Dynamic Transcriptional Response of *Escherichia coli* to Inclusion Body Formation

Faraz Baig
*Clemson University*, fbaig@g.clemson.edu

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DYNAMIC TRANSCRIPTIONAL RESPONSE OF *ESCHERICHIA COLI* TO INCLUSION BODY FORMATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Faraz Mirza Baig
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Accepted by:
Dr. Sarah W. Harcum, Committee Chair
Dr. Frank Alexis
Dr. Michael G. Sehorn
ABSTRACT

*Escherichia coli* is used intensively for recombinant protein production due to its many unique advantages, but one key challenge with the use of *E. coli* is the tendency of recombinant proteins to misfold and aggregate into insoluble inclusion bodies (IBs). The presence of IBs stresses cells and can hinder overall growth. Additionally, IBs contain high concentrations of recombinant protein in an inactive form and thus require recovery steps to salvage functional recombinant protein. Currently, no universally effective method exists to prevent IB formation in recombinant *E. coli*. Further research into the gene expression response to insoluble recombinant protein may provide insight into critical cellular mechanisms that could be leveraged to minimize IB formation. This study was focused on characterizing the dynamic transcriptional response of *E. coli* in the initial stages of IB formation, as previous studies have only characterized gene expression changes after IBs had accumulated.

In this study, DNA microarrays were used to compare the *E. coli* gene expression response to soluble and to insoluble recombinant protein production. Genes involved in many several cellular functionalities were differentially expressed due to the production of insoluble recombinant protein. As expected and previously reported, expression levels of many classical heat-shock genes increased, including protein folding chaperones and proteases. Additionally, cells increased expression levels of protein synthesis-related genes and of genes involved in energy-deriving pathways. Interestingly, expression levels decreased for many transmembrane transporter genes for many substances not found in the culture medium, while several genes involved in catabolic pathways for
these substances also decreased in expression. Additionally, over a third of the differentially expressed genes were classified as putative genes, indicating that IB stress regulates many genes that have not been extensively studied. Taken together, the results of this study indicate that IB formation in recombinant *E. coli* is a complex issue that not only induces the heat-shock genes but also directly causes the cells to increase protein and energy synthesis, while streamlining transport and catabolic processes. Further study of the differentially expressed putative genes could provide deeper insight into the dynamic response to IB formation.
DEDICATION

This work is dedicated to my parents, Mirza and Rana Baig. I could easily fill up an entire thesis with the many ways you two have positively impacted my life, and yet, I’d still inevitably fall short of capturing all that you’ve done for me. Thanks for everything, and I love you both very much.
ACKNOWLEDGMENTS

Many individuals were involved in the execution of this project. I would like to thank Dr. Sarah Harcum for her guidance and unwavering support throughout the entire process. I also would like to thank Drs. Frank Alexis and Michael Sehorn for their willingness to help and advise me. I would like to thank Mary Alice Salazar and Lawrence Fernando for their diligent efforts in developing this project from the early stages. Yogender Gowtham, Tom Caldwell, and Arthur Nathan Brodsky all shared a tremendous amount of knowledge and wisdom with me, and their unselfish contributions are much appreciated. Finally, I’d like to thank Shawn Backstrom for his assistance with the drawing of the gene classification graphic.

The pTVP1GFP plasmid was provided by E. García-Fruitós and A. Villaverde from the Autonomous University of Barcelona in Spain. The pTrcHis-GFP_{UV}/CAT plasmid was provided by W. E. Bentley from the University of Maryland, College Park. The GFPCAT plasmid was constructed by M. T. Morris from Clemson University. This work was partially supported by a grant from the National Science Foundation (SGER: 0738162) to Dr. Harcum. This research was also supported by an Institutional Development Award (IDEA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103444.
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CHAPTER ONE

PROJECT INTRODUCTION

This thesis describes the analysis of the dynamic genomic response of

*Escherichia coli* to recombinant protein insolubility. This project was initiated by Mary Alice Salazar. I began work on this project in February 2012. The majority of this thesis will be submitted as a peer-reviewed journal publication by the summer of 2013. The authors of the journal manuscript will be: Faraz Baig, Lawrence P. Fernando, Mary Alice Salazar, Rhonda Powell, Terri Bruce, and Sarah W. Harcum. The following describes the contributions of each author of the journal manuscript:

1. Mary Alice Salazar
   - Conducted growth experiments of *E. coli* pTVP1GFP and pGFPCAT cultures used to obtain the RNA for the DNA microarrays
   - Quantified the quality of RNA used for the DNA microarrays
   - Conducted the flow cytometry analysis of VP1GFP cultures

2. Dr. Lawrence Fernando
   - Isolated total RNA for the DNA microarrays
   - Quantified the total RNA isolated
   - Synthesized and quantified the cDNA for the DNA microarrays

3. Dr. Sarah W. Harcum (MS committee chair)
   - Isolated total RNA for DNA microarrays
   - Assisted with experimental planning
Assisted with journal manuscript preparation

4. Rhonda Powell and Dr. Terri Bruce

As part of future work, will obtain immunofluorescence images for *E. coli* pTVP1GFP and pGFPCAT cultures to localize the TVP1, GFP, and CAT proteins.

All DNA microarrays were processed at Florida State University’s NimbleGen Certified Microarray Facility. I was responsible for quantifying CAT activity in *E. coli* pGFPCAT cultures; importing the DNA microarray data into ArrayStar; analyzing the gene expression data; and preparing the journal manuscript.

This Master’s thesis is divided into six chapters. Chapter I briefly describes the project history and the responsibilities of the key contributors. Chapter II provides a background and the study objectives. Chapter III describes the materials and methods used and will be included in the journal manuscript. Chapter IV describes the results and discussion that will be included in the journal manuscript. Chapter V describes the conclusions of the gene expression analysis. Chapter VI describes future work in the immunofluorescence localization of the proteins which will be included in the journal manuscript. Appendix A contains a list of differentially expressed genes which will be included in the journal manuscript. Appendix B contains select gene expression profiles which will be included as supplementary material for the journal manuscript.
2.1 INTRODUCTION TO RECOMBINANT PROTEINS

Starting in the 1970s, scientists broke new ground and discovered how to intentionally manipulate cells at the genetic level. This advent led to the birth of genetic engineering. At its core, genetic engineering consists of isolating a gene of interest and recombining it with the DNA of another host. Once in the new host, the DNA is referred to as “recombinant DNA,” and the protein(s) the gene encode for are referred to as “recombinant protein(s).” The advent of recombinant DNA has revolutionized protein production methodologies. Proteins that previously could only be harvested from human and animal donors can now be safely mass-produced in fermenters (Shuler 2002, Chou 2007).

*How to Make a Recombinant Protein*

In order to make a recombinant protein, the first step is to obtain the gene of interest. One of the earliest and most common methods used to achieve this task involves the use of the enzyme reverse transcriptase to synthesize a DNA molecule. In this method, cells that generate the protein of interest are harvested, and the mRNA is isolated from these cells. Reverse transcriptase then reverse transcribes the mRNA, yielding a complementary of DNA (known as cDNA) (Shuler 2002). Other common methods for gene isolation include chemical synthesis of the exact nucleotide sequence (Hughes et al
and a method known as “shotgun cloning,” where restriction enzymes are used to fragment DNA from a host organism and subsequent screening methods filter impurities in order to yield the gene of interest (Shuler 2002). All of the various gene isolation methods differ in several factors, including specificity, speed, and costs. These factors must be evaluated when choosing a particular cloning method to utilize.

Once the gene of interest is obtained, it is joined onto a DNA carrier known as a “vector.” The vector allows the gene of interest to pass into a cell and to become incorporated into cellular DNA. Most commonly, plasmids are used as vectors, especially in bacterial cells, such as Escherichia coli. Plasmids are DNA molecules which replicate independently of a host genome. Since plasmids replicate independently of the host genome, there can be multiple copies of plasmids within a single cell, allowing for high expression levels of recombinant proteins. Several enzymes are used to incorporate the target gene into plasmids, including restriction enzymes, ligases, and polymerases. Once modified, the vectors are then inserted into host cells. Transformation is a frequently used method, where free DNA segments are taken up by the cells. Once the vectors are incorporated into the host cells, recombinant protein production can begin (Shuler 2002, Hanahan 1983).

Significance of Recombinant DNA Technology

Recombinant DNA technology allows for controlled manipulation of cellular DNA and DNA expression. Industrially, the main application of recombinant DNA technology has been to produce therapeutic proteins, such as human insulin, Factor VIII-
C, human growth hormone, erythropoietin, and various vaccines. Other uses for recombinant proteins include food processing, industrial catalysts, and animal husbandry (Shuler 2002).

To mass-produce proteins for industrial application, cells with recombinant DNA are grown in cultures, with the primary goal being to produce as much of the recombinant protein as possible, in as short of a time-frame as possible. The cells that are most frequently used to produce recombinant proteins are harvested from microbial sources (most notably, *E. coli*), Chinese hamster ovary (CHO) cells, plants, and yeasts. Various downstream process steps are then utilized to purify the protein (Chu and Robinson 2001, Doran 2000, Goodrick et al 2001, Wang et al 2001, Jana 2005).

2.2 **ESCHERICHIA COLI AND INCLUSION BODIES**

*E. coli* is a very popular organism to use for recombinant protein production because of its many unique advantages: high growth rates, high expression levels, low costs, simple growth medium, reasonably low levels of proteolytic degradation, and good overall safety. However, one of the biggest problems encountered with *E. coli*-produced recombinant proteins is that very often, the proteins do not fold properly and consequently become insoluble. Misfolded proteins are functionally compromised and tend to form dense, insoluble aggregates known as *inclusion bodies (IBs)* inside the cell. IBs have proven to be problematic for recombinant protein production (Shuler 2002, Baneyx 1999, Mergulhao 2005, Baneyx 2004, Kyratsous 2012). This study primarily focuses on issues related to the presence of IBs.
Pathophysiology of IBs

The average *E. coli* cell produces about 60,000 polypeptide chains per minute (Lorimer 1996). With such a rapid rate of protein production, protein folding can be a challenge. Large, multidomain proteins are particularly problematic due to more intensive folding requirements in comparison to smaller, simpler proteins. To cope with these challenges, *E. coli* cells are equipped with protein folding helpers. These include molecular chaperones, which assist in protein folding by providing a favorable environment for domains within proteins to interact appropriately, and folding catalysts, which are specialized enzymes that catalyze reactions necessary to fold polypeptide chains (Baneyx 2004).

Sometimes, the protein folding helpers of *E. coli* are not able to properly fold a protein. Recombinant proteins, in particular, tend to overwhelm the folding machinery due to high expression rates and due to lack of biological necessity for *E. coli* survival. This issue is exacerbated when strong promoters are used to increase protein expression rates in order to increase yields. One recent study suggested that on average, only about 30% of *E. coli*-produced recombinant proteins are expressed in soluble, folded form (Yang et al 2011). While terminally misfolded proteins can undergo proteolysis to prevent buildup, highly expressed recombinant proteins that misfold frequently tend to aggregate into IBs. Since IBs are often protease-resistant due to strong interactions between hydrophobic surfaces in the concentrated intracellular environment, cells only have a limited ability to remove IBs post-formation (Kyratsous 2012, Baneyx 2004).
Attempts to Control IB Formation

IBs pose a challenge to the recombinant protein production process. The presence of protease-resistant IBs induces stress-related responses in the cells and also can hinder overall cell growth (Ventura 2006, Chou 2007, Lesley 2002). Additionally, since IBs contain large amounts of the target recombinant protein, much effort goes into recovering these recombinant proteins from IBs. However, the recovery processes remain time-consuming and inefficient, with denaturation, refolding, and purification steps required to obtain properly folded recombinant protein (Choi 2004, Georgiou 1996). In addition to the added costs of these steps, yields at each step can be low: on average, only about 15-25% of the total recombinant protein contained in IBs is recovered as functional protein (Zhang et al 2009). For these reasons, many attempts have been made to prevent IB formation.

