from 1X, 0.76X, 1.91X, and 3.39X at each consecutive time point under heat shock. At 10 minutes H₂O₂ exposure *dnaK* transcription levels were significantly greater than when exposed to heat shock conditions (one-tailed Student’s t test, p=0.0001, α=0.05). Error bars represent standard error.
Figure 3.11. Relative transcription levels of *dnaK* during 10 mM H$_2$O$_2$ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *DnaK* transcript levels were observed at 1X, 1.53X, 2.00X, and 2.44X at 0, 10, 20, and 30 minutes. At 10 minutes H$_2$O$_2$ exposure *dnaK* transcription levels were significantly greater than when exposed to heat shock conditions (one-tailed Student’s t test, $p=0.0001$, $\alpha=0.05$). H$_2$O$_2$ treated *dnaK* levels were significantly higher than PQ treated levels at 10 minutes (one-tailed Student’s t test, $p=0.0008$, $\alpha=0.05$). Error bars represent standard error.
Figure 3.12. Relative transcription levels of *dnaK* during 50 mM PQ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *DnaK* transcripts were observed at 1X, 0.85X, 2.28X, and 1.95X at each consecutive time point. H$_2$O$_2$ treated *dnaK* levels were significantly higher than PQ treated levels at 10 minutes (one-tailed Student’s t test, p=0.0008, $\alpha$=0.05). Error bars represent standard error.
DnaJ expression in E. coli cells treated with H2O2 was 1X, 1.03X, and 0.89X, and 1.35X at 0, 10, 20, and 30 minutes of exposure (Figure 3.13, Figure 3.15). DnaJ levels under H2O2 exposure were significantly different from heat shock levels at 20, and 30 minutes (two-tailed Student’s t test, \( p=0.0000001 \) and \( p=0.02 \) respectively, \( \alpha=0.05 \)). Under heat shock conditions (Figure 3.14), dnaJ expression levels were 1X, 0.82X, 2.23X, and 0.74 at 0, 10, 20, and 30 minutes. Heat shock dnaJ levels were significantly different from PQ levels at 10 and 30 minutes (two-tailed Student’s t test, \( p=0.05 \), and \( p=0.002 \) at each point respectively, \( \alpha=0.05 \)). When exposed to 50 mM PQ, dnaJ expression was observed at 1X, 0.44X, 1.71X, and 4.68X at each consecutive time point (Figure 3.16). DnaJ levels for cells treated with 50 mM paraquat were significantly different from cells treated with H2O2 10, 20, and 30 minutes (two-tailed Student’s t test, \( p=0.004 \), \( p=0.045 \), and \( p=0.05 \) respectively, \( \alpha=0.05 \)).
Figure 3.13. Relative transcription levels of dnaJ during 10 mM H₂O₂, 50 mM PQ, and heat shock exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene gapA at each point. DnaJ levels under H₂O₂ exposure were significantly different from heat shock levels at 20, and 30 minutes (two-tailed Student’s t test, p=0.0000001 and p=0.02 respectively, α=0.05). Heat shock dnaJ levels were significantly different from PQ levels at 10 and 30 minutes (two-tailed Student’s t test, p=0.05, and p=0.002 at each point respectively, α=0.05). DnaJ levels for cells treated with 50 mM paraquat were significantly different from cells treated with H₂O₂ 10, 20, and 30 minutes (two-tailed Student’s t test, p=0.004, p=0.045, and p=0.05 respectively, α=0.05). Error bars represent standard error.
Figure 3.14. Relative transcription levels of *dnaJ* during heat shock at 42°C exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *DnaJ* transcript levels were 1X, 0.82X, 2.23X, and 0.74X at each time point under heat shock. *DnaJ* levels under H₂O₂ exposure were significantly different from heat shock levels at 20, and 30 minutes (two-tailed Student’s t test, p=0.0000001 and p=0.02 respectively, α=0.05). Heat shock *dnaJ* levels were significantly different from PQ levels at 10 and 30 minutes (two-tailed Student’s t test, p=0.05, and p=0.002 at each point respectively, α=0.05). Error bars represent standard error.
Figure 3.15. Relative transcription levels of *dnaJ* during 10 mM H$_2$O$_2$ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *DnaJ* transcripts were at 1X, 1.03X, 0.89X, and 1.35X at 0, 10, 20, and 30 minutes under H$_2$O$_2$ exposure. *DnaJ* levels under H$_2$O$_2$ exposure were significantly different from heat shock levels at 20, and 30 minutes (two-tailed Student’s t test, *p*=0.0000001 and *p*=0.02 respectively, *α*=0.05). *DnaJ* levels for cells treated with 50 mM paraquat were significantly different from cells treated with H$_2$O$_2$ 10, 20, and 30 minutes (two-tailed Student’s t test, *p*=0.004, *p*=0.045, and *p*=0.05 respectively, *α*=0.05). Error bars represent standard error.
Figure 3.16. Relative transcription levels of *dnaJ* during 50 mM PQ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *DnaJ* transcripts were observed at 1X, 0.44X, 1.71X, and 4.68X at each time point under PQ exposure. Heat shock *dnaJ* levels were significantly different from PQ levels at 10 and 30 minutes (two-tailed Student’s t test, p=0.05, and p=0.002 at each point respectively, α=0.05). *DnaJ* levels for cells treated with 50 mM paraquat were significantly different from cells treated with H₂O₂ 10, 20, and 30 minutes (two-tailed Student’s t test, p=0.004, p=0.045, and p=0.05 respectively, α=0.05). Error bars represent standard error.
qPCR studies of the H$_2$O$_2$-treated *E. coli* cells have shown that *groEL* expression in *E. coli* was 1X, 1.83X, and 1.27X, and 2.63 at 0, 10, 20, and 30 minutes of exposure (Figure 3.17, Figure 3.19). H$_2$O$_2$ *groEL* levels were significantly different than heat shock levels at 10 minutes (two-tailed Student’s t test, $p=0.01$, $\alpha=0.05$). Under heat shock conditions (Figure 3.18), *groEL* expression in *E. coli* shifts from 1X, 0.83X, 1.61X, and 2.50X at 0, 10, 20, and 30 minutes. Heat shock *groEL* levels were significantly different than PQ levels at 20 and 30 minutes (two-tailed Student’s t test, $p=0.01$ and $p=0.05$ at each point respectively, $\alpha=0.05$). When exposed to 50 mM PQ, *groEL* expression was observed at 1X, 0.83X, 3.24X, and 6.97X at each consecutive time point (Figure 3.20). *GroEL* levels for cells treated with 50 mM paraquat were significantly different than those treated with H$_2$O$_2$ at 10 and 20 minutes of exposure (two-tailed Student’s t test, $p=0.00001$ and $p=0.00005$ respectively, $\alpha=0.05$).
Figure 3.17. Relative transcription levels of *groEL* during 10 mM H$_2$O$_2$, 50 mM PQ, and heat shock exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. H$_2$O$_2$ *groEL* levels were significantly different than heat shock levels at 10 minutes (two-tailed Student’s t test, $p=0.01$, $\alpha=0.05$). Heat shock *groEL* levels were significantly different than PQ levels at 20 and 30 minutes (two-tailed Student’s t test, $p=0.01$ and $p=0.05$ at each point respectively, $\alpha=0.05$). *GroEL* levels for cells treated with 50 mM paraquat were significantly different than those treated with H$_2$O$_2$ at 10 and 20 minutes of exposure (two-tailed Student’s t test, $p=0.00001$ and $p=0.00005$ respectively, $\alpha=0.05$). Error bars represent standard error.
Figure 3.18. Relative transcription levels of \textit{groEL} during heat shock at 42°C exposure for 0, 10, 20, and 30 minutes in \textit{E. coli} cells. The transcript levels were normalized relative to the housekeeping gene \textit{gapA} at each point. \textit{GroEL} transcripts were at 1X, 0.83X, 1.61X, and 2.50X at 0, 10, 20, and 30 minutes under heat shock. H$_2$O$_2$ \textit{groEL} levels were significantly different than heat shock levels at 10 minutes (two-tailed Student’s t test, $p=0.01$, $\alpha=0.05$). Heat shock \textit{groEL} levels were significantly different than PQ levels at 20 and 30 minutes (two-tailed Student’s t test, $p=0.01$ and $p=0.05$ at each point respectively, $\alpha=0.05$). Error bars represent standard error.
Figure 3.19. Relative transcription levels of *groEL* during 10 mM H$_2$O$_2$ exposure for 0, 10, 20, and 30 minutes in *E. coli* cells. The transcript levels were normalized relative to the housekeeping gene *gapA* at each point. *GroEL* expression was at 1X, 1.83X, 1.27X, and 2.63X at each time point under H$_2$O$_2$ stress. H$_2$O$_2$ *groEL* levels were significantly different than heat shock levels at 10 minutes (two-tailed Student’s t test, $p=0.01$, $\alpha=0.05$). *GroEL* levels for cells treated with 50 mM paraquat were significantly different than those treated with H$_2$O$_2$ at 10 and 20 minutes of exposure (two-tailed Student’s t test, $p=0.00001$ and $p=0.00005$ respectively, $\alpha=0.05$). Error bars represent standard error.
Figure 3.20. Relative transcription levels of groEL during 50 mM PQ exposure for 0, 10, 20, and 30 minutes in E. coli cells. The transcript levels were normalized relative to the housekeeping gene gapA at each point. GroEL expression was at 1X, 0.83X, 3.24X, and 6.97X and 0, 10, 20, and 30 minutes under PQ exposure. Heat shock groEL levels were significantly different than PQ levels at 20 and 30 minutes (two-tailed Student’s t test, p= 0.01 and p=0.05 at each point respectively, α=0.05). GroEL levels for cells treated with 50 mM paraquat were significantly different than those treated with H2O2 at 10 and 20 minutes of exposure (two-tailed Student’s t test, p=0.00001 and p=0.00005 respectively, α=0.05). Error bars represent standard error.
3.1.2 Alternate Heat Shock Gene Detection: RNAseq

