ABSTRACT

Title of Thesis:	USING ANTISENSE MESSENGER RNA TO DOWNREGULATE LON MEDIATED PROTEOLYSIS IN ESCHERICHIA COLI
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The advent of metabolic engineering has instigated the introduction of foreign heterologous proteins into host cells, such as Escherichia coli. However, the metabolic burden incurred by the host cell to produce the desired recombinant protein elicits a cellular stress response that can result in reduced yields and degradation of the desired protein. In, lon is one of the major proteases responsible for this abnormal protein degradation, including recombinant proteins. Consequently, a variety of antisense strategies have been examined and shown to effectively control endogenous gene expression and function in E. coli.

For this investigation we explored using a 300 base pair sequence of the 5' coding region of E. coli lon gene, including the start codon, cloned into both the pSE420 and pTO plasmids in the antisense (reverse) orientation. We examined the ability of lon antisense RNA to inhibit the production of endogenous lon protease and increase the protein yield and activity of a model recombinant protein, organophosphorus hydrolase (OPH). Results indicate that the overproduction of lon antisense did effectively downregulate the production of endogenous lon. In addition, cultures induced for lon antisense also revealed higher OPH protein levels in the first hour of production and a 7-fold higher activity.

USING ANTISENSE MESSENGER RNA TO DOWNREGULATE LON MEDIATED PROTEOLYSIS IN ESCHERICHIA COLI

By

Karen Kimberly Carter

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DEDICATIONS

To my parents, Edward and Beverly Carter, who had faith in me, loved me and supported me throughout this arduous journey. You believed there was an end, even when I could not see it and you did not really understand it. You always kept me going regardless, and for that, I am eternally grateful.

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I would like to thank my advisor Dr. William E. Bentley for his guidance, support, and continued encouragement throughout my research experience. I tell you again, if you were not the way you are I may not have gotten to this point.

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CHAPTER 1

INTRODUCTION

1.1 Recombinant Protein Technology

Increasingly sophisticated molecular techniques have facilitated a shift in focus from the external environment to the physiology of the individual host cell. By employing these techniques to manipulate and transform the host cell's biological systems, scientists can now make and modify novel products for industrial applications, medical and pharmaceutical developments, and environmental practices. Some of the most promising of these contributions have been made in the area of metabolic engineering.

Metabolic engineering is the improvement of cellular activities by manipulation of the enzymatic, transport and regulating functions of the cell to produce new compounds, improve the production of existing compounds, or improve the degradation of a compound (Bailey, 1991; Keasling, 1999). One of most basic metabolic engineering approaches for improving cellular activities is using recombinant DNA technology. Cloning and expression of heterologous proteins can serve to extend existing pathways to obtain new chemical products, or alter posttranslational protein processing, but the protein must avoid

proteolysis by the host organisms' defense mechanisms (Bailey, 1991). Issues such as proteolysis can be addressed with methods like antisense technology, but the technology will still rely on advancements in metabolic applications and analyses (Stephanopoulos, 1998).

Metabolic engineering employs concepts from various scientific backgrounds and will continue to be a crucial tool in our understanding and improvement of microorganism systems. Finally as the demand for recombinant proteins increases, metabolic engineering will continue to make breakthroughs in gene therapy (i.e. engineered vaccines, enzymes, and antibiotics) (Arbige et al., 2001), the causality of disease and drug design (Laskowski and Chan, 2002), and bioremediation (Kang et al., 2002). In addition, these advancements will also serve to broaden the arsenal of informational databases, and create diverse molecular species for enabling science and engineering in the next generation, particularly at the interface of bioinformatics and molecular biology (Klade, 2002; Satagopan and Panageas, 2003).