One common approach to controlling IB formation is to simply down-regulate protein expression to ease the workload on the cellular protein-folding machinery. Down-regulated protein expression can be accomplished in many ways, including through the use of different transcription promoters, through culturing cells in less nutrient-dense growth medium, or through lower cell culture temperatures that slow gene transcription/translation. Overall, while down-regulating protein expression does reduce the occurrence of IBs, it comes at the expense of not being able to fully exploit the cellular protein-producing capabilities (Baneyx 2004, Chou 2007).
In order to reduce IB formation without sacrificing final protein yield, many attempts have been made to improve recombinant protein solubility. Manipulation of secretory pathways, fusion of soluble proteins, growth supplementation with essential cofactors, and substitution of specific amino acid residues have all had varying degrees of success in improving recombinant protein solubility, but there still are no universally applicable methods (Mergulhao 2005, Baneyx 2004, Choi 2004, Hammarstrom 2002, Ghosh 2002). An additional approach to improving recombinant protein solubility is through the increase of folding chaperones. For example, co-expression of folding chaperones with the desired recombinant protein can increase protein solubility. Also, certain chemicals added to the growth medium can increase chaperone expression by inducing a heat-shock-like response, consequently enhancing the solubility of IB-prone proteins. Most notably, ethanol can be used to achieve this effect (Chou 2007, Oganesyan 2007). While strategies that increase the expression of chaperones can reduce IB formation, there have been instances of overexpressed chaperones yielding undesirable proteolytic effects on the protein of interest because some chaperones, such as the DnaKJ and GroELS protein complexes, have dual roles of modulating protein folding and enhancing proteolysis (Kyratsous 2012, Choi 2004, Martínez-Alonso 2010, de Marco 2007, Thomas and Baneyx 1997, Sherman and Goldberg 1992). Thus, overexpressing chaperones is not yet a comprehensively applicable method.

Over the last 20 years, many advancements have been made in improving recombinant protein solubility and thereby controlling IB formation. However, there have been no unanimously effective methods. Since *E. coli* is simply a living organism
responding to an environmental stressor (in this case, insoluble recombinant protein), further research into the bacterial genomic response to insoluble recombinant protein expression would represent a key step towards devising practical methods to minimize IB formation.

2.3 GENE EXPRESSION ANALYSIS WITH DNA MICROARRAYS

Measurements of gene expression levels for an organism (also known as gene expression profiling or transcriptome analysis) can provide a wealth of knowledge the genomic behavior, elucidating the underlying mechanisms for the observed phenotypic behavior of an organism at macroscopic levels. When it comes to producing recombinant proteins, knowledge of how a cell responds to different environments allows one to manipulate the cellular genome and/or the culture environment in a manner conducive to maximizing protein production (Chou 2007, Yeung 2002).

Gene Expression Analysis Methods

Many methods can be used to analyze the gene expression of an organism. Most of these methods rely on quantifying nucleotide hybridization pairing between DNA-DNA or DNA-RNA sequences. Approaches to gene expression analysis can typically be classified as either “low-throughput” or “high-throughput” based on the number of genes directly measured. “Low-throughput” methods are used to quantify the behavior of a few targeted genes, such as a set of genes known to be activated by a specific stressor or the genes involved with a specific metabolic process. Low-throughput methods can be used
to analyze these limited-in-scope gene sets. Gel-based assays (such as Northern blots and Southern blots), quantitative PCR (polymerized chain reaction) primer extension, and RNase protection assays are all common approaches and all operate by estimating the quantity of mRNA or protein being expressed (Brown 2001, Taniguchi 2001, Spurgeon 2008, Nuber 2005). However, when it is desirable to measure the expression levels of the entire genome of an organism, low-throughput gene expression methods are not feasible. For simultaneously estimating expression levels of thousands of genes, “high-throughput” methods must be employed. In the 1990s, DNA microarrays were introduced as a high-throughput approach to gene expression analysis and have since gained widespread usage in the field (Nuber 2005).

**DNA Microarrays**

DNA microarrays are an arrangement of unique DNA molecules, known as probes, organized onto a small chip. To analyze the gene expression levels in cells, the total RNA is harvested from the cells. The mRNA in the total RNA is converted to complementary DNA (cDNA), which is much more stable than the mRNA. The cDNA is contacted with the probes on the microarray chip. When a cDNA strand encounters a probe with a complementary base-pair sequence, hydrogen bonds are formed, resulting in a stable conformation; this process is known as “hybridization.” Various labeling techniques can be used to determine the hybridization levels of the various probes. A higher signal from a particular probe is indicative of a higher quantity of cDNA with the complementary base sequence of that probe, which correlates back to high gene
expression levels in the cells. A single microarray chip contains hundreds to thousands of different probes and can therefore be used to quantitatively measure gene expression levels of a large portion of the genome of an organism (Baldi and Hatfield 2002).

**Conducting a DNA Microarray Experiment**

In order to conduct a DNA microarray experiment, the mRNA must first be harvested from the cells of interest. Many standard procedures exist for harvesting RNA, including protocols specific for total RNA and mRNA (Baldi and Hatfield 2002, Reddy and Gilman 2001). The RNA extraction process must be performed with the utmost care due to the liable nature of RNA. Many factors can degrade the harvested RNA, including prolonged contact with cellular enzymes (particularly RNases); temperature and osmotic variations during processing steps, and contamination of samples with exogenous RNase from the environment. Accordingly, the best approach for RNA isolation includes quick preservation of cellular material. Several RNA isolation methods exist to preserve RNA from cells and to separate the RNA from cellular components, particularly genomic DNA. There are many commercial RNA purification kits, such as the RNeasy Protect Bacteria Minikit from Qiagen™ and the TRIzol® Plus RNA Purification System, that facilitate total RNA isolation by inhibiting RNase activity, lysing cells, solubilizing cellular components, and precipitating total RNA (Rio et al 2010, Simms et al 1993, Masuda and Church 2002, Baldi and Hatfield 2002). Additional steps are used to isolate mRNA, which is usually reverse transcribed to cDNA.
Transcript Quantification

In order to quantify the mRNA level of a specific gene, the nucleotide targets (mRNA or cDNA) are typically labeled with fluorophores. After hybridization of the target nucleotides to the probes on the DNA microarray, an energy source can be used to excite the fluorophores. The subsequent emission energies, which are proportional to the amount of fluorophore attached to the probes, can then be detected and digitized into a gene expression value (Baldi and Hatfield 2002). After fluorescent emission energies of the probes are quantified, data analysis is needed to interpret the intensity signals from the multiple probes that represent a single gene. Software programs employ algorithms to generate gene expression values from the detected emission energies. These algorithms account for various sources of expected variability and background noise. The DNA microarray software program then generates raw signal intensity data for each gene on the array. Next, these raw data intensities can be imported into interpretation software, such as ArrayStar™ (DNASTar, Inc.) and GeneSpring (Agilent Technologies, Inc.). The interpretation software allows for statistical analysis and for visualization of the data (Baldi and Hatfield 2002).

2.4 STATISTICAL ANALYSIS TOOLS

After the raw DNA microarray hybridization data are obtained, numerous statistical tools are available to identify significantly regulated gene behavior. These statistical tools allow researchers to take a disciplined, data-driven approach in determining a population’s characteristics, and it removes much of the subjectivity
inherent in the decision-making process (Ott 2010). With gene expression studies, statistical procedures aim to balance the rate of identifying false positives and false negatives. False positives represent genes that were not significantly regulated but were identified as being significantly regulated, also known as a Type I error. False negatives represent genes that were significantly regulated but were identified as unchanging, also known as a Type II error. The next section outlines different statistical tools that are applicable to microarray data.

Analyzing the Behavior of a Gene Between Two Different Conditions

To determine if the expression level for a particular gene is different between two conditions, a common non-statistical approach is to simply examine the fold change of the gene between the two conditions. For example, the mean expression level of Gene A might be 3.5-fold higher at Time X relative to Time Z. While examining fold changes provides a very quick and simple analysis, the obvious drawback is that it doesn’t take into account sample variability; it simply analyzes the average values. Therefore, the results obtained using only fold changes can be misleading in many instances (Baldi and Hatfield 2002).

A simple statistical approach for comparing gene expression levels between two different conditions is the Student’s t-test. The Student’s t-test compares mean gene expression levels after normalizing the data and estimating the pooled standard deviation. Note that the Student’s t-test can be used with log-transformed gene expression levels. The Student’s t-test yields more statistically relevant results than the fold change analysis
approach; however, the Student’s t-test does have limitations. Specifically, sample sizes used in gene expression studies are usually small. As a result, findings from the Student’s t-test are not as accurate as other statistical tests for population studies. Many probabilistic models have been developed for population studies which improves confidence in these types of studies (Baldi and Hatfield 2002, Ott 2010, Nadon and Shoemaker 2002).

*Analyzing the Behavior of a Gene Between More Than Two Different Conditions*

If samples are collected from more than two conditions, a Student’s t-test would have to be performed between every single possible pairing to determine differential expression between conditions. This approach can clearly become very tedious for studies with many conditions. More importantly, if a Student’s t-test is used with a confidence level of 95%, there is a 5% chance of making a Type I error. If two Student’s t-tests are run at 95% confidence, each individual test has a 5% probability of a Type I error, so the probability that a Type I error has been made on at least one of the two tests is actually greater than 5%. As the number of tests performed increases, the likelihood of making at least one Type I error increases dramatically (Ott 2010).

To circumvent the issue of high Type I error probability with multiple comparisons, Analysis of Variance (ANOVA) tests can be used. ANOVA tests compare mean values in multiple conditions at a set confidence level. ANOVA tests operate by comparing the sum of variances between sample means with the sum of variances within individual samples. The premise of the ANOVA test is that if the variability between
sample means is high relative to the variability within samples, then it is very unlikely that all of the conditions being compared have the same mean value. If expression data is collected for a gene over multiple conditions, ANOVA tests can quickly determine if the gene has differential behavior in at least one of the conditions relative to the others (Ott 2010, Nuber 2005).

While an ANOVA test can determine if at least one condition has a different mean from the rest of the conditions tested, post-hoc tests are required to determine specifically which conditions are different. When an ANOVA test determines that one condition has a different mean value than the remaining conditions, a pairwise comparison can be made between the means of any two conditions. If the difference between the two conditions’ means is greater than a threshold value, then the two conditions are statistically different. Post-hoc tests, such as the Tukey’s W and the Fisher’s LSD procedures, can be used to determine this threshold value (Ott 2010).

Linear Regression

Linear regression is a statistical tool used to analyze the relationship between an explanatory variable and a response variable. A linear regression model plots an explanatory variable (x) and a response variable (y) on an x-y axis. The regression model then makes use of a “least-squares method” to determine if a linear correlation exists between the explanatory and the response variable. Frequently, studies are performed where time is the explanatory variable (Ott 2010). Linear regression is a valuable tool for
gene expression analysis because it allows for the identification of genes with subtle
time-dependent changes in response to a stimulus.

*Specifying the Significance Level of Statistical Tests*

All of the statistical tests discussed are conducted using a specified significance
level. The significance level of statistical tests quantifies the strength of evidence needed
to make significant conclusions. Statistical tests conducted with lower significance levels
require stronger evidence for significant results, and consequently, Type I errors are less
likely to occur with lower significance levels. Very commonly, a significance level (p-
level) of 0.05 is used (which corresponds to a 5% chance of making a Type I Error), and
any statistical test with a p-value less than 0.05 (p ≤ 0.05) would yield a significant result;
however, 0.05 is not an appropriate level for all statistical tests. Different significance
levels can be used based on many factors, including the experimental conditions, the
goals of the study, and the potential consequences of making Type I errors. The context
of the study determines the optimal significance level. Significance levels ranging from
0.01 to 0.1 are most frequently used (Ott 2010).

2.5 SUMMARY AND STUDY OBJECTIVE

Recombinant protein technology has allowed genetic engineers to produce a vast
array of proteins in large quantities. *E. coli* has been one of the preferred cellular
factories for producing recombinant proteins, but one persistent issue with *E. coli* cells is
the tendency of its recombinant proteins to misfold and form IBs. IBs stress the cells, can
hinder overall growth, and are a source of inefficiency in the recombinant protein production process. An improved understanding of the dynamic transcriptional response of *E. coli* to insoluble recombinant protein could help formulate methods to prevent IB formation.