Quantitative real-time PCR detection of mRNA expression of *rpoH*-dependent heat shock genes allows for the study of transcription of genes on an individual basis. Whole transcriptome sequencing would yield similar information and additional data on all genes transcribed under given stress conditions. Given the qPCR findings, it was expected that *rpoH* and the heat shock genes it regulates would be differentially transcribed during oxidative stress versus heat shock in the RNAseq analysis.

RNAseq analysis was unsuccessful during the course of this study. When RNA was extracted from experimental *E. coli* cells and analyzed for quality, concentrations were 701.34, 801.15, and 2350.01 ng/µL for PQ, no treatment, and HS treated cells. Following ribosomal RNA depletion, RNA concentrations decreased to 27.28, 42.79, and 180.49 ng/µL for PQ, no treatment, and HS treated RNA. Samples were converted to cDNA and analyzed for quality. The quality of cDNA samples were all able to yield a library dilution of 210 X10^6 molecules of template per 20 µL recommended by the Ion total RNAseq kit protocol (LifeTechnologies). The paraquat treated samples had 27% loading of sample with 19% usable sequences. The heat-shocked samples had 64% loading of samples with no usable sequence in order to perform sequence analysis of these samples.

3.2 Modification of DnaK C-Terminus and Effects on σ^{32} Activity

Reporter studies were performed to support the mechanism for posttranslational modification of Lys residues by oxidative stress of DnaK. These studies were carried out by
constructing plasmids containing lacZ that is under the control of pGroE, a $\sigma^{32}$-dependent heat shock protein promoter. The constructs from Table 1 were exposed to heat shock, oxidative stress, or no treatment in triplicate. X-gal was added to each sample in order to detect beta-galactosidase and samples were plated as shown in (Figure 3.21).
Figure 3.21. Beta-galactosidase assay plating. The WT DnaK complement, modified DnaKV634X complement, empty ΔdnaK control, lacZ reporter control, reporter-less DnaK control, and reporter-less DnaKV634X control were plated in triplicate following stress treatment.
Absorbance measurements were collected at 635 nm for detection of the blue color produced when beta-galactosidase cleaves X-gal, as well as at 550 nm, which was used to quantify cell growth. To obtain beta-galactosidase expression in units, the absorbance at 635 nm was divided by growth at 550 nm.