1.2 E. coli Expression System

The high demand for the production of quality heterologous proteins requires the development of efficient protein expression systems and optimal bioprocesses. For both large- and small-scale

protein productions, Escherichia coli (E. coli) is one of the most widely used host organisms. E. coli is a Gram-negative bacterium utilized largely due to the fact its genetics are extremely well characterized (the entire E. coli genome sequence is readily available). This system also supports low cost, rapid, and high-density cell growth. There are also a large number of mutant host strains and cloning vectors available. The disadvantages encountered using the E. coli system include the inability to glycosylate and phosphorylate recombinant proteins, to post-transcriptionally modify or secrete proteins, and the sometimes avoidable formation of insoluble inclusion bodies. However, improved genetic tools are increasing the fundamental understanding of E. coli transcription, translation, and protein folding, allowing many of these limitations to be addressed (Baneyx, 1999; Gilbert and Albala, 2002).

1.3 Stress Responses

Genetic engineering involves introducing foreign DNA encoding for recombinant proteins into a host organism, which in turn uses its own cellular machinery and resources for the synthesis of the foreign protein. This is often accomplished by positioning the foreign DNA into an expression vector containing a promoter that can be induced by a chemical signal to control gene transcription levels and expression upon transformation into the host (Lewin, 1990; Shuler, 1992). One such

chemical signal is the synthetic galactose analogue, isopropyl-β-D-thiogalactopyranoside (IPTG) that derepresses the lactose (lac) operon depicted in Figure 1 (Dale, 1998).

Inducing foreign protein expression in a host organism creates cellular changes in the cell environment adding to its normal regulatory and housekeeping functions. The host reacts to the extra physical load with its evolved survival mechanism, the stress response (Alberts et al., 1994; Neidhardt et al., 1996). The stress response may elicit the upregulation of additional proteins as part of a regulon, controlled by a regulatory gene, to adapt to and survive the external stress (Gottesman, 1983; Gottesman and Neidhardt, 1984). A regulon consists of an operon network in which a number of operons are associated with the same pathway. In an operon, bacterial genes with related functions are often located together in a group. The group responds to the same promoter site which transcribes all the genes into a single polycistronic mRNA molecule carrying information for all of the proteins in the group (Dale, 1998). Conditions evoking stress responses in E. coli include heat shock (Neidhardt and VanBogelen, 1987; Yamamori and Yura, 1980), oxygen stress (Han et al., 1998), chemical addition (Blom et. al., 1992; Cha et. al., 1999; Van Dyk et al., 1994), DNA damage (e.g. UV exposure), nutrient limitations (Neubauer et. al., 1995), amino acid