The objective of this study was to determine the dynamic transcriptional response of *E. coli* to IB formation. DNA microarrays were used to characterize gene expression changes due to IB formation. The gene expression changes due to expression of an IB-prone protein were directly compared to gene expression changes due to expression of a soluble recombinant protein. Since the addition of ethanol has been shown to increase the solubility of IB-prone proteins, the effects of ethanol on the gene expression response to IB formation was also examined.
3.1 BACTERIAL STRAIN AND PLASMIDS

*E. coli* MG1655 strains were obtained from the American Type Culture Collection (ATCC). The plasmid pTVP1GFP (gift from A. Villaverde) encodes the VP1 capsid of foot-and-mouth disease (Liu et al. 2006) fused to a GFP (García-Fruitós et al. 2007). The pGFPCAT plasmid was constructed from the pTrcHis-GFP<sub>UV</sub>/CAT plasmid (gift from W. E. Bentley) (Cha et al. 2000), where the GFP<sub>UV</sub> was replaced with the GFP from the pTVP1GFP plasmid (Salazar et al., submitted). *E. coli* MG1655 were transformed with either pTVP1GFP or pGFPCAT plasmid. Both plasmids are ampicillin-resistant and isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible through a *trc* promoter.

3.2 CULTURE CONDITIONS

*E. coli* MG1655 transformed with the pTVP1GFP or pGFPCAT plasmid were cultured in a minimal medium described previously (Sharma et al. 2007, Korz et al. 1995). Frozen stock (1 mL, stored at -80°C) were thawed and added to the minimal medium containing 40 µg/mL ampicillin (Hyclone). Cells were grown overnight in a shaker incubator (C24, New Brunswick Scientific, Inc.) at 37°C and 250 rpm to approximately 2.5 OD. ODs were obtained at 600 nm with a spectrophotometer (Spectronic 20 Genesys), where 1 OD is equivalent to 0.50 g dry cell weight per L. Samples were diluted with deionized water to obtain absorbance readings in the linear
range (0–0.25 OD). The overnight cultures were used to inoculate the experimental flasks. Cells were added to 500 mL shake flasks (120 mL working volume). These cultures were placed at 37°C in a water bath shaker at 200 rpm (C76, New Brunswick Scientific).

Cultures were induced in the mid-exponential phase (OD of 0.5) with 1 mM IPTG. Uninduced cultures were run in parallel. For the ethanol treated cultures, ethanol was added when the cultures reached 0.25 OD to a final concentration of 3%. Samples were collected prior to induction (time 0) and 5, 20, 40, and 60 minutes post-induction for the induced cultures. Samples at time 0 and 60 minutes were collected for the uninduced cultures and the ethanol treated cultures. Samples were removed while flasks continued to shake at 37°C. Samples were immediately stabilized in RNA Protect Bacteria Reagent (Qiagen, Inc.) and processed as per manual instructions. The RNA Protect solution was removed by centrifugation (14,500 x g, 10 minutes, Hermle Labnet Z383K centrifuge), and the cell pellets were stored in -80°C until used for RNA isolation. All culture conditions were conducted in biological triplicates.

3.3 PROTEIN EXPRESSION ANALYSIS

To confirm VP1GFP production, the fluorescence signal was measured quantitatively. Samples were harvested and immediately assayed with the Influx Cell Sorter flow cytometer (BD, Inc.) with a 488 nm Argon excitation laser and a 530/40 nm emission filter. Fluorescence levels from 100,000 cells were averaged to obtain the fluorescent intensity of the sample. (García-Fruitós et al. 2007). CAT activity was
quantified using the kinetic assay described by Rodriguez and Tait (Rodriguez and Tait 1983), and adapted to a 96-well plate format (Sharma et al. 2007). Additionally, CAT activity was confirmed by growing cells on minimal medium agar plates containing 0.61 mM chloramphenicol.

3.4 RNA ISOLATION AND CHARACTERIZATION

Total RNA isolation was performed using RNeasy Protect Bacteria Kits (Qiagen, Inc.). A Nanodrop spectrophotometer (ND 1000 from Thermo Scientific, Inc.) was used to quantify RNA. The Agilent 2100 Bioanalyzer with Expert Software (Version B.02.07.SI532) with Prokaryote Total RNA series II assay settings was used to obtain RNA integrity numbers (RIN) with the RNA 6000 Nanochip Kit. Total RNA was used to synthesize the first strand cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Inc) as per the Nimblegen instruction manual (Version 3.2). The RNA 6000 Nanochip Kit was also used to quantify mRNA (using the mRNA protocol) after second strand synthesis.

3.5 DNA MICROARRAYS

The custom *Escherichia coli* DNA microarrays (12 arrays per slide x 135K probes per array) with probes (45-60mer, 10 probes per target, 3 copies of each probe on array) for 4,281 *E. coli* genes and probes for mGFP, TVP1, ampicillin resistance gene (Amp’), and CAT were prepared by Roche NimbleGen. The DNA microarrays were processed at Florida State University’s NimbleGen Certified Microarray Facility in Tallahassee,
Florida. NimbleGen’s NimbleScan software normalizes the gene expression levels with a quantile normalization method in order to reduce obscuring variation between samples. The software uses a Robust Multichip Average (RMA) algorithm to generate Calls files (_RMA.calls) that contain normalized average gene expression values.

The DNA microarray data was imported into ArrayStar™ from the RMA.call files. Technical replicate expression levels were scaled using the “global averaging” data transformation. An ANOVA test (p ≤ 0.10) was conducted on the gene expression values for all of the culture conditions analyzed. A total of 14 sets of biological triplicates (i.e., three biological replicates) and one set of six biological replicates (Time 0 of the VP1GFP ethanol-treated culture) were analyzed. Tukey’s W post-hoc testing (p ≤ 0.05) and regression analysis (p ≤ 0.05) were subsequently applied to identify genes with significant differences between these conditions.

Gene annotations were obtained from the ASAP database of the University of Wisconsin-Madison on January 5, 2013 for Escherichia coli MG1655 Version m56 (Glasner et al. 2003). For genes annotated with unknown products or function (i.e., genes labeled predicted, putative, or conserved), EcoCyc (Version 16.5) was used (accessed January 7, 2013) to provide additional information about the gene product or function.
CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 CELL GROWTH AND PROTEIN PRODUCTION

_E. coli_ MG1655 pTVP1GFP and pGFPCAT were cultured in synchronized shake flasks to produce either the insoluble VP1GFP or the soluble GFPCAT proteins, respectively. Overnight cultures were synchronized to an OD of approximately 0.1 at 600 nm, where 1 OD is equivalent to 0.50 g dry cell weight per L. One set of VP1GFP and GFPCAT cultures was induced with IPTG, while parallel control cultures were not induced (uninduced). Cultures were induced in the mid-exponential phase (OD of 0.5) with 1 mM IPTG. Additionally, VP1GFP cultures treated with ethanol were examined for both induced and uninduced conditions. Samples taken for DNA microarray analysis were VP1GFP and GFPCAT induced cultures 5, 20, 40, and 60 minutes post-induction, VP1GFP and GFPCAT uninduced cultures at 0 and 60 minutes relative to the induced cultures, VP1GFP ethanol-treated uninduced cultures at 0 and 60 minutes, and VP1GFP ethanol-treated induced cultures at 60 minutes post-induction; in all, 15 conditions with at least 3 biological replicates were analyzed by DNA microarrays (48 total arrays). The cell density profiles for the triplicate cultures are shown in Figure 4.1A. The addition of IPTG did not change the observed growth rate for any of the cultures, as shown by the lines which represent exponential growth rates of 0.55 h\(^{-1}\) and 0.45 h\(^{-1}\) for VP1GFP/GFPCAT and ethanol-treated VP1GFP, respectively. For the ethanol-treated VP1GFP cultures, the growth rate was lower due to the ethanol addition; however, the IPTG addition did not further alter the growth rate.
Figure 4.1: Growth and protein expression profiles for *E. coli* pTVP1GFP and pGFPCAT. A) Cells were cultured in minimal medium with and without induction and with and without ethanol treatment. VP1GFP (●, ○), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊). Uninduced (○, △, ◊) and Induced (●, ▲, ♦). B) Fluorescent profiles for *E. coli* VP1GFP (□, ●, ▼) and ethanol-treated *E. coli* VP1GFP (♦, ▲, △) cultures induced at Time 0 hours. Triplicate data are shown. C) Specific CAT activity profiles for *E. coli* GFPCAT Uninduced (△) and Induced (▲) cultures.
Flow cytometry was used to confirm GFP expression due to IPTG-induction in both the untreated and the ethanol-treated VP1GFP cultures. As shown in Figure 4.1B, fluorescent intensity increased linearly post-induction in both untreated and ethanol-treated VP1GFP cultures (p ≤ 0.05); triplicate culture data is shown. Also, the fluorescent intensity for the ethanol-treated induced cultures was higher than the untreated induced cultures. Since the addition of 3% ethanol to the VP1GFP protein does not change the fluorescent intensity, these data indicate that either more VP1GFP was being expressed or a higher fraction of soluble VP1GFP was being expressed in the cells. Previous researchers have also suggested the fraction of soluble protein is increased by ethanol (Thomas and Baneyx 1997). García-Fruitós correlated higher fluorescence intensity to more soluble protein VP1GFP (García-Fruitós et al. 2005).

GFPCAT expression was quantified via the CAT enzyme assay (Rodriguez and Tait 1983). Figure 4.1C shows the CAT enzyme assay results for both induced and uninduced cultures. The specific CAT activity of the uninduced cultures was approximately 34 U/mg (as expected) (Sharma et al. 2007). The induced culture had a linear increase in specific CAT activity over 4 hours post-induction (p ≤ 0.05). At 4 hours post-induction, the specific CAT activity of the induced GFPCAT culture was approximately 23-fold higher than that of the uninduced cultures, which is very consistent with CAT activity observed for non-fused GFPCAT fusions (Sharma et al. 2007b). Additionally, induced GFPCAT cultures displayed significant growth on 0.61 mM chloramphenicol minimal media plates. Therefore, the GFP did not appear to significantly alter CAT activity. The fold increase was also observed to be approximately
23-fold for the induced VP1GFP cultures (Figure 4.1B), whereas the ethanol-treated VP1GFP induced cultures had approximately 35-fold higher fluorescence intensity than the ethanol-treated VP1GFP uninduced cultures at 4 hours post-induction. Thus, the relative induction of the VP1GFP and GFPCAT, both of which were controlled by a trc promoter, were similar.