When unstressed (Figure 3.22) the DnaK complemented strain and modified DnaKV634X complement strains are not significantly different (two-tailed Student’s t test, \( p=0.81 \), \( \alpha=0.05 \)). Both strains approach 1 unit of beta-galactosidase output. Similarly, both strains display significantly lower beta-galactosidase activity than the lacZ reporter control (one-tailed Student’s t test, \( p=1.3\times10^{-8} \) and \( p=1.8\times10^{-8} \) for the wild type DnaK complement and the modified DnaKV634X complement strains, \( \alpha=0.05 \)).
Figure 3.22. Normalized beta-galactosidase activity (absorbance 635 nm/ OD) of DnaK constructs under normal growth (no stress treatment) conditions. The WT DnaK complement, modified DnaKV634X complement, empty ΔdnaK control, lacZ reporter control, reporter-less DnaK control, and reporter-less DnaKV634X control were plated in triplicate following no treatment and the addition of X-gal. Error bars represent standard error.
When exposed to heat shock conditions (Figure 3.23), the DnaKV634X strain displays slightly higher beta-galactosidase activity relative to the wild type DnaK strain, though it is not statistically greater (one-tailed Student’s t test, $p=0.14$, $\alpha=0.05$). Both strains display significantly lower beta-galactosidase activity than the lacZ reporter control (one-tailed Student’s t test, $p=0.001$ and $p=0.008$ for the wild type DnaK complement and DnaKV634X complement strains, $\alpha=0.05$) though the DnaKV634X strain approaches one unit of beta-galactosidase activity at the end of the assay.
Figure 3.23. Normalized beta-galactosidase activity (absorbance 635 nm/ OD) of DnaK constructs under heat shock conditions. The WT DnaK complement, modified DnaKV634X complement, empty ΔdnaK control, lacZ reporter control, reporter-less DnaK control, and reporter-less DnaKV634X control were plated in triplicate following heat shock conditions (42°C for 10 minutes) and the addition of X-gal. Error bars represent standard error.
In the presence of oxidative stress (10 mM H$_2$O$_2$ for 10 minutes) (Figure 3.24), the wild type DnaK complement strain displays significantly higher beta-galactosidase activity than the DnaKV634X strain (one-tailed Student’s t test, $p=6.3 \times 10^{-5}$, $\alpha=0.05$). Under oxidative stress the wild type DnaK strain displays statistically similar beta-galactosidase activity to the reporter control (two-tailed Student’s t test, $p=0.83$, $\alpha=0.05$). While the DnaKV634X strain displays statistically lower beta-galactosidase activity than the reporter control (two-tailed Student’s t test, $p=7.3 \times 10^{-5}$, $\alpha=0.05$).
Figure 3.24. Normalized beta-galactosidase activity (absorbance 635 nm/ OD) of DnaK constructs under H$_2$O$_2$ stress. The WT DnaK complement, modified DnaKV634X complement, empty $\Delta$dnaK control, lacZ reporter control, reporter-less DnaK control, and reporter-less DnaKV634X control were plated in triplicate following oxidative stress conditions (10 mM H$_2$O$_2$ for 10 minutes) and the addition of X-gal. Error bars represent standard error.
In the presence of PQ induced oxidative stress (50 mM for 10 minutes) (Figure 3.25), the wild type DnaK complement strain displays significantly higher beta-galactosidase activity than the DnaKV634X strain (one-tailed Student’s t test, $p=6.3\times10^{-9}$, $\alpha=0.05$). Under PQ stress the wild type DnaK strain approaches the beta-galactosidase activity of the reporter control but is still significantly different (two-tailed Student’s t test, $p=0.001$, $\alpha=0.05$). The DnaKV634X strain displayed statistically lower beta-galactosidase activity than the reporter (two-tailed Student’s t test, $p=0.00004$) but it was slightly higher than the reporter-less controls.
Figure 3.25. Normalized beta-galactosidase activity (absorbance 635 nm/ OD) of DnaK constructs under oxidative stress conditions. The DnaK WT complement, modified DnaKV634X complement, empty ΔdnaK control, lacZ reporter control, complement control, and modified DnaKV634X complement control were plated in triplicate following oxidative stress conditions (50 mM PQ for 10 minutes) and the addition of X-gal. Error bars represent standard error.
3.2.1 Detection of Lipid Peroxides in *E. coli* and *R. palustris* by Exposure to ROS

These experiments characterize the type of reactive oxygen species that modifies DnaK in an organism capable of lipid peroxidation. DnaK was investigated because the eukaryotic homologue HSP70 is a target for lipid peroxide products (Reed 2008). TBARS assay was used to determine if exposure to reactive oxygen species hydrogen peroxide or paraquat would induce lipid peroxides in *E. coli* and *R. palustris*, respectively. Lipid peroxide products are highly unstable and readily degrade to lipid aldehyde by-products such as MDA. MDA concentration was estimated by comparison of a color reagent to an MDA standard curve. Cells were exposed to H$_2$O$_2$ or paraquat concentrations of 1, 10, 50, and 100 mM.

A TBARS study determined that MDA or a similarly reactive substance was formed at increasing concentrations of paraquat exposure in *R. palustris*. Putative MDA concentrations increased to approximately 2.7 $\mu$M at 50 mM paraquat (Figure 3.1). The addition of 100 mM paraquat did not increase MDA concentration relative to cells exposed to 50 mM paraquat. These findings indicate that 50 mM paraquat treatment generates the maximal amount of lipid peroxidation stress. This concentration was used as the exposure concentration in subsequent ROS studies of *R. palustris*. In *E. coli* 1 mM hydrogen peroxide produced a putative MDA concentration of 0.21 $\mu$M. MDA content increased to 1.16 $\mu$M with exposure to 10 and 50 mM hydrogen peroxide then decreased with exposure to 100 mM hydrogen peroxide (Figure 3.2). 10 mM H$_2$O$_2$ was used to induce oxidative stress in *E. coli* in subsequent studies. Alternate GC/MS detection of lipid peroxides