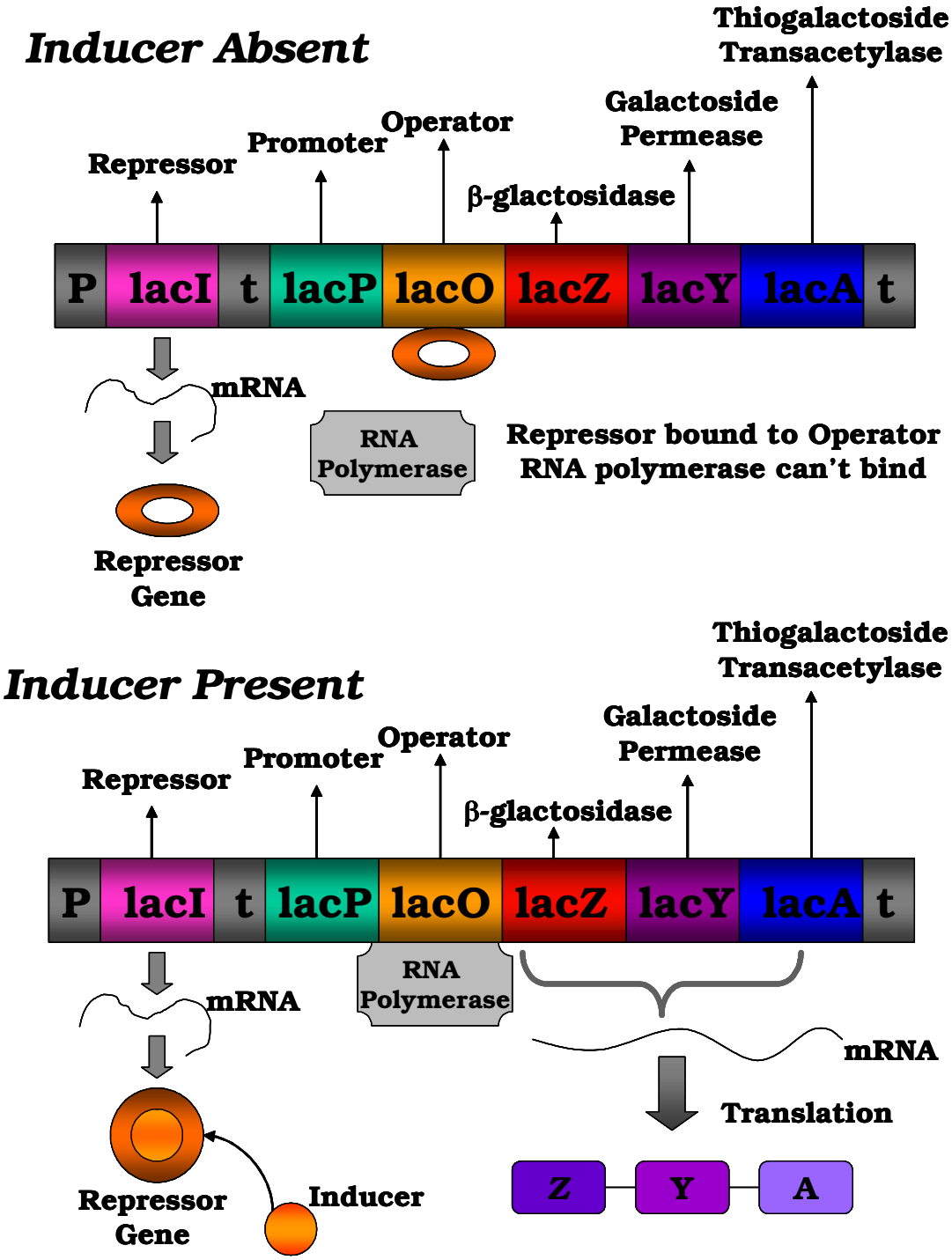


Figure 1. Inducible Lac Operon Promoter System

When the inducer is absent the repressor binds to the operator and there is no gene expression. When inducer is present, the repressor experiences a conformational change allowing the RNA polymerase to bind to the promoter resulting in protein expression.

starvation (Cashel et al., 1996), and recombinant protein induction (Bentley et. al., 1990; Kanemori et al., 1994; Yamada et al., 1988). One or more of the following regulons monitors each of these conditions, the htpR controlled heat shock regulon, the oxyR controlled oxidation stress regulon, the lexA controlled SOS regulon and the stringent regulon (Harcum and Bentley, 1999; VanBogelen et. al; 1987).

1.4 Heat Shock Response and Sigma 32 (σ^{32})

Of the many stress responses, the one often invoked by a variety of external stresses, is the heat shock response. The heat shock response was first discovered in Drosophila upon exposure to an abrupt upward shift in temperature, but was soon found to be a universal response in most organisms in both the prokaryotic and eukaryotic kingdoms (Niederhardt et al., 1996). The heat shock response is often used interchangeably with the stress response to describe general cell stress. The term now refers to other external stresses such as ethanol stress (Niederhardt et al., 1996; Straus et al., 1987) and recombinant protein induction (Bentley et al., 1990; Thomas and Baneyx, 1996) that also elicit the expression of heat shock proteins.

Specifically, the heat shock response in E. coli is regulated by an alternate sigma factor, Sigma 32 (σ^{32}), a product of the rpoH gene (Grossman et al., 1984; Straus et al., 1987; Yura et al., 1993; Yura,

1996). During stress conditions, σ^{32}, a 32-kilodalton (kD) σ subunit, binds to the ribonucleic acid polymerase core (RNAP) (also denoted by E) to form the holoenzyme ($E\sigma^{32}$), instead of the normal binding σ subunit, Sigma 70 (σ^{70}). The holoenzyme is then directed to the consensus promoter sequence transcribing for the heat shock genes (Blaszczak et al., 1995; Cowing et al., 1985; Lesley et al., 1987). Even during normal growth conditions σ^{32} is required for cell growth. E. coli mutants lacking σ^{32} are not viable at temperatures above 20°C (Kusukawa and Yura, 1988; Zhou et al., 1988).