4.2 GENE EXPRESSION ANALYSIS

An ANOVA analysis (p ≤ 0.10) of all culture conditions and time points was used to identify 961 genes with significant differences between at least two conditions. Tukey’s W post-hoc pairwise comparisons were used to identify genes that had differential expression between all Time 0 and Time 60 pairings. Results of the pairwise comparisons are shown below in Table 4.1. Many of the pairwise comparisons were not biologically meaningful, such as the comparisons between GFPCAT uninduced cultures and ethanol-treated VP1GFP induced cultures. These types of comparisons are indicated with an “X” following the number of differentially expressed genes. Additionally, some of the pairwise comparisons had more than one condition difference (i.e., confounding effects). For example, the induced VP1GFP 60-minute cultures compared to the uninduced ethanol-treated VP1GFP cultures. These comparisons are indicated with a “C” following the number of differentially expressed genes. Four comparisons captured differentially expressed genes that may be attributed to the solubility state of the recombinant protein. These four comparisons included: 1) 0-minute to 60-minute for the induced VP1GFP cultures; 2) uninduced to induced for the VP1GFP 60-minute cultures;
3) VP1GFP to GFPCAT for the induced 60-minute cultures; and 4) ethanol-treated to untreated for the induced VP1GFP 60-minute cultures. These four comparisons are identified in Table 4.1 by bold-faced numbers. Additionally, since the *E. coli* response to insoluble recombinant protein is similar to the heat-shock response, the numbers of heat-shock genes identified as differentially expressed for the four comparisons are also noted in Table 4.1.
Table 4.1: Tukey's W pairwise comparisons. Tukey's W pairwise comparisons (p ≤ 0.05) were used to identify genes with differential expression between culture conditions; the 961 significant genes previously identified by the ANOVA analysis (p ≤ 0.10) were analyzed. The solubility-sensitive pairwise comparisons are indicated in **bold**. The numbers of heat shock (HS) genes identified by a comparison are indicated. Pairwise comparisons with limited biological meaning (X) or with multiple condition differences *(i.e., confounding effects)* (C) are indicated. Self-comparisons form the diagonal, which is blank.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>VP1GFP Time 0 Uninduced</th>
<th>VP1GFP Time 60 Uninduced</th>
<th>VP1GFP Time 60 Induced</th>
<th>GFCAT Time 0 Uninduced</th>
<th>GFCAT Time 60 Uninduced</th>
<th>GFCAT Time 60 Induced</th>
<th>VP1GFP Time 0 Ethanol Uninduced</th>
<th>VP1GFP Time 60 Ethanol Uninduced</th>
<th>VP1GFP Time 60 Ethanol Induced</th>
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<tbody>
<tr>
<td>VP1GFP Time 0 Uninduced</td>
<td>2</td>
<td>22 (5 HS)</td>
<td>2</td>
<td>0 (C)</td>
<td>1 (X)</td>
<td>1</td>
<td>1</td>
<td>1 (C)</td>
<td>1 (C)</td>
</tr>
<tr>
<td>VP1GFP Time 60 Uninduced</td>
<td></td>
<td>162 (11 HS)</td>
<td>0 (X)</td>
<td>0 (C)</td>
<td>1 (C)</td>
<td>1 (X)</td>
<td>2</td>
<td>5 (C)</td>
<td></td>
</tr>
<tr>
<td>VP1GFP Time 60 Induced</td>
<td></td>
<td></td>
<td>65 (X)</td>
<td>45 (C)</td>
<td>126 (10 HS)</td>
<td>164 (C)</td>
<td>76 (C)</td>
<td>9 (0 HS)</td>
<td></td>
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<tr>
<td>GFCAT Time 0 Uninduced</td>
<td></td>
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<td>GFCAT Time 60 Uninduced</td>
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<td>GFCAT Time 60 Induced</td>
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<tr>
<td>VP1GFP Time 0 Ethanol Uninduced</td>
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<td>VP1GFP Time 60 Ethanol Uninduced</td>
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<tr>
<td>VP1GFP Time 60 Ethanol Induced</td>
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</table>
Since a key objective of this study was to identify the dynamic transcriptional events related to IB formation, regression analysis (p ≤ 0.05) was used to identify genes with time-dependent behavior. The 0-, 5-, 20-, 40- and 60-minute samples were analyzed for both the VP1GFP and GFPCAT cultures, where the 0-minute samples were taken just prior to IPTG addition. The regression analysis identified 33 genes with time-dependent behavior for the VP1GFP cultures and 92 genes with time-dependent behavior for the GFPCAT cultures. The uninduced VP1GFP and GFPCAT and the uninduced and induced ethanol-treated cultures did not have a sufficient number of time points to perform regression analysis. In order to identify a comprehensive set of genes that were affected by recombinant protein solubility, a union of the four Tukey-identified and the two regression-identified, time-dependent gene sets was compiled. This union identified a total of 318 differentially expressed genes. In Table A.1 (Appendix A), all of the 318 differentially expressed genes are listed alphabetically by gene name with fold changes for the four Tukey comparisons (p ≤ 0.05) and with the slope direction (positive or negative) for genes with significant regression results (p ≤ 0.05).

4.3 GENE BEHAVIOR—HEAT-SHOCK GENES

In wild-type *E. coli*, there are 35 classical heat-shock genes that are known to be up-regulated in response to elevated culture temperatures (*clpABPX, dnaJK, gapA, groLS, grpE, hflB, hscA, hslJRU, htaA, htpGX, htrABCE, ibpAB, ldhA, lon, lysU, pspA, rfaD, rlmE, rpoDEH, yrfI*) (Gross 1996; Richmond et al. 1999). Many of these heat-shock genes are also known to be up-regulated in the presence of misfolded recombinant
proteins (*clpBP, dnaJK, groLS, grpE, hslRUV, htpGX, ibpAB, lon, rlmE, rpoD, yrfI*) (Harcum and Haddadin 2006; Lesley et al. 2002; Smith 2007). The ANOVA analysis (p \( \leq 0.10 \)) identified 18 heat-shock genes as significantly regulated, and 14 of these 18 heat-shock genes were identified by the union of the Tukey’s W and regression analyses. All 18 significantly regulated classical heat-shock genes (ANOVA p \( \leq 0.10 \)) are listed in **Table 4.2** with statistically significant (p \( \leq 0.05 \)) fold changes and regression slope directions.
Table 4.2: Classical heat-shock gene behavior. Classical heat-shock genes that were identified by ANOVA analysis (p ≤ 0.10) as significantly regulated. The regression analysis slopes (p ≤ 0.05) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. GO terms are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced. C = VP1GFP 60-minute induced vs. GFPCAT 60-minute induced.] The fold changes observed by Lesley et al. (2002) and Smith (2007) for insoluble protein culture conditions relative to control culture conditions are indicated.

<table>
<thead>
<tr>
<th>bname</th>
<th>Gene</th>
<th>Gene Description</th>
<th>GO Term</th>
<th>VPI-GFP</th>
<th>GFP-CAT</th>
<th>Fold changes: Current Study</th>
<th>Fold changes: Past Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
<td>b2592</td>
<td>clpB</td>
<td>protein disaggregation chaperone</td>
<td>Protein Folding</td>
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<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>b0437</td>
<td>clpP</td>
<td>proteolytic subunit of ClpA-ClpP and ClpX-ClpP ATP-dependent serine proteases</td>
<td>Proteolysis</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>b0015</td>
<td>dnaJ</td>
<td>chaperone Hsp40, co-chaperone with DnaK</td>
<td>Protein Folding</td>
<td></td>
<td></td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>b0014</td>
<td>dnaK</td>
<td>chaperone Hsp70, co-chaperone with DnaK</td>
<td>Protein Folding</td>
<td></td>
<td></td>
<td>1.9</td>
<td>1.9</td>
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<tr>
<td>b3178</td>
<td>ftsH</td>
<td>subunit of integral membrane ATP-dependent zinc metalloprotease</td>
<td>Proteolysis</td>
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<tr>
<td>b1779</td>
<td>gapA</td>
<td>glyceraldehyde-3-phosphate dehydrogenase A</td>
<td>Energy Metabolism</td>
<td>+</td>
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<tr>
<td>b4143</td>
<td>groL</td>
<td>Cpn60 chaperonin GroEL, large subunit of GroESL</td>
<td>Protein Folding</td>
<td>+</td>
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<tr>
<td>b4142</td>
<td>groS</td>
<td>Cpn10 chaperonin GroES, small subunit of GroESL</td>
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<tr>
<td>b3931</td>
<td>hslU</td>
<td>molecular chaperone and ATPase component of HslU protease</td>
<td>Protein Folding</td>
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<td>1.2</td>
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<tr>
<td>b3932</td>
<td>hslV</td>
<td>peptidase component of the HslU protease</td>
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<tr>
<td>b0473</td>
<td>hspG</td>
<td>molecular chaperone HSP90 family</td>
<td>Protein Folding</td>
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<tr>
<td>b1829</td>
<td>hspX</td>
<td>heat shock protein, integral membrane protein</td>
<td>Proteolysis</td>
<td></td>
<td></td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>b0439</td>
<td>hsp</td>
<td>DNA-binding ATP-dependent protease La</td>
<td>Proteolysis</td>
<td></td>
<td></td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>b3179</td>
<td>rlmE</td>
<td>23S rRNA U2352 ribosome 2'-O-methyltransferase</td>
<td>RNA Methylation</td>
<td></td>
<td></td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>b3400</td>
<td>rlmR</td>
<td>ribosome-associated heat shock protein Hsp15</td>
<td>RNA Methabolism</td>
<td></td>
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<td>28.3</td>
<td>8.2</td>
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<tr>
<td>b3686</td>
<td>rlpB</td>
<td>heat shock chaperone</td>
<td>Protein Folding</td>
<td></td>
<td></td>
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<tr>
<td>b4129</td>
<td>lrpE</td>
<td>lysine-tRNA synthetase, inducible</td>
<td>Aminoacyl-tRNA Synthetase</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>b2573</td>
<td>rpoE</td>
<td>RNA polymerase, sigma 24 (sigmaE) factor</td>
<td>Transcription Regulation</td>
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</tbody>
</table>
This study identified the same heat-shock genes that Lesley et al. (2002) and Smith (2007) observed in cells expressing IBs, except the response magnitudes observed in both of those studies were significantly higher. Table 4.2 lists the fold changes of classical heat-shock genes in response to IB stress observed in the current study, as well as fold changes observed in Lesley et al. (2002) and Smith (2002). The fold changes observed by Lesley et al. (2002) and Smith (2007) for the heat-shock genes were between 2- to 40-fold higher due to insoluble recombinant protein expression; however, in the current study, fold changes were only between 1.5- to 2.2. Several factors likely contributed to these differences: 1) In this study, a minimal medium was used to slow growth rates to 0.55 h\(^{-1}\), whereas the LB medium used by Lesley et al. (2002) and Smith (2007) commonly has growth rates of approximately 2.0 h\(^{-1}\); 2) Lesley et al. (2002) used 6x His-tags on all proteins, whereas in this study, only GFPCAT had a 6x His-tag; 3) Smith (2007) expressed recombinant proteins in *E. coli* BL21, whereas in this study, *E. coli* K-12 strain was used as the host; and 4) Both Lesley et al. (2002) and Smith (2007) used strong T7-based promoters, whereas in this study, the weaker trc promoter was used. The significantly higher growth rates in LB medium compared to a minimal medium would allow for more cell doublings within 60 minutes, and thus, the turnover rates for mRNA species would be higher, allowing for more accumulation to be observed. Despite the magnitude difference in the heat-shock responses, the present study identified the same heat-shock genes as Lesley et al. (2002) and Smith (2007). Thus, the dynamic behavior obtained by this study for heat-shock and other differentially expressed genes will be representative of insoluble protein effects but with much lower
magnitudes. Additionally, the dynamic behavior has not previously been reported (Lesley et al. 2002; Smith 2007).

In order to better understand the time-dynamic behavior of the heat-shock genes, time profiles for each of the 14 differentially expressed heat-shock genes was examined for the VP1GFP and GFPCAT cultures (Figure 4.2). Additionally, the time profiles for \( tig \), the trigger factor that constitutes one of the three major protein folding chaperones in \( E. coli \) (along with \( dnaK \) and \( groLS \)), were included. The heat-shock genes identified by the regression analysis with positive slopes for VP1GFP are shown in the first two rows (\( clpB, dnaK, gapA, groLS, htpG \)). The time profiles of these six genes indicate that these genes responded early to the synthesis of VP1GFP, whereas the other heat-shock genes responded later, between 40 and 60 minutes post-induction, and seemed to be responding more to the presence of VP1GFP than to the synthesis of VP1GFP. Specifically, the chaperone genes responded immediately to the synthesis of VP1GFP with a linear increase in expression. In contrast, the protease genes responded later in time, after the insoluble VP1GFP protein had begun to accumulate within the cells.
Figure 4.2: Classical Heat Shock and \textit{tig} gene expression profiles. The dynamic gene expression profiles for \textit{E. coli} VP1GFP and GFPCAT heat-shock genes and the protein-folding chaperone gene \textit{tig}. VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (●, ◆); Uninduced (□, △, ◆) and Induced (■, ▲, ●) are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray. Standard error bars are shown.
Within the DnaK-DnaJ-GrpE chaperone system, also known as the Hsp70 chaperone system, *dnaJ*, a protein folding chaperone gene, was not identified by regression analysis on VP1GFP cultures and instead displayed a delayed response similar to the heat-shock proteases, while *grpE* was not identified to have statistically significant behavior. The *dnaK* gene expression level immediately increased due to VP1GFP synthesis. These behavior differences are expected mechanistically. The Hsp70 chaperone system is powered by ATPase activity and is always in an ATP-bound state or an ADP-bound state. In the ATP-bound state, the substrate-binding pocket is open and allows for binding of misfolded protein domains, while in the ADP-bound state, the substrate binding pocket is closed and allows for the chaperone to interact with and fold a misfolded protein. Hydrolysis of ATP is usually the rate-limiting step for the Hsp70 chaperone system, and DnaK provides the primary ATPase activity for the Hsp70 chaperone system (Bukau and Horwich 1998, Keseler et al 2012). Thus, it is expected that *dnaK* would be up-regulated in response to misfolded protein prior to *dnaJ* and *grpE*.