The validity of the TBARS assay has been criticised as a method of detection due to the violent acid boiling step that can artificially form malondialdehyde and overrepresent lipid
peroxides. Other methods of detection of lipid peroxides include fluorescence, light emission, high-performance liquid chromatography, antibodies, and gas chromatography-mass spectrometry (GC-MS). Liu et al. determined that the GC-MS method of detection was two- to six-fold more sensitive to actual oxidation of unsaturated acids than the TBARS assay (Liu 1997). A GC/MS pilot study was performed using selected ion monitoring to detect the lipid aldehydes malondialdehyde and 4-hydroxynonenal. The initial run of \textit{R. palustris} showed large peaks of a compound similar to malondialdehyde with the ions 161, 181, and 255 in both paraquat treated and untreated cells. Malondialdehyde peaks would have an expected m/z of 181 and 250. HNE and the deuterated HNE internal standard were not detected (Figure 3.26).
Figure 3.26. *R. palustris* sample 1. Chromatogram (top) is representative of all PQ treated and untreated samples in run 1. Both PQ treated and untreated samples contained large peaks of a compound similar to MDA with ions present (bottom) at 161, 181, and 255. HNE and the deuterated HNE internal standard were not detected in any of the samples. The absence of the internal standard, led to changes in solvent from acetone to DMF in subsequent trials.
Samples from the initial run were brought into solution prior to derivatization with 100 µL of acetone. Acetone contains double bonds that are susceptible to derivatization. In the second sample run DMF was used to bring samples into solution. As in the initial run, an MDA-like compound was detected containing the ions 161, 181, and 255. HNE and the deuterated HNE internal standard were not detected in run 2 (Figure 3.27).
Figure 3.27. *R. palustris* sample run 2. Chromatogram is representative of all PQ treated and untreated samples. In this second GC/MS run, samples were brought to solution in DMF to correct for any false positives due to the use of acetone. Like in the previous experiment, a large MDA-like peak with m/z 161, 181, and 255 of was detected in both PQ treated and untreated samples. HNE and the deuterated HNE internal standard were still undetected in any samples.
In order to determine why the deuterated HNE internal standard had failed to be detected in all standards, the internal standard was added in the next run at three points in the extraction protocol where the internal standard could be lost. Internal standard was added to the dried cells pre-wash and centrifugation at 4°C. Internal standard was also added post-wash, and post extraction, prior to being brought into solution by addition of DMF. A method blank composed of water and internal standard was included to determine any matrix effects. *R. palustris* samples still showed the MDA-like peak eluting at ~10 minutes. The internal standard was not detected in the *R. palustris* samples or the method blank (Figure 3.28).
Figure 3.28. (a) *R. palustris* sample from run 3 with internal standard added to the dried sample cells pre-wash step and centrifugation at 4°C. Chromatogram is representative of both treated and untreated *R. palustris* samples. (b) A method blank composed of water with internal standard added pre-wash was included in case the cells were interfering with the detection of the lipid aldehyde compounds. The MDA-like peak was still eluting at ~10 minutes in this run of experiments and the deuterated HNE internal standard was not detected in the *R. palustris* samples or the H2O method blank.
When the internal standard was added post-wash, it was not detected in the *R. palustris* samples but was detected in the method blank (Figure 3.29). When the internal standard was added post-extraction it was detected in both the *R. palustris* samples and the method blank though it was barely detected in the *R. palustris* sample (Figure 3.30). The detection of the internal standard following post-extraction addition indicates that this step was where putative aldehydes were lost using this method.
Figure 3.29. (a) *R. palustris* sample from run 3 with internal standard added post-wash. Sample is representative of both treated and untreated *R. palustris* samples. (b) Method blank with internal standard added post-wash. When the deuterated HNE internal standard was added at the post-wash step, it was not detected in the *R. palustris* samples but was detected in the method blank.
Figure 3.30. (a) *R. palustris* sample from run 3 with internal standard added post-extraction. Sample is representative of both treated and untreated *R. palustris* samples. (b) Method blank with internal standard added post-extraction. When the internal standard was added at the post-extraction step it was detected in both the method blank and *R. palustris* samples, indicating that this was the step at which the internal standard and potential aldehydes were lost in the initial trial.
CHAPTER 4

DISCUSSION

Understanding the interaction between $\sigma^{32}$ and its regulator DnaK may inform the discussion on neurodegenerative diseases in which oxidative modification of stress proteins may play a critical role. The importance of stress proteins to environmental and clinically relevant systems has been demonstrated in numerous studies (Romero 1998; Sayre 2006; Reed 2008; Woodrick 2014). This project sought to investigate three principal questions. First, what are the transcriptional responses of key heat shock proteins under oxidative and heat shock stress? Next, I sought to determine the relationship between $\sigma^{32}$ and DnaK working under the hypothesis that a post-translational modification occurs at the Lys-rich C-terminus of DnaK during oxidative stress that prevents the binding of DnaK to $\sigma^{32}$ in a manner distinct to the stress response observed during heat shock. Lastly, the possible mode of action of oxidative injury was investigated.

4.1 Quantification of Transcriptional Responses of Key Heat Shock Genes rpoH, dnaK, dnaJ, and groEL Under Heat Shock and Oxidative Stress

4.1.1 RpoH Response is Greater during Oxidative Stress by Hydrogen Peroxide

Under oxidative stress by hydrogen peroxide, rpoH mRNA transcript levels increase steadily, albeit with a dip in transcript levels at 20 minutes in both E. coli. RpoH levels under heat shock conditions demonstrated a similar dip at 20 minutes; however, the level of rpoH transcripts was significantly less under heat shock conditions than under hydrogen peroxide stress (Figure 3.3) (one-tailed Student’s t test, p=0.00009 for all time points, $\alpha=0.05$). Previous
studies have demonstrated that following heat shock, levels of $\sigma^{32}$ increase quickly by five to six minutes of exposure. However, by 15 minutes of exposure to 42°C, $\sigma^{32}$ levels decreased to be only slightly higher than unstressed controls (Straus 1987). These findings correspond with an increase in $rpoH$ mRNA synthesis in response to heat shock leading to “increased message translation” and increased synthesis of $\sigma^{32}$ (Straus 1987). The observed decrease in $\sigma^{32}$ in the literature corresponds with the dip in $rpoH$ levels observed during both oxidative stress and heat shock conditions at 20 minutes in the present work. When an organism undergoes heat shock, the salt concentrations, cellular chemistries, and pH all become inappropriate for protein stability. As a result, proteins become denatured, exposing hydrophobic regions that are normally folded within the protein. These hydrophobic regions are attracted to each other and tend to aggregate in an attempt to refold. This effect can create nonsense proteins with no function and can also lead to cytotoxic proteins. The heat shock response is an organism’s attempt to refold denatured proteins before they are degraded by proteases and prevents formation of inappropriate protein aggregates. Heat shock response signals for the quick increase in the production of heat shock proteins (HSPs) by $\sigma^{32}$ followed by a slow decrease in HSPs as the cell adapts to the higher temperature and their presence is no longer appropriate.