The concentration level and activity of σ^{32} is the limiting step for transcription of heat shock genes and is attributed to an increase in σ^{32} synthesis and/or stability (Grossman et al., 1987; Kanemori et al., 1994; Straus et al., 1987; Straus et al., 1989; Tilly et al., 1989). σ^{32} is normally a very unstable protein, having a half-life of ~1 minute. During stress conditions, this time is increased to ~10 minutes when σ^{32} is stabilized. This increase in σ^{32} synthesis and stability seems to be regulated by a cycle of binding and release of the DnaK, DnaJ, and GrpE chaperone proteins (Blaszczak et al., 1999; Gamer et al, 1992; Gamer et al., 1996; Straus et al., 1990). The cycle begins with an association between DnaJ (J) and σ^{32}. In the presence of this complex DnaK (K), which usually only interacts transiently with σ^{32} in its adenosine triphosphate (ATP)-bound form, stimulates ATP hydrolysis

and targets σ^{32}. DnaK binds to σ^{32} resulting in a new DnaJ-DnaK-σ^{32} complex. GrpE (E) can associate to DnaK and forms a quaternary GrpE-DnaJ-DnaK-σ^{32} complex, upon triggering nucleotide release adenosine diphosphate (ADP). σ^{32} then becomes the degradation target of HflB and HslUV, the genes that encode for the FtsH and ClpQY proteases respectively (Blaszczak et al., 1999; Kanemori et al., 1997). Upon induction of the heat shock response, the cell sequesters DnaK to bind and repair damaged or misfolded proteins, leaving σ^{32} unbound and active to associate with the RNAP core and upregulate the production of heat shock proteins (Bukau, 1993; Craig and Gross, 1991: Yura et al., 1993). After damage repair by protease degradation, DnaK is again free to bind with σ^{32} and the cycle continues as shown in Figure 2 (Herman et al., 1995; Kanemori et al., 1997; Tomoyasu et al., 1995). Other proteins that cooperate with DnaK and DnaJ in preventing protein misfolding and aggregation are GroEL and GroES. GroEL and GroES are chaperonins also transcribed by σ^{32} during mediated heat shock response (Gottesman and Hendrickson, 2000; Gragerov et al., 1992; Parsell and Lindqist, 1993; Kusukawa and Yura, 1988).