In addition to the heat-shock genes, an additional 30 genes regulated by the heat-shock transcription factor *rpoH*, or σ32, were included in the 318 differentially expressed genes (*aaeB, dcuR, entD, hcaR, kdgR, lexA, lrp, lspa, lysR, mlrA, nadR, nhaR, nor, phoQ, proB, rcnR, rhaR, rsmI, sdiA, yaiO, ybeX, ybeZ, ydeO, yeeJ, yfiR, yghQ, yjfp, yjfZ, yqeG, yqf*). The *rpoH* gene was not significantly changed due to IBs, which is consistent with previous observations (Harcum and Haddadin 2006; Lesley et al. 2002; Smith 2007). Recombinant protein overexpression and insolubility cause similar, but not
identical, responses to heat stress. Also, the recombinant cultures in this study were not exposed to elevated temperatures

4.4 GENE BEHAVIOR—GENE ONTOLOGIES

In order to identify gene functions that were sensitive to IBs, the 318 genes with differential expression were grouped by common functionalities using gene ontology (GO) terms from the ASAP database and EcoCyc. Most genes had several GO term entries. In those cases, the dominant function of the encoded protein was used for classification so that no single gene is represented twice. For example, *lon* is listed with 13 GO terms, ranging from “response to stress” to “DNA-binding.” The protein product of *lon* is the protease La, which is a well-characterized protease in *E. coli* that degrades abnormal proteins. Thus, *lon* was grouped under Proteolysis. **Figure 4.3** shows the gene classifications, with the number of genes with a particular GO term indicated. **Table A.2** (Appendix A) lists all genes grouped by classification. For the 14 differentially expressed heat-shock genes, eight genes were grouped with Protein Folding (*clpB, dnaJK, groLS, hslUV, and htpG*), four genes were grouped with Proteolysis (*clpP, fitsH, htpX*, and *lon*), one gene was grouped with Energy Metabolism (*gapA*, a glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis), and one gene was grouped with RNA Methylation (*rlmE*, a 23S rRNA methyltransferase).
Figure 4.3: Classification of differentially expressed genes. The chart showing the number of genes within each classification for the 318 differential expressed genes (p ≤ 0.05). Genes have been grouped by function using gene ontology (GO) terms from EcoCyc and annotations from the ASAP database.
For the 318 genes with differential expression, the largest fraction of genes was classified within Metabolic Process (152 genes), as shown in Figure 4.3. The metabolic process group contained the following sub-classifications (with number of genes indicated in parentheses): Biosynthesis (38 genes), RNA Metabolism (33 genes), Catabolism (26 genes), Energy Metabolism (23 genes), Protein Metabolism (16 genes), and DNA Metabolism (5 genes); and eleven metabolic process genes did not align with these sub-classifications (*aphA*, *deoA*, *frc*, *glpK*, *guaD*, *hyuA*, *pntA*, *pps*, *sufS*, *yicI*, and *yihQ*). After metabolic process, Putative genes were the next largest first-level classification (111 genes), representing 35% of the differentially expressed genes. Since only approximately 14% of the *E. coli* genome is considered to be of unknown function (Riley et al. 2006), this high proportion of putative genes indicates that *E. coli* responds to insoluble recombinant proteins using many genes that have not been well-studied. The remaining first-level classifications were Transmembrane Transport (33 genes) and Transcription Regulation (17 genes). Of the 318 differentially expressed genes, five genes did not align with these classifications (*asr*, *fimFG*, *tsr*, *yeeJ*), and were mainly cell wall components. These gene classifications were subsequently used to identify coordinated behavior within cellular functionalities due to insoluble recombinant protein expression (IB formation).

**Protein Metabolism**

Protein metabolism genes encode for chaperones that assist with protein synthesis and proteases that degrade misfolded or unneeded proteins. Within the protein
metabolism group, nine protein folding and seven proteolysis genes were identified. The nine protein folding genes include eight classical heat-shock genes (\textit{clpB}, \textit{dnaJK}, \textit{groLS}, \textit{hslUV}, and \textit{htpG}), plus \textit{tig}. All of the protein-folding genes increased in expression due to VP1GFP production, including \textit{tig}. The time profile for \textit{tig} is shown in Figure 4.2 with the classical heat-shock genes; although \textit{tig} is not a classical heat-shock protein, it is a chaperone. The observed behavior of the \textit{tig} gene, however, was more similar to the heat-shock protease genes (\textit{clpP}, \textit{ftsH}, \textit{htpX}, and \textit{lon}) as opposed to the heat-shock folding chaperone genes (\textit{i.e.}, \textit{tig} expression did not increase immediately due to VP1GFP production). The seven proteolysis genes identified included four classical heat-shock genes (\textit{clpP}, \textit{ftsH}, \textit{htpX}, and \textit{lon}), plus \textit{ompX} and \textit{pepBN}. The \textit{ompX} and \textit{pepBN} gene time profiles are very similar to the heat-shock protease genes, indicating that the accumulation of insoluble recombinant protein increases protease gene expression more so than the initial production of insoluble recombinant protein.

\textbf{Protein Synthesis-Related Gene Classifications}

Many of the differentially expressed genes were involved with protein synthesis, including genes involved with ribosomal subunits (\textit{rplACEILNQR}, \textit{rpmDIJ}, and \textit{rpsAFGHLMNPU}), aminoacyl-tRNA synthetases (\textit{asnS}, \textit{leuS}, \textit{pheT}, \textit{thrS}, and \textit{tyrS}), and amino acid synthesis (\textit{aroAG}, \textit{dadX}, \textit{gdhA}, \textit{hisGH}, \textit{lrp}, \textit{metH}, \textit{proB}, \textit{thrC}, and \textit{trpCD}). These genes are shown in Table 4.3 with gene ontologies. The most coordinated group was the ribosomal subunit genes. Twenty ribosomal subunit genes were identified, including both 30S ribosomal subunit genes (\textit{rpsAFGHLMNPU}) and 50S ribosomal
subunit genes (\textit{rplACEJLNQR} and \textit{rpmDIJ}). The behavior of these 20 genes was very similar within cultures (Figure 4.4ABC). Thus, average time profiles represent well the dynamic behavior of a coordinated gene group (Figure 4.4D). The average time profiles for the ribosomal subunit genes are also shown in Figure 4.5A with the other protein-synthesis-related genes. There are 55 ribosomal subunit genes in \textit{E. coli}; however, the remaining 35 ribosomal subunit genes did not meet statistical significance, although the time profiles were very similar to the 20 ribosomal subunit genes that were identified (Figure B.1 in Appendix B). The ribosomal subunit gene expression levels increased due to protein expression: soluble GFPCAT expression resulted in a linear increase in expression; VP1GFP expression initially resulted in a similar linear increase, but between 40 and 60 minutes post-induction, a dramatic increase was observed. Interestingly, the ribosomal subunit gene expression profiles of the ethanol-treated VP1GFP cultures resembled the time profiles of the GFPCAT cultures more so than the time profiles of the untreated VP1GFP cultures, indicating that ethanol dampened the ribosomal subunit response to the high level of VP1GFP synthesis and to the presence of VP1GFP, as the ethanol-treated VP1GFP cell contained very high levels of VP1GFP (see Figure 4.1B). Past studies have indicated that the expression of ribosomal proteins and other protein synthesis machinery can be down-regulated in high-expression recombinant protein systems, presumably due to aminoacyl-tRNA deprivation (Dong et al. 1995; Gallant 1979; Rinas 2008). The seemingly contradictory results of this study may indicate that cells grown in minimal medium and analyzed predominantly in the early-phases of recombinant protein production (\(\leq 1\) hour post-induction) had not yet encountered a
significant shortage of amino acids or charged tRNAs. Interestingly, the addition of ethanol decreased the expression of the ribosomal subunit genes, and the ribosomal subunit expression remained lower after induction. Ethanol decreased cell growth, a characteristic consistent with past studies (Thomas and Baneyx 1997), where inhibited cell growth is consistent with a decrease in overall protein synthesis (Figure 4.1A).
Table 4.3: Protein synthesis-related genes with differential expression. Protein synthesis-related genes that were identified as differentially expressed. The regression analysis slopes ($p \leq 0.05$) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons ($p \leq 0.05$) are indicated. GO terms are indicated. [For fold changes, $A = VP1GFP$ 60-minute induced vs. $VP1GFP$ 0-minute uninduced. $B = VP1GFP$ 60-minute induced vs. $VP1GFP$ 60-minute uninduced. $C = VP1GFP$ 60-minute induced vs. GFPCAT 60-minute induced.]

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Figure 4.4: Time profiles for ribosomal subunit genes. The individual gene time profiles for 20 differentially expressed ribosomal subunit genes are shown for the A) VP1GFP, B) GFPCAT, and C) Ethanol-treated VP1GFP cultures. The average time profiles for all 20 differentially expressed ribosomal subunit genes are shown by culture (D). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray. Standard error bars are shown.
Figure 4.5: Gene expression profiles for protein synthesis-related genes with differential expression for *E. coli* pTVP1GFP and pGFPCAT. A) Ribosomal subunit genes (average of 20 genes). B) Aminoacyl-tRNA Synthetase genes (average of 5 genes). C) Amino Acid Synthesis genes (average of 12 genes). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray. Standard error bars are shown.
The ribosomal subunit gene expression levels were relatively high at Time 0 in VP1GFP cultures relative to ethanol-treated VP1GFP and GFPCAT cultures. By examining the raw data by biological replicate, it was determined that biological replicate B was particularly high for many genes, even when normalized to the average gene on the DNA microarray. For example, the ribosomal subunit gene expression levels are shown for replicates A, B, and C for VP1GFP Time 0 in Figure 4.6. An ANOVA test with Bonferroni multiple testing was used on the three biological replicates of VP1GFP; however, the number of differentially expressed genes was not greater than the false positive rate. Thus, there was not enough statistical evidence to warrant exclusion of any replicates of VP1GFP Time 0, and replicate B was utilized for all data analysis.
Figure 4.6: Gene expression levels of ribosomal subunit genes across biological replicates. Data for three biological replicates of VP1GFP Time 0 cultures are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray. The error bars represent 95% confidence intervals.
Amino acid synthesis genes (*aroAG, dadX, gdhA, hisGH, lrp, metH, proB, thrC, trpCD*) encode for enzymes involved in the various pathways used to generate amino acids. The amino acid synthesis genes shown in Figure 4.5B had similar expression profiles to the ribosomal subunit genes shown in Figure 4.5A. The amino acid synthesis gene expression levels increased due to protein expression for both the soluble and insoluble proteins, but were increased to a greater extent by VP1GFP expression. The similar responses of the ribosomal subunit genes and amino acid synthesis genes support the observed increase in protein synthesis machinery levels in response to recombinant protein expression.

Aminoacyl-tRNA synthetase genes encode for the enzymes responsible for covalently binding amino acids to tRNA molecules for protein synthesis (*i.e.*, aminoacyl-tRNA charging). For the aminoacyl-tRNA synthetase genes identified in this study as differentially expressed (*asnS, leuS, pheT, thrS, and tyrS*), the most pronounced increase in expression was observed for induced VP1GFP 60-minute cultures post-induction. Time profiles for aminoacyl-tRNA synthetase genes are shown in Figure 4.5C and are very similar to the heat-shock proteases. The observed delay in increased expression may indicate that the aminoacyl-tRNA synthetase genes responded to the accumulation of VP1GFP more so than to the initial synthesis of VP1GFP.