When exposed to the redox cycler, paraquat, $rpoH$ levels steadily increased at 10, 20 and 30 minutes in *E. coli*. In *R. palustris* there was also a steady increase in $rpoH$ transcripts with a peak at 20 minutes. The steady increase in $rpoH$ can be explained by the slower activation of stress response to redox cycling. During redox cycling, PQ and its reduced pair, reduce molecular oxygen, forming a superoxide anion. This process continues to cycle until all the reductants are used. Superoxide radicals readily injure DNA by oxidizing guanine bases and
the mispairing resulting from this injury leads to point mutations in the DNA. ROS also
commonly target cysteine, methionine, and hydrophobic residues of proteins. These oxidized
regions will aggregate in the cell and facilitate further cell injury and eventual cell death.
However, there are DNA repair mechanisms and molecular chaperones such as DnaK that
prevent protein aggregation. The continued increase of rpoH in the presence of PQ stress could
be a result of key chaperones themselves being oxidized and the intrinsic interaction with σ32
weakens (Wang 2009). Superoxide has also been shown to be converted to hydrogen peroxide
that will then react with iron compounds to form hydroxyl radicals that would perpetuate cell
damage (Tamarit 1998). The ability of PQ to incite multiple avenues of ROS stress, could
explain the additive effect of this stress on rpoH levels.

To determine the effect that increased levels of σ32 has on the transcription of heat
shock proteins in this system, dnaK, dnaJ, and groEL transcription levels were observed by
qPCR. Increased transcription of these key heat shock genes was observed with dips in
transcription between 10 and 20 minutes of H2O2 and heat shock exposure. The decreases at
10 and 20 minutes in dnaJ and groEL correspond to decreases in rpoH levels. This supports
what is known about RpoH controlling the transcription of heat shock proteins under multiple
stress conditions (Cowing 1985; Cabiscole 2000; Wang 2003).

There are two main chaperone systems in bacteria, the GroE machine and the DnaK
machine. The GroE machine that includes GroEL and GroES provides a protective environment
for protein refolding. While ATP does facilitate the movement of an unfolded protein through
the GroE machine, the refolding process is fairly passive in contrast to the DnaK machine
includes DnaK, DnaJ, and GrpE. The DnaK machine uses active binding to protect denatured or
nascent proteins while refolding occurs (Yura 2000; Chattopadhyay 2002; Ulrich Hartl 2002). DnaK is also considered the first line of defense during stress conditions with GroE facilitating folding of proteins that cannot be folded by DnaK (Ulrich Hartl 2002). dnaK transcripts do appear to be present at higher levels than groEL during H2O2 (one-way Student’s t test, p=0.005, p=3.7x10^{-6}, and p=0.02, 10, 20, and 30 minutes, α=0.05) and heat shock at 20 and 30 minutes (one-way Student’s t test, p=0.00008 and p=0.0005 for 20, and 30 minutes, α=0.05). However, under PQ stress, groEL is present at higher levels at 20 and 30 minutes indicating a larger role during this stress (one-way Student’s t test, p=0.001 and p=0.0004, for 20 and 30 minutes, α=0.05). Expression of the co-chaperone, dnaJ, is significantly different from dnaK at 10, 20, and 30 minutes of H2O2 exposure (two-tailed Student’s t test, p=0.003, p=0.00004, and p=0.003, α=0.05). Though DnaJ plays a vital role in the refolding of proteins in the DnaK machine, its transcription levels do not equal that of DnaK under stress, underlining the importance of DnaK over its co-chaperones. DnaK, dnaJ, and groEL all experienced increases in transcription under the control of rpoH in response to H2O2, heat shock, and PQ exposure. While dnaK was present at higher levels under H2O2 and heat shock, groEL showed increased activity under PQ stress.

4.2 The Relationship Between σ^{32} and DnaK

Reactive carbonyls formed from oxidation of carbohydrates, amino acids, and in the process of lipid peroxidation are a major contributor to protein modification in cells. Lipid peroxidation yields reactive carbonyls that include MDA that is routinely detected via the TBARS assay (Shao 2010). In studies of the cholesterol efflux pathway, apolipoprotein A-I was shown to be selectively modified by MDA at Lys residues. The Lys damage in this protein took
place at the C-terminus, leading to MDA-Lys, and Lys-MDA-Lys adducts. The modified C-terminus was postulated to undergo cross-linking that could affect the conformation and conformational adaptability of the protein. Furthermore, the authors suggest that positively charged amino acid residues that juxtapose the Lys residues in the protein favor the formation of MDA-Lys adducts (Shao 2010). When in a complex with DnaJ, the C-terminal Lys residues of DnaK would be directly opposite the J-domain of DnaJ that contains both Arg and His residues (Greene 1998). These positive residues could assist in the preferential modification of the Lys residues at DnaK’s C-terminus.

When exposed to hydrogen peroxide and paraquat stress, DnaK has been shown to form protein carbonyls. The authors postulate that as a chaperone protein DnaK would interact with damaged (or oxidized) proteins at a higher rate (Tamarit 1998). When exposed to 10 mM H$_2$O$_2$, in the TBARS assay, 1.16 µM of MDA or a similarly reactive substance was detected. This reactive carbonyl is capable of damaging the Lys-rich C-terminus of Dnak and causing the formation of cross-linkages that could alter the activity of the protein.

When the interaction of DnaK with $\sigma^{32}$ was assayed in the beta-galactosidase experiments, there was no significant difference in beta-galactosidase activity between the wild type DnaK and DnaKV634X strains under no treatment conditions (two-tailed Student’s t test, p=0.81, $\alpha=0.05$). This suggests that when unstressed, both strains are functional and able to bind $\sigma^{32}$ at the same rate, resulting in the same low beta-galactosidase output. Though the beta-galactosidase activity did not reach levels close to the lacZ reporter control, there was a slight increase that may be physiologically relevant. Under heat shock conditions, the DnaKV634X strain displays the same beta-galactosidase activity as the wild type DnaK strain.
(one-tailed Student’s t test, \( p=0.14, \alpha=0.05 \)). During heat shock response, both wild type DnaK and DnaKV634X become occupied by chaperoning duties and are unable to bind \( \sigma^{32} \), resulting the same rate of beta-galactosidase output. When subjected to \( \text{H}_{2}\text{O}_2 \) induced oxidative stress, the wild type DnaK strain produced significantly higher amounts of beta-galactosidase than the DnaKV634X strain (one-tailed Student’s t test, \( p=0.000002, \alpha=0.05 \)) and even approached the same level of output as the \( \text{lacZ} \) reporter control (two-tailed Student’s t test, \( p=0.34, \alpha=0.05 \)). Under oxidative stress by \( \text{H}_{2}\text{O}_2 \) the wild-type form of DnaK putatively undergoes Lys modification at the C-terminus, preventing the protein from binding to \( \sigma^{32} \), effectively increasing the activity of the sigma factor, and resulting in dramatically higher beta-galactosidase activity than DnaKV634X. Under PQ stress, the WT DnaK strain displays slightly higher beta-galactosidase activity and approaches the level of the \( \text{lacZ} \) reporter control. The DnaKV634X strain has been engineered to be truncated prior to the Lys repeats at the C-terminus so that it remains insensitive to oxidative modification. This form of DnaK would continue to bind to \( \sigma^{32} \) so that the sigma factor could not promote the production of beta-galactosidase in this \( \text{lacZ} \) reporter construct. These findings demonstrate that under oxidative stress conditions, DnaK is oxidatively modified and prevented from hindering the activity of \( \sigma^{32} \). The increased activity of \( \sigma^{32} \) during oxidative stress is distinct from what occurs during heat shock where DnaK is occupied by chaperoning duties resulting in an increase in \( \sigma^{32} \). This is demonstrated by the magnitude of beta-galactosidase output as a result of \( \sigma^{32} \) activity under oxidative stress conditions that approaches that of the \( \text{lacZ} \) reporter control that is constitutively producing beta-galactosidase.
4.3 Routes of Oxidative Modification to DnaK