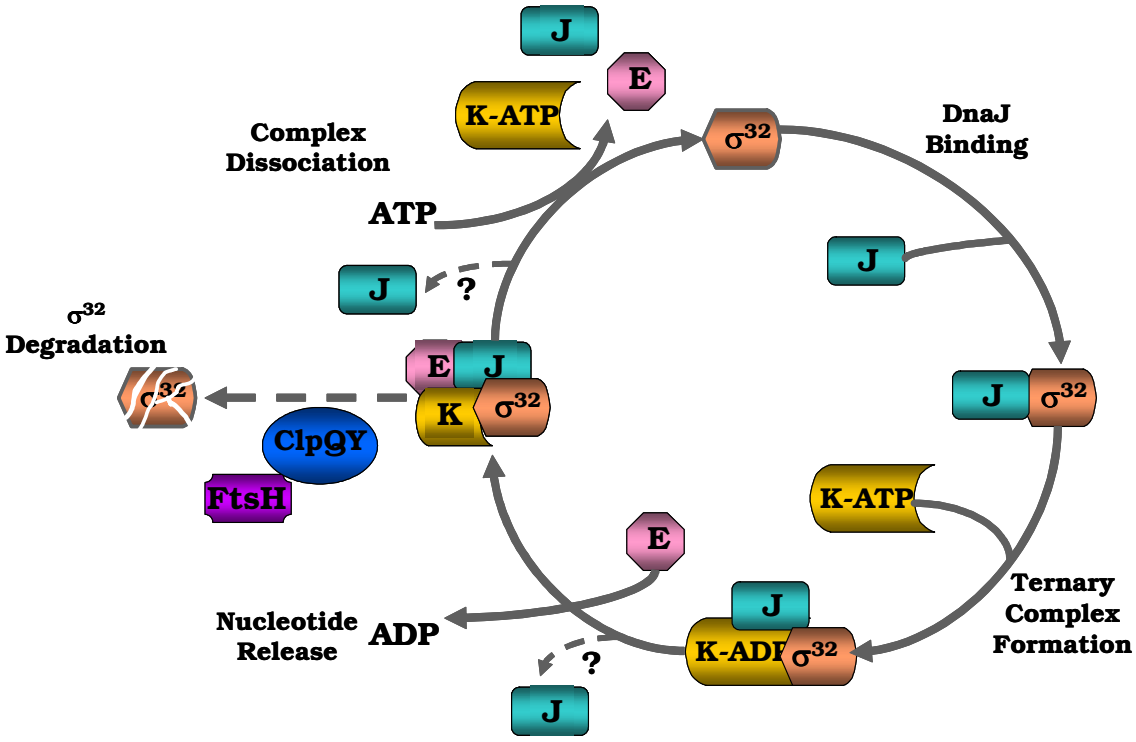


Figure 2: σ^{32}-DnaJ-DnaK-GrpE Binding/Release Cycle

The association begins between DnaJ (J) and σ^{32}. DnaK (K) stimulates ATP hydrolysis and targets and binds to the DnaJ- σ^{32} complex. The resulting DnaJ-DnaK- σ^{32} then associates with GrpE (E) upon nucleotide release resulting in a quaternary complex. σ^{32} is then targeted for degradation by FtsH and ClpQY. When DnaK and DnaJ are sequestered to bind and repair damaged or misfolded proteins, σ^{32} exists in its unbound, active free form it can assemble with RNAP to direct heat shock transcription. (Figure adapted from Gamer et al., 1996, and Kanemori et al., 1997.)

1.5 Lon (La) Protease

In addition to directing increased transcription and sequestering of chaperone proteins (Bukua, 1993; Yura et al., 1993), the heat shock response stimulates the transcription of a host of degradation proteases for cellular proteolysis (Gottesman, 1989; Gottesman and Maurizi, 1992). Proteolysis is required for maintaining homeostasis in the cellular environment, regulating levels of specific proteins and eliminating damaged, unstable and abnormal proteins (e.g. from overexpression of cloned foreign recombinant proteins) (Maurizi, 1992). A list of some proteases in E. coli is included in Table 1 (Gottesman, 1996; Maurizi, 1992). "Abnormal" proteins also include incomplete peptides, misfolded proteins and even native proteins during stress responses. The accumulation of inclusion bodies occurs when synthesis of abnormal proteins surpasses protease degradation (Goldberg, 1976; Thomas and Baneyx, 1996).

Lon (La) was the first ATP dependent protease discovered in E. coli. Along with the Clp proteases, Lon has the primary role of degrading 70-80% of the cells abnormal proteins, as well as, a few unstable regulatory proteins (Chin et al., 1988; Goff and Goldberg, 1987; Maurizi, 1992). This group of regulatory proteins is comprised of SulA, which inhibits cell division during the SOS response to DNA damage (Sonezaki et al., 1995), RcsA, which positively regulates capsule

Table 1. ATP dependent proteases of Escherichia coli.