Lesley *et al.* (2002) and Smith (2007) did not observe coordinated changes in the expression for the amino acid synthesis or aminoacyl-tRNA synthetase genes in response to insoluble recombinant protein (Lesley *et al.* 2002; Smith 2007). These previous studies were conducted in LB medium, which provides high levels of amino acids to the
cells. In contrast, growth in minimal medium requires the cells to synthesize all amino acids from glucose and ammonium. The observed increased expression of the ribosomal subunit, amino acid synthesis, and aminoacyl-tRNA synthetase genes in this study indicates that the cells were adapting to increased protein synthesis rates for both VP1GFP and GFPCAT cultures; however, the response was greater for the VP1GFP cultures, indicating there may be a solubility related response too.

Transmembrane Transport

Transmembrane transport genes encode for proteins that control cellular import and export of medium components. In the current study, 33 transmembrane transport genes were identified as having differential expression. Within these 33 transmembrane transport genes, 13 carbohydrate transport genes (actP, bglF, dgoT, fucP, glpF, gntT, lamB, malF, opgH, ugpAB, uhpT, and ulaA) and eight amino acid transport genes (dppBC, dtpB, eamB, gltS, gspDL, and tdcC) were identified, as well as 12 transmembrane transport genes that were not further sub-classified (aaeB, acrBF, blc, btuE, cysP, lspa, modB, ompF, pstS, purP, and tsgA). Eleven carbohydrate transport genes, six amino acid transport genes, and six of the unclassified genes decreased significantly due to VP1GFP production, whereas these effects were not observed in the ethanol-treated VP1GFP and GFPCAT cultures. These 23 genes are listed in Table 4.4. The average time profiles for these 23 transmembrane transport genes with decreased expression due to VP1GFP production are shown in Figure 4.7A. The dynamic response of these time profiles indicates that the transporter genes appeared to be affected by
VP1GFP synthesis initially and further by the accumulation of VP1GFP. Lesley et al. (2002) and Smith (2007) also noted the decreased expression of several membrane transporters due to the presence of insoluble recombinant protein, including various sugar and metal ion transporters (chaA, fecB, feoA, fruA, glpF, lamB, rbsC, setA, ychM) (Lesley et al. 2002; Smith 2007). This study identified the decreased expression of several transporters previously not associated with IBs (aaeB, acrFP, bglF, blc, dgoT, dtpB, eamB, fucP, gttS, gntT, gspDL, malF, modB, purP, tdcC, tsgA, ugpB, uhpt, ulaA).

Interestingly, for the 23 transmembrane transporters that had decreased expression due to the presence of VP1GFP, 13 genes are directly regulated by the cyclic AMP receptor protein, Crp, (including 10 of the 11 carbohydrate transport genes). These 13 genes are indicated in Table 4.4. The Crp protein is known to regulate catabolite repression, or the preferential utilization of certain carbon sources over others (Görke and Stülke 2008; Gosset et al. 2004). The Crp-controlled genes that decreased in expression facilitated entry of a variety of substrates, such as actP (glycolate), bglF (methyl-β-D-glucoside-6-phosphate and arbutin-6-phosphate), dgoT (galactonate), fucP (fucose), glpF (glycerol), gntT (gluconate), lamB/malF (β-maltose), and uhpt (hexose phosphate); however, in this study, glucose was the sole carbon source. Thus, the decreased expression of these Crp-controlled transporters may indicate that VP1GFP production shifts metabolic efforts onto specific substrates by down-regulating unnecessary transporter proteins or that IB stress induces a physical change at the cellular membrane.
Table 4.4: Transmembrane transport genes with decreased expression levels due to IB stress. Transmembrane transport genes that were identified for decreased expression due to VP1GFP production. GO terms are indicated. Many of these genes are regulated by Crp, which is indicated. The regression analysis slopes (p ≤ 0.05) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced. C = VP1GFP 60-minute induced vs. GFP-CAT 60-minute induced.]

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<tr>
<td>b3496</td>
<td>dipB</td>
<td>proton-dependent peptide transporter</td>
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<td>cysteine and O-acetylserine exporter</td>
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<td>b3653</td>
<td>gltS</td>
<td>glutamate transporter</td>
<td>Amino Acid Transport</td>
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<td>b3322</td>
<td>gspD</td>
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<td>Amino Acid Transport</td>
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<td>gspL</td>
<td>general secretory pathway component, cryptic</td>
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<td>b3116</td>
<td>tdcC</td>
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<tr>
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<td>acetP</td>
<td>acetate permease</td>
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<td>b3722</td>
<td>bgIF</td>
<td>PTS system beta-glucoside-specific EIIIBCA component</td>
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<td>dgoT</td>
<td>D-galactonate transporter</td>
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<td>0.8</td>
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<td>b2801</td>
<td>fucP</td>
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<td>b3927</td>
<td>glpF</td>
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<td>gntT</td>
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<tr>
<td>b4036</td>
<td>lamB</td>
<td>maltose outer membrane porin (maltoporin)</td>
<td>Carbohydrate Transport</td>
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<tr>
<td>b4033</td>
<td>malf</td>
<td>membrane component of an ABC superfamily maltose transporter</td>
<td>Carbohydrate Transport</td>
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(Table 4.4 continued on next page)
Table 4.4 (continued)

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<td>b3453</td>
<td>ugpB</td>
<td>periplasmic-binding component of an ABC superfamily glycerol-3-phosphate transporter</td>
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<tr>
<td>b3666</td>
<td>hupT</td>
<td>hexose phosphate transporter</td>
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<tr>
<td>b4193</td>
<td>ulaA</td>
<td>L-ascorbate-specific enzyme IIC component of PTS</td>
<td>Carbohydrate Transport</td>
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<tr>
<td>b3240</td>
<td>aaeB</td>
<td>p-hydroxybenzoic acid efflux pump subunit AaeB</td>
<td>Transmembrane Transport</td>
<td>Yes</td>
<td>+</td>
<td>0.9</td>
</tr>
<tr>
<td>b3266</td>
<td>acrF</td>
<td>multidrug efflux system protein</td>
<td>Transmembrane Transport</td>
<td>No</td>
<td>0.8</td>
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<tr>
<td>b4149</td>
<td>bIC</td>
<td>outer membrane lipoprotein (lipocalin)</td>
<td>Transmembrane Transport</td>
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<td>0.9</td>
<td>0.9</td>
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<tr>
<td>b0764</td>
<td>modB</td>
<td>molybdate transporter subunit; membrane component of ABC superfamily</td>
<td>Transmembrane Transport</td>
<td>Yes</td>
<td>0.9</td>
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<tr>
<td>b3714</td>
<td>purP</td>
<td>predicted inner membrane protein</td>
<td>Transmembrane Transport</td>
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<td>-</td>
<td>0.9</td>
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<tr>
<td>b3364</td>
<td>tagA</td>
<td>predicted transporter</td>
<td>Transmembrane Transport</td>
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Figure 4.7: Gene expression profiles for transmembrane transport and catabolism genes with differential expression for *E. coli* pTVP1GFP and pGFPCAT. A) Transmembrane transport genes with decreased expression due to VP1GFP (average of 23 genes). B) Catabolism genes with decreased expression due to VP1GFP (average of 19 genes). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, △, ♦) are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray. Standard error bars are shown.
Catabolism

Catabolism reactions in cells control the breakdown and recycling of cellular building blocks. The catabolism classification mainly identified carbohydrate and amino acid degradation genes with differential expression. For carbohydrate degradation, 12 of the 13 genes identified decreased due to VP1GFP expression (bglB, dgoDK, glcD, gudD, maoC, mtlD, rhBM, treF, uxB, and yiaS). Additionally, three amino acid degradation genes (dtd, tdh, and tnaA) and four unclassified catabolism genes (caiB, chiA, cpdB, and nudE) decreased due to VP1GFP expression. These 19 catabolic genes are listed in Table 4.5, and the average time profiles for these 19 genes are shown in Figure 4.7B. These catabolism genes had time profiles similar to the transmembrane transport genes; genes appeared to be affected by VP1GFP synthesis initially and further by the accumulation of VP1GFP.

For the 19 transmembrane transporters that had decreased expression due to the presence of VP1GFP, 9 genes are directly regulated by Crp. These 9 genes are indicated in Table 4.5. Taken in conjunction with the coordinated decrease in expression of Crp-controlled transmembrane transport genes, these results seem to indicate that catabolite repression may have been occurring in the cells. Interestingly, several of transmembrane transporter genes with decreased expression due to VP1GFP acted upon the same target substrate as many catabolic genes with decreased expression due to VP1GFP. For example, the dgoT gene encodes the D-galactonate transporter and the dgoDK genes encode enzymes that degrade D-galactonate (Karp et al. 2010). Table 4.6 lists the catabolic genes and transmembrane transporter genes with identical target substrates.
These catabolic and transmembrane transporter genes all had decreased expression due to VP1GFP production but were all unaffected by GFPCAT production and by VP1GFP production in the ethanol-treated cultures. Lesley (2002) and Smith (2007) did not observe coordinated expression between catabolism genes and transmembrane transporter genes. These results indicate that cells respond to the onset of IB stress through a coordinated down-regulation of transporters and metabolic degraders of a variety of substrates and that the cells may be undergoing catabolite repression to a degree.
Table 4.5: Catabolic genes with decreased expression levels due to IB stress. Catabolism genes that were identified for decreased expression due to VP1GFP production. GO terms are indicated. Many of these genes are regulated by Crp, which is indicated. The regression analysis slopes (p ≤ 0.05) are indicated (+/-, positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced. C = VP1GFP 60-minute induced vs. GFPCAT 60-minute induced.]

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<td></td>
<td></td>
<td>VP1-GFP</td>
<td>GFP-CAT</td>
</tr>
<tr>
<td>b3887</td>
<td>did</td>
<td>D-Tyr-tRNA(Tyr) deacylase</td>
<td>Amino Acid Degradation</td>
<td>No</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>b3616</td>
<td>tdh</td>
<td>threonine 3-dehydrogenase, NAD(P)-binding</td>
<td>Amino Acid Degradation</td>
<td>No</td>
<td>+</td>
<td>0.8</td>
</tr>
<tr>
<td>b3708</td>
<td>tnaA</td>
<td>tryptophanase-L-cysteine desulphhydrase, PLP-dependent</td>
<td>Amino Acid Degradation</td>
<td>Yes</td>
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<td></td>
</tr>
<tr>
<td>b3721</td>
<td>bgIB</td>
<td>cryptic phospho-beta-glucosidase B</td>
<td>Carbohydrate Degradation</td>
<td>Yes</td>
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<tr>
<td>b4478</td>
<td>dgoD</td>
<td>galactonate dehydratase</td>
<td>Carbohydrate Degradation</td>
<td>No</td>
<td>0.8</td>
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<tr>
<td>b3693</td>
<td>dgoK</td>
<td>2-oxo-3-deoxygalactonate kinase</td>
<td>Carbohydrate Degradation</td>
<td>No</td>
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<tr>
<td>b2979</td>
<td>gldD</td>
<td>glycolate oxidase subunit, FAD-linked</td>
<td>Carbohydrate Degradation</td>
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<td>-</td>
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<tr>
<td>b2787</td>
<td>gudD</td>
<td>D-glucarate dehydratase</td>
<td>Carbohydrate Degradation</td>
<td>No</td>
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<tr>
<td>b1387</td>
<td>maoC</td>
<td>enoyl-CoA hydratase</td>
<td>Carbohydrate Degradation</td>
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<tr>
<td>b3600</td>
<td>mldD</td>
<td>mannitol-1-phosphate dehydrogenase, NAD(P)-binding</td>
<td>Carbohydrate Degradation</td>
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<tr>
<td>b3904</td>
<td>rhaB</td>
<td>rhamnulokinase</td>
<td>Carbohydrate Degradation</td>
<td>Yes</td>
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<tr>
<td>b3901</td>
<td>rhaM</td>
<td>L-rhamnose mutarotase</td>
<td>Carbohydrate Degradation</td>
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<tr>
<td>b4323</td>
<td>wuxB</td>
<td>D-mannone oxidoreductase, NAD-binding</td>
<td>Carbohydrate Degradation</td>
<td>Yes</td>
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<tr>
<td>b4323</td>
<td>uxaB</td>
<td>D-mammonate oxidoreductase, NAD-binding</td>
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<td>Yes</td>
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<td>-</td>
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<tr>
<td>b3583</td>
<td>ykaS</td>
<td>L-ribulose-5-phosphate 4-epimerase</td>
<td>Carbohydrate Degradation</td>
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<tr>
<td>b0038</td>
<td>catB</td>
<td>crotonobetanyl CoA:carnitine CoA transferase</td>
<td>Catabolism</td>
<td>Yes</td>
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<tr>
<td>b3338</td>
<td>chiA</td>
<td>periplasmic endochitinase</td>
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<tr>
<td>b4213</td>
<td>cdpB</td>
<td>2',3'-cyclic-nucleotide 2'-phosphodiesterase</td>
<td>Catabolism</td>
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<td>b3397</td>
<td>nudE</td>
<td>ADP-ribose diphosphatase</td>
<td>Catabolism</td>
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<td>b3519</td>
<td>treF</td>
<td>cytoplasmic trehalase</td>
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Table 4.6: Transmembrane transporters and catabolic genes with common substrates. These genes had decreased expression due to VP1GFP production. The connections were determined by the PathwayTools software Version 13.0 (Karp et al. 2010).