The beta-galactosidase assays demonstrate that the C-terminus of DnaK is modified during oxidative stress. The most likely causative agents of oxidative modification of DnaK are reactive carbonyls. The route of protein injury and putative generation of reactive carbonyls merits discussion as these processes could mirror oxidative injury that has been implicated in various diseases. With H₂O₂ as the primary oxidative stress, there are two viable routes of DnaK modification. In the first scenario, a hydroxyl radical is generated from H₂O₂ by the Fenton reaction. This reaction requires a divalent metal ion such as iron and a reducing equivalent like NADH (Tamarit 1998; Cabiscol 2000; Boelsterli 2009). The hydroxyl radical could then cause direct injury to the C-terminus of DnaK (Figure 4.1).
Figure 4.1. Oxidative modification of DnaK by direct injury. In this route of protein injury, the Fenton reaction, facilitated by electron transfer from a compound such as NADPH, converts H$_2$O$_2$ into highly reactive •OH. •OH proceeds to oxidatively modify the C-terminus of proteins such as DnaK.
In a recent review of the molecular mechanisms of oxidative stress in *E. coli*, Imlay maintains that lipid peroxidation is unlikely in most bacteria because, “in the standard model of lipid peroxidation, the propagation step of the chain reaction requires that lipids be unsaturated” and bacteria tend to contain only saturated and monounsaturated fatty acids (Imlay 2013). Evidence for lipid peroxidation in *E. coli* has been reported (Gonzalez-Flecha 1997; Semchyshyn 2005; Arenas 2011). In these studies TBARS is used as a measure for membrane damage while damage to proteins is assessed through the amount of carbonylated proteins. It is asserted that the level of “damage products accumulated” is indicative of the intensity of oxidative stress, where TBARS is a measure of the initial lipid injury and the concentration of carbonylated proteins demonstrates the intensity of the damage (Semchyshyn 2005). The present study has also detected lipid peroxidation through TBARS in an organism that contains only saturated and monounsaturated fatty acids, *E. coli*. In *E. coli* putative MDA content increased with increasing concentrations of hydrogen peroxide until 100 mM of hydrogen peroxide (Figure 3.2). Since TBARS is a measure of the initial lipid injury, it is postulated that the intensity of carbonyl damage to proteins would also be markedly intense when exposed to 10 mM hydrogen peroxide.

The route of injury to DnaK via lipid peroxidation would start with the hydroxyl radical being generated through Fenton chemistry. The hydroxyl radical would then abstract a hydrogen from lipids in the membrane to form lipid radicals (Figure 1.5). Lipid peroxides are extremely unstable and readily form carbonyl by-products (Figure 1.6). These reactive carbonyls would then target the Lys-rich C-terminus of DnaK in a way that alters the activity of the protein. This route of damage mirrors that observed in eukaryotic organisms and is the
suspected etiology of numerous diseases (Romero 1998) including Alzheimer’s disease (Reed 2008), cholesterol (Shao 2010), and cancer (Winczura 2014; Woodrick 2014). This work supports the use of bacterial models such as *E. coli* for the study of the effects of lipid peroxidation and frames the importance of DnaK in stress response to lipid peroxidation injury.

4.4 Factors Affecting Variation in this Project

4.4.1 RNA Sample Collection and Treatment

There are several factors contributing to poor quality RNA for qPCR analysis. These factors include inadequate sample storage, nucleic acid quality, and variability in yields. RNA is readily degraded by RNases during sampling, RNA purification, and RNA storage (Pfaffl 2004). While it has been argued that tissue samples can be stored on ice with little to no major effects on RNA quality or concentration, not all samples have the same physical properties, bacterial samples being especially sensitive to environmental effects (Bustin 2009). The samples in this study were processed for RNA extraction immediately after retrieval to minimize any negative effects from storage conditions. Contaminating genomic DNA was removed by DNase treatment in this study. Purity of the RNA samples and subsequent cDNA samples was assessed through 260/280 and 260/230 ratios. The MIQE guidelines state that regardless of the method for determining nucleic acid quality used, “it is advisable to measure all samples with a single method only” (Bustin 2009). In this study, all samples were assessed using UV absorbance. For RNA samples 260/260 ratios between 1.75 and 2.0 and 260/230 ratios between 2.0 and 2.2 were considered acceptable (ThermoScientific 2008). The reverse transcription step in cDNA
synthesis can be the source of the most variability in the qPCR process because of the putatively variable amount of RNA added to each reaction (Livak 2001; Pfaffl 2004). RNA concentrations were normalized to 300 ng/µL prior to cDNA synthesis. The secondary and tertiary structures of mRNA could also affect priming efficiency (Stahlberg 2004; Stahlberg 2004). When the same priming strategy and reaction conditions are observed in the conversion of mRNA to cDNA in all experiments, the same total amount of RNA is assumed (Stahlberg 2004; Stahlberg 2004). To address variability of cDNA yields, cDNA sample concentrations were normalized to 100 ng/µL for each qPCR reaction.