Protease	Substrate	ATP Energy Dependency	Type
Lon (La)	SulA, RcsA, λN, abnormal proteins	Yes	Serine
ClpAP (Ti, Clp)	abnormal proteins	Yes	Serine
ClpXP	RpoS, λO,	Yes	Serine
ClpQY	RpoH, abnormal proteins	Yes	Serine
FtsH (HflB)	RpoH, λcII	Yes	Metalloprotease

synthesis (Torres-Cabassa and Gottesman, 1987), and the lambda N (λN) protein (Maurizi, 1987). In addition to reduced cell viability, lon mutants also display several phenotypic alterations, such as, overproduction of capsular polysaccharide (mucoidy), increased UV and ionizing radiation sensitivity, filament formation resulting from cell division inhibition, reduced lysogenation by bacteriophages lambda and P1, and reduced degradation of both normal and abnormal proteins. These alterations are directly attributed to the stability of the aforementioned regulatory proteins (Fu et al., 1997; Gayda et al., 1985; Maurizi, 1992; Phillips et al., 1984). Lon has the ability to specifically select these substrates from among hundreds of other nonsubstrate proteins in a cell. This discriminator activity is accredited to a conserved domain in the lon structure which is still being investigated (Ebel et al., 1999).

Lon is a tetramer composed of four identical subunits of 87 kilodaltons (kD), each consisting of three functional domains: the proteolytic C-terminal containing a catalytically active serine residue, a centrally located ATPase binding domain, and a highly charged N-terminal domain with a yet to be determined function (Ebel et al., 1999; Vasilyeva et al., 2002). Lon requires ATP hydrolysis for degradation of large proteins; however, ATP hydrolysis is not necessary for shorter polypeptides (Maurizi, 1992; Menon, 1987). ATP is thought to facilitate

the unfolding of protein substrates and to transport them to the proteolytic chamber (vanMelderen and Gottesman, 1999). Lon hydrolyzes two to four ATPs for each peptide bond cleaved in the protein substrate (Chin et al., 1988; Goldberg, 1992). Degradation by lon generates polypeptides of 5-20 amino acids (aa), which are further degraded by other ATP independent cellular proteases and peptidases (Menon et Goldberg, 1987). Lon was long assumed to have nonspecific DNA-binding activity, but mounting evidence suggests that it is site specific (Fu et al., 1997; Hilfinger et al., 1993).

1.6 Protein Synthesis

Gene expression, or protein synthesis, requires the transfer of information encoded within the DNA genome into proteins via RNA. This process occurs in two steps, transcription (DNA to RNA) and translation (RNA to protein). Double stranded DNA is the cell component that carries all of the cells genetic information. DNA is composed of 2' deoxyribose sugar that forms a nucleotide polymer chain via linking alternating phosphate residues that create phosphodiester bonds. A DNA double helical structure is formed when two nucleotide chains are coupled through complementary base pairing. The two DNA strands run in opposite directions of each other (5’ to 3’ and 3’ to 5’). Each nucleotide, in addition to a sugar and a phosphate, also contains

a base purine (adenosine (A) or guanine (G)) or pyrimidine (thymine (Y) or cytosine (C)). The double helix can only maintain its correct conformation when two DNA strands are linked by hydrogen bonding between complementary bases, A and T, or G and C (one purine opposite one pyrimidine at each position) (Alberts et al., 1994; Dale, 1998).

During transcription the information encoded in one of the DNA strands (the coding strand) is synthesized into a single mRNA strand. The RNA is similar to the copied DNA but uses a sugar ribose instead of the deoxyribose. In addition, RNA contains the base uracil (U) instead of thymine (T). RNA transcription begins when an enzyme, RNA polymerase (RNAP or E), recognizes and binds to a promoter site. The RNAP opens short regions of the DNA helix and uses one strand for a template, base pairing with incoming ribonucleotide triphosphate monomers that form the mRNA chains as illustrated in Figure 3. This process continues in the 5' to 3' direction until the RNAP reaches a termination signal. RNA polymerase consists of five subunits, the coenzyme ($\alpha_2\beta\beta'$), and a sigma subunit (σ), to create the holoenzyme ($E\sigma$). It is the σ subunit that directs the holoenzyme to the specific site to transcribe genes for the cell's current conditions. For example, σ^{70}, directs the holoenzyme to transcribe the genes necessary for normal cell