<table>
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<th>Substrate</th>
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<th>Gene in Catabolic Pathway with Decreased Expression Due to VP1GFP Production</th>
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<tr>
<td>D-galactonate</td>
<td>\textit{dgoT}</td>
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<td>Glycolate</td>
<td>\textit{actP}</td>
<td>\textit{glcD}</td>
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<tr>
<td>Arbutin-6-phosphate</td>
<td>\textit{bglF}</td>
<td>\textit{bglB}</td>
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<tr>
<td>Methyl-β-D-glucoside-6-phosphate</td>
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<td>Threonine</td>
<td>\textit{tdcC}</td>
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<td>Cysteine</td>
<td>\textit{eamB}</td>
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**Cofactor Synthesis**

Cofactors are compounds required by a protein to function properly and often modulate enzyme activity. Within the cofactor synthesis gene classification, nine genes increased in expression level due to VP1GFP synthesis, whereas these effects were not observed in the ethanol-treated VP1GFP and GFPCAT cultures (*dxs, folC, hemL, ispEFG*, and *thiCMS*). These nine genes are listed in **Table 4.7**, and the time profiles are shown in **Figure B.2 (Appendix B)**. The *dxs* and *ispEFG* genes encode enzymes in the methyldihydroxypropane phosphate pathway, which primarily synthesizes membrane glycolipids, peptidoglycans, and quinols. Taken in conjunction with the regulation of many transmembrane transporters, the increased expression of the *dxs* and *ispEFG* genes may indicate that VP1GFP accumulation induces a physical change of the cell membrane and that the cells were attempting to repair or reinforce the cell membrane. The *thiCMS* genes encode enzymes involved in thiamine diphosphate synthesis, an essential cofactor for both pyruvate dehydrogenase and transketolase. Pyruvate dehydrogenase is involved with decarboxylating pyruvate into acetyl-CoA in order to feed the TCA cycle. Transketolase is involved with catabolizing sugars in the pentose phosphate pathway. Thus, the increased expression of the *thiCMS* genes may indicate that cells were attempting to increase energy production in response to VP1GFP accumulation. Interestingly, the genes encoding for pyruvate dehydrogenase (*aceEF* and *lpd*) and transketolase (*tktA*) were not identified statistically as differentially expressed but had time profiles that were similar to the time profiles of *thiCMS* and the other identified cofactor synthesis genes (**Figure B.3 in Appendix B**). Overall, these nine cofactor
synthesis genes had time profiles similar to the time profiles of the heat-shock proteases shown in Figure 4.2, indicating that these genes are sensitive to VP1GFP accumulation more so than to the initial synthesis of VP1GFP.
Table 4.7: Cofactor synthesis genes with increased expression levels due to IB stress.
Cofactor synthesis genes that were identified for increased expression due to VP1GFP accumulation. The regression analysis slopes (p ≤ 0.05) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced uninduced. C = VP1GFP 60-minute induced vs. GFP CAT 60-minute induced.]

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<td>b0420</td>
<td>dxs</td>
<td>1-deoxyxylulose-5-phosphate synthase, thiamine-requiring, FAD-requiring</td>
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<td>b2315</td>
<td>folC</td>
<td>bifunctional folylpolyglutamate synthase/dihydrofolate synthase</td>
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<td>b0154</td>
<td>hemL</td>
<td>glutamate-1-semialdehyde aminotransferase (aminomutase)</td>
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<tr>
<td>b1208</td>
<td>ispE</td>
<td>4-diphosphocytidyl-2-C-methylerthritol kinase</td>
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<tr>
<td>b2746</td>
<td>ispF</td>
<td>2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase</td>
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<td>b2515</td>
<td>ispG</td>
<td>1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase</td>
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<tr>
<td>b3994</td>
<td>thiC</td>
<td>thiamin (pyrimidine moiety) biosynthesis protein</td>
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</tr>
<tr>
<td>b2104</td>
<td>thiM</td>
<td>hydroxyethylthiazole kinase</td>
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<td>b4407</td>
<td>thiS</td>
<td>sulfur carrier protein</td>
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Energy Metabolism

Energy metabolism genes are responsible for extracting metabolic energy (usually in the form of ATP, NADH, or quinols) from carbon sources, such as glucose. In this study, 23 energy metabolism genes were identified as significantly changed. Six TCA cycle genes had increased expression levels due to both VP1GFP and GFPCAT synthesis (\textit{acnB, gltA, sdhAB,} and \textit{sucBC}), but were increased to a greater extent by VP1GFP expression. Ethanol treatment reduced the magnitude of the increase of these six TCA cycle genes. These six TCA cycle genes are listed below in Table 4.8, and the time profiles for these six TCA cycle genes are shown in Figure 4.8. Additionally, 12 other TCA cycle genes (\textit{acnA, fumABC, icd, lpd, mdh, mqo, sdhCD,} and \textit{sucAD}) that did not meet statistical significance had time profiles similar to the six identified TCA cycle genes (Figure B.4 in Appendix B). The coordinated increased expression of the TCA cycle genes supports previous observations that protein synthesis is an energy intensive process (Glick 1995); however, insoluble VP1GFP protein accumulation creates a greater need for energy that is mitigated by the addition of ethanol.
**Table 4.8: TCA cycle genes with differential expression.** TCA cycle genes that were identified as differentially expressed. The regression analysis slopes (p ≤ 0.05) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced. C = VP1GFP 60-minute induced vs. GFPCAT 60-minute induced.]

<table>
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<tr>
<th>bname</th>
<th>Gene</th>
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<th>GFP-CAT</th>
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<th>C</th>
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<td>b0118</td>
<td>acnB</td>
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<td>sucB</td>
<td>dihydrolipoyltranssuccinase</td>
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<tr>
<td>b0728</td>
<td>sucC</td>
<td>succinyl-CoA synthetase, beta subunit</td>
<td></td>
<td></td>
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</table>
Figure 4.8: Gene expression profiles for TCA cycle genes with differential expression for *E. coli* pTV1GFP and pGFPCAT. A) TCA Cycle (average of 6 genes). VP1GFP (■,□), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray. Standard error bars are shown.
In addition to the TCA cycle genes, two ATP synthase genes (atpFI) were observed to have differential expression due to GFPCAT and VP1GFP expression. The atpF gene expression profile increased due to both GFPCAT and VP1GFP expression similar to that observed for the ribosomal subunit genes (Figure B.5 in Appendix B). The atpF gene encodes for subunit b of the ATP synthase F0 sector, which is critical to proton translocation. There are seven additional genes that encode for subunits of the F1F0 ATP synthase complex (atpABCDEGH). The gene expression profiles of these seven genes were similar to atpF; however, these seven genes did not meet statistical significance (Figure B.5 in Appendix B). Interestingly, atpI gene is considered to be unessential for the function of the ATP synthase complex (Keseler et al. 2012) and was observed to be decreased due to GFPCAT and VP1GFP expression. The increased expression of ATP synthase genes is consistent with increased cellular ATP needs due to recombinant protein expression.

Of the remaining energy metabolism genes, eight genes involved in the electron transport chain were increased due to VP1GFP accumulation (appC, dld, hyaDE, napD, narW, and nuoCG). These eight genes encode for components of the NADH oxidoreductase, cytochrome oxidase, nitrate reductase, hydrogenase 1, and lactate dehydrogenase complexes (Keseler et al. 2012). These eight genes are listed in Table 4.9, and the time profiles are shown in Figure B.6 (Appendix B). The electron transport chain is a key energy generating pathway, where electron transfer is coupled with transmembrane proton translocation and results in a proton gradient to produce ATP via ATP synthase (Weber and Senior 2003). Overall, these eight genes involved with the
electron transport chain had time profiles similar to the time profiles of the heat-shock proteases shown in Figure 4.2, indicating that these eight genes are sensitive to VP1GFP accumulation more so than to the initial synthesis of VP1GFP. Lesley (2002) and Smith (2007) did not observe coordinated changes in expression for electron transport genes. Most likely, the multiple carbon sources present in LB medium mitigated this response. The increased gene expression involved in electron transport processes is consistent with the higher metabolic burden associated with recombinant protein expression (Glick 1995) and additionally the energy-intensive processes needed to cope with misfolded proteins (Bukau and Horwich 1998).
Table 4.9: Eight genes involved in electron transport chain with increased expression levels due to IB stress. Genes involved in electron transport chain that were identified for increased expression due to VP1GFP accumulation. The regression analysis slopes (p ≤ 0.05) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced. C = VP1GFP 60-minute induced vs. GFPCAT 60-minute induced.]

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<td>GFP-CAT</td>
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<td>cytochrome bd-II oxidase, subunit I</td>
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<tr>
<td>b2133</td>
<td>dld</td>
<td>D-lactate dehydrogenase, FAD-binding, NADH independent</td>
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<td>b0975</td>
<td>hyaD</td>
<td>protein involved in processing of HyaA and HyaB proteins</td>
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<td>hyaE</td>
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<td>b2207</td>
<td>napD</td>
<td>assembly protein for periplasmic nitrate reductase</td>
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<td>narW</td>
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<td>nucO</td>
<td>NADH:ubiquinone oxidoreductase, chain G</td>
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Transcription Regulation

Transcription regulation genes control transcription rates. Altered levels of a transcription regulation gene can have a strong impact on several related cellular functions. In this study, 17 transcription regulation genes were identified as differentially expressed. Nine transcription regulation genes were observed to decrease in response to VP1GFP accumulation (atoS, dcuR, hcaR, lexA, lysR, nadR, rcnR, rfaH, and rhaR), and one gene (sdiA) was observed to decrease in expression for the VP1GFP cultures relative to the ethanol-treated VP1GFP cultures. These ten genes with decreased expression due to VP1GFP production are listed in Table 4.10, and the time profiles are shown in Figure B.7 in Appendix B. The time profiles for these transcription regulation genes are similar to the transmembrane transport genes in Figure 4.7A. The differentially expressed transcription regulation genes control several cellular functionalities. The atoS, hcaR, nadR, and rhaR genes all control various catabolic pathways, including catabolic pathways for short-chain fatty acids (atoS), rhamnose (rhaR), NADP (nadR), and hydrocinnamic acid (hcaR). The decreased expression of genes that regulate catabolic pathways supports the observed decreased gene expression for catabolic pathways discussed earlier. Additionally, the lexA gene is known to repress genes involved in DNA repair which are needed for the SOS response; decreased lexA levels would result in higher DNA repair (Fernández de Henestrosa et al. 2000). The decreased expression of lexA may indicate that the accumulation of VP1GFP resulted in DNA damage. Finally, the sdiA gene controls several genes that promote cell division; decreased sdiA levels would inhibit cell division (García-Lara et al. 1996, Keseler et al.
2012). The decreased expression of sdiA may be indicative of cells slowing growth in response to the accumulation of VP1GFP.
Table 4.10: Transcription regulation genes with decreased expression levels due to IB stress. Transcription regulation genes that were identified for decreased expression due to VP1GFP accumulation. The regression analysis slopes (p ≤ 0.05) are indicated (+/-; positive/negative slopes). Fold changes for Tukey’s W pairwise comparisons (p ≤ 0.05) are indicated. [For fold changes, A = VP1GFP 60-minute induced vs. VP1GFP 0-minute uninduced. B = VP1GFP 60-minute induced vs. VP1GFP 60-minute uninduced. C = VP1GFP 60-minute induced vs. GFPCAT 60-minute induced.]