4.4.2 qPCR and Probe Variation

Primer design and PCR efficiency play a significant role in the success of a qPCR assay because of the sensitive nature of this detection method. Three sets of primers were designed for each target. Using the melting temperature from the initial primer design as a guide, the optimal annealing temperature for each pair was determined by performing a gradient PCR assay with a genomic DNA template. PCR efficiency for each pair was determined by a standard curve in which a dilution series of known template concentration was used. The most efficient primer set was chosen for each gene to be used in the duration of the study. Melt curves were included for every assay in order to monitor for potential primer dimers that can generate false positives in SYBR Green assays (Bustin 2009).

In addition to the optimization protocols mentioned above, no template controls (NTCs) were included on every plate in these experiments. The NTC is routinely used to detect PCR contamination and distinguish primer dimers from intended PCR products (Bustin 2009).
Furthermore, in order for 2 genes to be compared by the Livak method, where the differences between a calibrator/ reference gene such as gapA are compared to the stress genes of this study (dnaK, dnaJ, groEL, and rpoH), the PCR efficiencies must be high (>98%) and nearly equal (within 1%) (Pfaffl 2004; Bustin 2009). If PCR efficiency fell below this standard, the assay was assessed for errors by examining the melt curves and NTCs and repeated.

4.4.3 Variation in Growth of Clones in Beta-Galactosidase Assays

In preliminary trials it was noted that the wild type DnaK and DnaKV634X mutants took up to 24 hours longer to reach OD= 0.3, indicative of exponential phase, than the other E. coli strains used in this study. In order to compensate for this variation in growth rate, a continuous culture of the wild type DnaK and DnaKV634X mutants were maintained close to exponential phase. Prior to each experiment, OD readings were taken for all E. coli strains, cultures were then diluted to just under OD= 0.3 (approximately OD=0.2). Cultures were incubated until OD=0.3 was reached then the experimental conditions were applied. Absorbance readings were taken after exposure to oxidative stress, heat shock, or no treatment for 10 minutes. It is possible that in the time it took to pipette the individual samples to the 96 well assay plate, some E. coli strains achieved exponential phase as a higher rate than others, leading to potential variation in the final estimate of beta-galactosidase activity. However, all strains were normalized to their respective growth rates, so any variation in the final estimate of beta-galactosidase activity attributed to growth rate should be eradicated by this normalization technique.
Another source of influence of this experiment could be attributed to the nature of each stress. In this study, organisms were plated for the assay directly after incubation with exposure to each stress response without change in the media. While this methodology allowed for a representative view cells when exposed to continuous stress under oxidative stress conditions like H$_2$O$_2$ and PQ. It is not representative of what a cell undergoes during continuous heat shock, instead, heat shock and recovery is represented in these experiments as the cells were incubated at 37°C for the duration of the assay. This may account for the diminished response of the dnaK complemented strains under heat shock in comparison to oxidative stress.
CHAPTER 5
CONCLUSIONS

The role of oxidative modification to stress response may be critical to the understanding of bacterial stress response and could contribute to the treatment of multiple diseases that have oxidative stress as causative agents. This work sought to elucidate the relationship between a key regulator of bacterial stress response, RpoH and the molecular chaperone that controls its activity, DnaK. This work supports the hypothesis that a post-translational modification occurs at the Lys-rich C-terminus of DnaK during oxidative stress that obstructs the interaction with RpoH. The inability to bind RpoH increases rpoH transcription and triggers stress response by promoting transcription of the heat shock genes as demonstrated by the qPCR assays. Furthermore, the marked increase in beta-galactosidase output under H2O2 stress of the wild type DnaK strain while the DnaKV634X strain maintained low beta-galactosidase output, support the assertion that the Lys-rich C-terminus as a target for modification under oxidative modification. Lastly, detection of putative lipid aldehydes provides a potential mechanism for oxidative modification to occur in this system.

Future work would delve further into the type of oxidative modification that could occur at the Lys-rich C-terminus of DnaK and other proteins. Identification and quantification of carbonylated proteins would aid in defining the route of injury that occurs during oxidative modification of DnaK. The conserved function of HSP70s like DnaK includes preventing the formation of protein aggregates and ensuing toxicity. This work would demonstrate a novel mechanism for the control of stress response through oxidative modification and subsequent gene regulation. The conserved nature of this protein allows for the work in this system to
translate other organisms in a manner beneficial to the treatment of diseases or development of environmental technologies.

The role of lipid peroxides and oxidative modification has been shown to be particularly relevant at the onset of human disease. Elevated amounts of lipid peroxides have been detected in both chronic Hepatitis C (Romero 1998) and amnestic mild cognitive impairment patients (Reed 2008). It has been speculated that inflammation associated with disease, promotes oxidative stress, accounting for the elevated levels of lipid peroxides. If detection of lipid peroxides and modified proteins like DnaK were measured at each stage of the disease, a novel marker that could aid in clinical management and treatment of these diseases could be developed. A marker for modified proteins would be particularly useful for chronic Hepatitis C patients that currently require repeated biopsies to monitor their disease.

Accumulation of lipid peroxides at the onset of disease has also been implicated in disease progression. The lipid aldehyde HNE has been shown to modify proteins and affect enzymatic activity. Glucose-6-phosphate dehydrogenase activity has been shown to decrease in the presence of the lipid aldehyde HNE by modification of an active-site lysine residue (Romero 1998). The present study demonstrates how lysine modification can affect the activity of DnaK with downstream effects on the deployment of the heat shock system. HSP70, the human homologue of DnaK has been shown to be modified by HNE in mild cognitive impairment patients (Reed 2008).

Oxidatively modified DnaK could be directly adding to the onset of Alzheimer’s disease from the mild cognitive impairment stage if the oxidative modification shown in this study has similar effects in human cells. Atypical deployment of the heat shock proteins that would normally
repair proteins under stress due to the oxidative modification of HSP70, could contribute to the
deterioration of individuals afflicted with this disease. The work in the present study provides a
solid starting point for research in higher order systems that could lead to better monitoring
and treatment schemes.
APPENDIX A

GENE SEQUENCES
R. palustris rpoH sequence

ATGGCCCGTGCTGCTACTCTCCGATTCTCAACGGAGAACATTCCGGCGTTGCTGCTACCTT
GCAGAACATCCCGGAATTTCCGAGTCTCCAGCCTCAACAAGAGTACATGTGCTCGCAAGCGC
TGCCCAAAGATCAGAGGCTCGGATGCTCACGACGCGGATCCGGGAGATGACCGCTGTTGTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT

R. palustris gapDH sequence

ATGGCCCGTGCTGCTACTCTCCGATTCTCAACGGAGAACATTCCGGCGTTGCTGCTACCTT
GCAGAACATCCCGGAATTTCCGAGTCTCCAGCCTCAACAAGAGTACATGTGCTCGCAAGCGC
TGCCCAAAGATCAGAGGCTCGGATGCTCACGACGCGGATCCGGGAGATGACCGCTGTTGTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT
GCGGAAATCCGCAAGTTTCCGATGCTGATGACTCTCCGAAAGTCTGGCTGCTGCTACCTT

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E. coli rpoH sequence

ATGACTGACAAGATGTTAGTCGAGTTGCAATCGTAGTATTCTACATCCGGG
CAGCTAAGGCTGGCCGAGATTTTGGCCGCTAGGAGGAGCCGCGGCTGCAAAAGACTGCAATTACCAT
GGCGATCTGGAAGACGATTTAACAGGATCATTGCTGGAGGCTTGCCTAGATGAAATGCTGAGGACG
CCGCTTTCAACCCGAGGACGTTGCTGCAAGGGCTTCTCCGCGCTTCCTCGCTGACGGAATCCAGAGATC
CAGCAATACGTCTTCCGTAAGTGGAATGCTGAAAGTACCAAGCAGCAGCAGCAGATCTGTTCCTGCTG
TTAACCACTTCTGGAATCCCAGGACGACGACGACGACGACGACGACGACGACAGCTTCAAGGCTCC
TGGCTGCAGGGAGTAAGGTAAAGAATTGCGTGCTGCCATTGAAGCGTAA

E. coli gapA sequence

ATGGGATTTTTTGTTACGCTCTGGTTTCTTTTGTTGTAAGGCTTCCATTGAGCCGCTGAAAGGCTGAG
TTGAAGAGCTGGTACAGACTCGCAACCAGGGCGACCATCTGCTGCACAGCACCGTAAGCAGGTTGAA
GAAGCAGGCGACAAACTGCCGGCTGACGACAAAACTGCTATCGAGTCTGCGCTGACTGCACTGGAAAC
108
TGCTCTGAAAGGTGAAGACAAAGCCGCTATCGAAGCGAAAATGCAGGAACTGGCACAGGTTTCCCAGA
AACTGATGGAAAATCGCCCCAGCAGCAACATGCCCAGCAGAAGCTGCGGCTGATGTTCTGGCAAAACA
ACGCGAAAGATGACGATGTTGTCGACGCTGAATTGAAGAAGTCAAAGACAAAAAATAA

E. coli dnaJ sequence

ATGGCCTAAGCAAGATTATTATCAGAAGTATTTTAGGCTTCCAAAAACACGGGAAGACGCGTAAATCAGAAA
GGCCTCACAACGCCTCGCCATGAAAATACACACCAGCCGACCAGTAAACCAGGTTGAACAAGAGAGGCCGAGGCGA
AATTAAAGAGATCAAGGAAACAGCTATTAGTGAAGTGGTGACGCTGAAATTTGAAGAAGTCAAAGACAAAAAATAA

E. coli groEL sequence

ATGGCAGCTAAAGACGTAAAATTCGGTAACGACGCTCGTGTGAAAATGCTGCGCGGCGTAAACGTACT
GGGCTGCAGATGTTGAAAGATGGGTTTCCGTTGCTCGTGAAATCGAACTGGAAGACAAGTTCGAAAATAT
GGGTGCGCAGATGGTGAAAGAAGTTGCCTCTAAAGCAAACGACGCTGCAGGCGACGGTACCACCACTG
CAACCGTACTGGCTCAGGCTATCATCACTGAAGGTCTGAAAGCTGTTGCTGCGGGCATGAACCCGATGG
ACCTGAAACGTGGTATCGACAAAGCGGTTACCGCTGCAGTTGAAGAACTGAAAGCGCTGTCCGTACCAT
GCTCTGACTCTAAGCGATTGCTAGTGTGTTTCCGTTGCTCGTGAAATCGAAGCTGGGCTAGCTGCGGGGC
ACCGTGATCTCTGAAGAGATCGGTATGGAGCTGGAAAAAGCAACCCTGGAAGACCTGGGTCAGGCTAA
ACGTGTTGTGATCAACAAAGACACCACCACTATCATCGATGGCGTGGGTGAAGAAGCTGCAATCCAGG
GCCGTGTTGCTCAGATCCGTCAGCAGATTGAAGAAGCAACTTCTGACTACGACCGTGAAAAACTGCAGG

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AACGCGTAGCGGAAACTGGCAGGCGGCGTTGCAGTTATCAAAGTGTTGGTGCTGCTACCAGAAGTTGAAATGAAAGAGA
AAGAGAACGACGCGTGAAGATGCCCTGCACGCGACCCTGCTGCGGTAGAAGAAGGCGTGGTTGCTGGT
GGTTTGCGCTGATCCGCGTAGCTCTAAACTGGCTGACCTGCTGTTGCTGACAGAAGG
ACCAGAAGCTGTGGGTATGAAAGTTGCAACTGCCGTGCAATGGAAGCTCCG
CTGCTGACCTGCTGCTGAGATCGTATTGAACTGCGGCGAACCGTCTGTTGTTGGCGTACTAACG
ACGCGGCGACGGCAACTACGGTTACAAAACGCGAAACCGGCAACATGACATGGGTATCCTG
TAGATCACCACCGAATGCATGGTTACCGACCTGCGGCTG
AACGATGCGATCAGCTATAGGCCTGCTGGCGGTATGGGACGGATGGGTGCGCATGGGCGCATGATGTA
APPENDIX B

PRIMER DESIGN
B1. Oligo Calc Oligonucleotide properties calculation results for rpoH primers in *R. palustris* RPAL rpoH FWD (top) and RPAL rpoH REV (bottom).
B2. Oligo Calc Oligonucleotide properties calculation results for rpoH primers in *E. coli* rpoH EC2F (top) and rpoH EC2R (bottom).
B3. Oligo Calc: Oligonucleotide properties calculation results for *dnaK* primers in *E. coli* dnaK EC1F (top) and dnaK EC1R (bottom).
B4. Oligo Calc Oligonucleotide properties calculation results for dnaJ primers in *E. coli* dnaJ EC3F (top) and dnaJ EC3R (bottom).
B5. Oligo Calc Oligonucleotide properties calculation results for groEL primers in *E. coli* groEL EC1F (top) and groEL EC1R (bottom).
B6. Oligo Calc Oligonucleotide properties calculation results for gapA primers in *E. coli* gapA EC1F (top) and gapA EC1R (bottom).
REFERENCES