<table>
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<td>nadR</td>
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Three transcription regulation genes were observed to increase due to VP1GFP production, but were unaffected by GFPCAT production (kdgR, nusA, and phoQ). The time profiles for these three genes are similar to the heat-shock proteases (Figure B.8 in Appendix B). The nusA gene plays a key role in ribosomal RNA synthesis by modifying RNA polymerase in order to promote effective transcription of 5S, 16S, and 23S rRNA (Keseler et al 2012, Quan et al 2005). The increased nusA levels may indicate a need for greater protein synthesis in response to VP1GFP accumulation, which is consistent with the increased levels of many protein synthesis-related genes also observed in this study (Tables 4.3 and Figure 4.5ABC). The phoQ gene is known to increase under magnesium starvation (Kato et al. 1999); thus, the increased phoQ levels may indicate that the IB stress caused a greater degree of magnesium utilization. This magnesium starvation may have been brought on by increased protease activity in response to VP1GFP accumulation; at least two proteases in E. coli are magnesium-dependent, and both are associated with IB formation. The clpP gene was observed to increase in expression due to insoluble recombinant protein production in this study and by Lesley et al. (2002) and Smith (2007); clpP encodes for a magnesium-dependent protease (Gross 1996). Additionally, there is at least one more unidentified magnesium-dependent protease in E. coli, where protease function decreases under low magnesium concentration (Jordan and Harcum 2002).
**Effects of Ethanol on IB Stress Response**

The effects of ethanol were also analyzed in this study. Since ethanol is known to improve recombinant protein solubility (Thomas and Baneyx 1997), the stress response to IBs should theoretically be dampened when cells are grown in ethanol. Surprisingly, only nine genes were differentially expressed between the untreated and the ethanol-treated VP1GFP cultures, compared to 126 differentially expressed genes between VP1GFP and GFPCAT cultures. The similar transcriptome profiles between untreated and ethanol-treated VP1GFP cultures seem to indicate that ethanol did not mitigate IB stress dramatically. However, by examining the time profiles for genes identified to be sensitive to VP1GFP production, it is clear that ethanol affects gene expression dynamics and mitigates many of the responses associated with insoluble protein accumulation. These results may suggest that ethanol-treated medium relieves IB stress by altering expression levels of genes related to heat-shock (**Figure 4.2**), ribosomal subunits (**Figure 4.5A**), aminoacyl-tRNA synthetases (**Figure 4.5B**), transmembrane transporters (**Figure 4.7A**), and catabolism genes (**Figure 4.7B**). Due to the lack of statistical differences observed between VP1GFP and ethanol-treated VP1GFP gene expression levels, further research is needed to clarify the effects of ethanol treatment on the stress responses caused by IB formation.
CHAPTER FIVE

CONCLUSION

With *E. coli*, the tendency for recombinant proteins to misfold and form inclusion bodies (IBs) represents a major roadblock to large-scale production processes. This study characterized the dynamic transcriptional behavior of *E. coli* in the early stages of insoluble recombinant protein production. As expected, classical heat-shock genes had increased expression due to IB formation. Additionally, several protein-folding and protease genes not associated with the classical heat-shock response had increased expression levels due to IB formation. The increased levels for genes involved in protein-folding and in proteolysis indicates that the cells attempt to alleviate this stress by increased synthesis of chaperones to assist with protein folding and by increased synthesis of proteases to remove misfolded proteins.

It was also observed that components of the cellular protein synthesis machinery had increased expression due to recombinant protein expression but were more severely affected by IB formation, including ribosomal subunit genes and genes involved with amino acid synthetic pathways. Additionally, the aminoacyl-tRNA synthetase gene increased specifically due to IB formation. Thus, the cells appeared to respond to the early onset of IB stress by increasing cellular protein synthesis machinery. In contrast, several substrate-specific transmembrane transport and catabolism genes were decreased due to IB formation. Additionally, most of the affected transmembrane transport and catabolism genes are regulated by Crp. Confounding these observations is the fact that these substrates were not present in the growth medium. Thus, decreased expression of
these genes may serve to alleviate the metabolic burden required to synthesize the cell membrane components and enzymes needed for import and catabolism of these substrates. The increased level of energy metabolism genes and electron transport chain genes suggests that the cells were attempting to generate more ATP by enhancing the electrochemical gradient required by ATP synthase; additionally, thiamine diphosphate, an essential cofactor for energy metabolism enzymes, production was increased. Also, over a third of genes identified as differentially expressed by IB formation are classified as putative, or unknown, functionality. Taken together, the results of this study indicate that IB formation in recombinant *E. coli* is a complex issue that not only induces the heat-shock chaperones and proteases, but also directly causes the cells to increase protein and energy synthesis while streamlining transport and catabolic processes; furthermore, ethanol treatment mitigates all of these effects. Further study of the differentially expressed putative genes could provide deeper insight into the dynamic response to IB formation.
CHAPTER SIX

FUTURE WORK

While the majority of the objectives of this study have been completed, future work will include microscopy imaging of the VP1GFP and GFPCAT cultures to independently confirm localization of CAT, GFP, and VP1. While VP1 and CAT are both well-studied proteins immunofluorescence imaging provide proof of protein localization in the IB and throughout the cell. Rhonda Powell and Dr. Terri Bruce will conduct the immunofluorescence imaging experiments.
APPENDICES
APPENDIX A

DIFFERENTIALLY EXPRESSED GENES

Table A.1: Genes with differential expression listed in alphabetical order by gene name (318 genes)

Table A.2: Genes with differential expression grouped by ontology terms (318 genes)

For both Table A.1 and Table A.2, the regression analysis slopes ($p \leq 0.05$) are shown (+/−; positive/negative slopes). The Tukey’s W pairwise comparisons fold changes ($p \leq 0.05$) are shown. Gene ontology (GO) terms are based on the classifications shown in Figure 4.3.

For Tukey’s W pairwise comparison fold changes:

A = VP1GFP 60-minute induced versus VP1GFP 0-minute uninduced

B = VP1GFP 60-minute induced versus VP1GFP 60-minute uninduced

C = VP1GFP 60-minute induced versus GFPCAT 60-minute induced

D = Untreated VP1GFP 60-minute induced versus Ethanol-treated VP1GFP 60-minute induced
Table A.1: Genes with differential expression listed in alphabetical order by gene name (318 genes)
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<th>Regression Slope</th>
<th>Gene GO terms</th>
<th>Gene Description</th>
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Table A.2: Genes with differential expression grouped by ontology terms (318 genes)

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APPENDIX B

DYNAMIC GENE PROFILES

The dynamic gene expression profiles for selected gene groups referenced in the thesis for *E. coli* VP1GFP and GFPCAT cultures. VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100, which represents the “average” gene expression intensity on the DNA microarray.
Figure B.1: Gene expression profiles for 35 ribosomal subunit genes that did not meet statistical significance. Gene expression profiles are shown for 35 ribosomal subunit genes that did not meet statistical significance by ANOVA (p ≤ 0.10) and by subsequent regression analysis (p ≤ 0.05) and/or Tukey’s W comparisons (p ≤ 0.05) (average of 35 genes: rplBDFIKMOPSTUVWX, rpmABCEFGR, rpsBCDEIJKQOQRST, and sra). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100.
**Figure B.2:** Gene expression profiles for cofactor synthesis genes that were identified for increased expression due to VP1GFP accumulation. Gene expression profiles are shown for cofactor synthesis genes that were identified for increased expression due to VP1GFP accumulation (average of nine genes: *dxs*, *folC*, *hemL*, *ispEFG*, and *thiCMS*). All genes were identified by ANOVA analysis (p ≤ 0.10) and then were further identified by regression analysis (p ≤ 0.05) and/or by Tukey’s W comparisons (p ≤ 0.05). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100.
Figure B.3: Gene expression profiles for genes of pyruvate dehydrogenase complex and of transketolase. Gene expression profiles are shown for genes of pyruvate dehydrogenase complex (aceEF and lpd) and for transketolase (tktA) genes. VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, △, ♦) are shown. Gene expression levels were normalized to 100.
Figure B.4: Gene expression profiles for nine TCA cycle genes that were not differentially expressed. Gene expression profiles are shown for nine TCA cycle genes that were not differentially expressed (acnA, fumA, icd, lpd, mdh, sdhCD, sucAD). These nine genes did not meet statistical significance by ANOVA (p ≤ 0.10) and by subsequent regression analysis (p ≤ 0.05) and/or Tukey’s W comparisons (p ≤ 0.05). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100.
Figure B.5: Gene expression profiles for ATP synthase genes. Gene expression profiles are shown for ATP synthase genes. The *atp*F gene was identified by ANOVA analysis (p ≤ 0.10) and then further identified by regression analysis (p ≤ 0.05), and the remaining ATP synthase genes did not meet statistical significance (*atp*ABCDEGH).

VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100.
Figure B.6: Gene expression profiles for eight differentially expressed genes involved in the electron transport chain. Gene expression profiles are shown for eight differentially expressed genes (*appC, dld, hyaDE, napD, narW, nuoCG*) involved in the electron transport chain. All genes were identified by ANOVA analysis (p ≤ 0.10) and were further identified by regression analysis (p ≤ 0.05) and/or by Tukey’s W comparisons (p ≤ 0.05). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◇); Uninduced (□, △, ◇) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100.
Figure B.7: Gene expression profiles for ten differentially expressed transcription regulation genes with decreased expression due to VP1GFP production. Gene expression profiles are shown for ten differentially expressed transcription regulation genes with decreased expression in response to VP1GFP production (average of ten genes: atoS, dcuR, hcaR, lexA, lysR, nadR, rcnR, rfaH, rhaR, and sdiA). All genes were identified by ANOVA analysis (p ≤ 0.10) and were further identified by regression analysis (p ≤ 0.05) and/or by Tukey’s W comparisons (p ≤ 0.05). VP1GFP (■, □), GFPCAT (▲, △), and ethanol-treated VP1GFP (♦, ◊); Uninduced (□, △, ◊) and Induced (■, ▲, ♦) are shown. Gene expression levels were normalized to 100.
Figure B.8: Gene expression profiles for three differentially expressed transcription regulation genes with increased expression due to VP1GFP production. Gene expression profiles are shown for three differentially expressed transcription regulation genes with increased expression due to VP1GFP production (kdgR, nusA, phoQ). All genes were identified by ANOVA analysis (p ≤ 0.10) and were further identified by regression analysis (p ≤ 0.05) and/or by Tukey’s W comparisons (p ≤ 0.05). VP1GFP (■,□), GFPCAT (▲, △), and ethanol-treated VP1GFP (◦, ○); Uninduced (□, △, ○) and Induced (■, ▲, ◦) are shown. Gene expression levels were normalized to 100.
REFERENCES


19. de Marco, A, E Deuerling, A Mogk, T Tomoyasu, and B Bukau. "Chaperone-based procedure to increase yields of soluble recombinant proteins produced in E. coli." BMC Biotechnology. 7.32 (2007) (No page numbers from the journal)