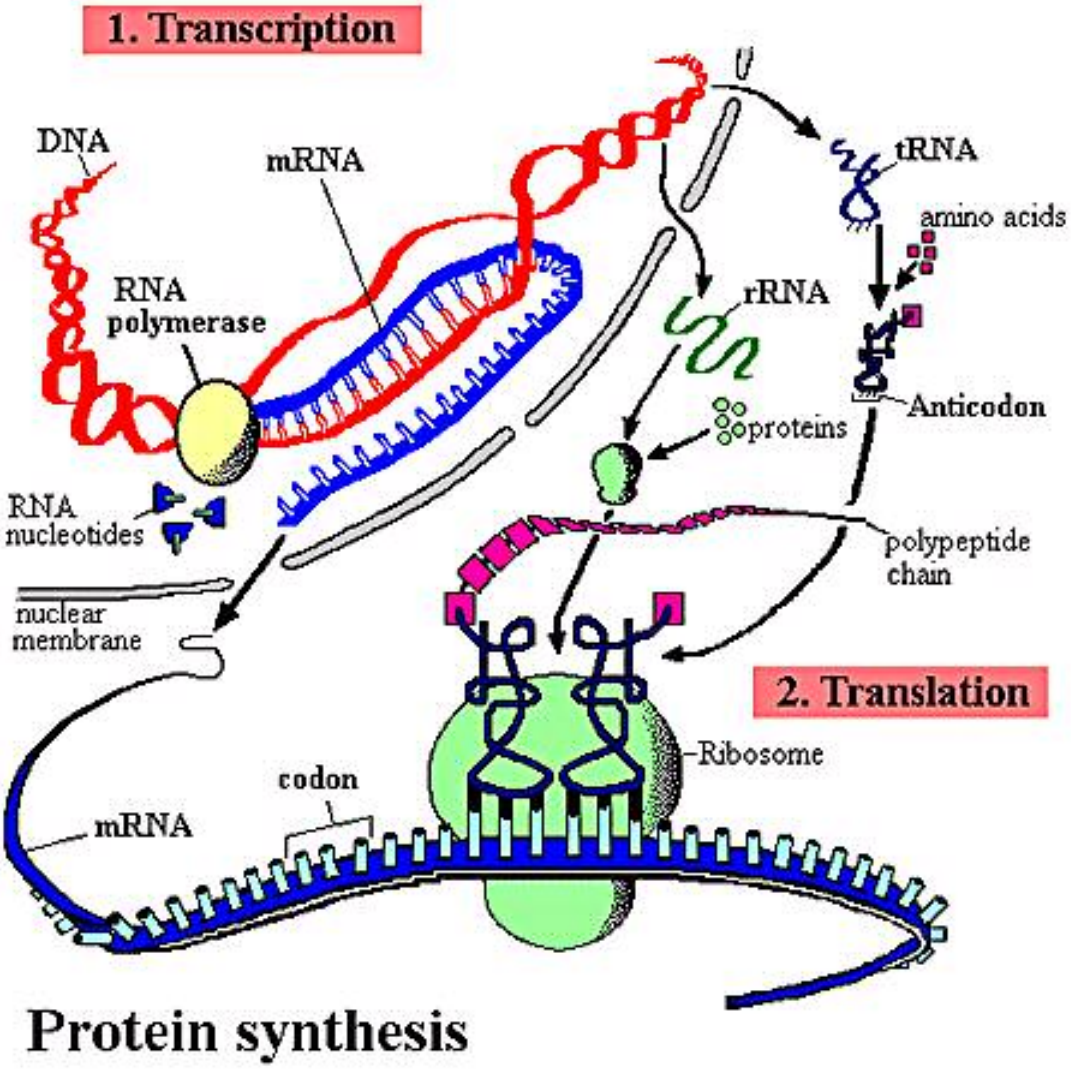


Figure 3: Protein Synthesis

In transcription DNA is the template for RNA synthesis. The mRNA is then translated into a protein. Figure reprinted from www.accessexcellence.org/AB/GG/protein_synthesis.html.

functions. However, during heat shock, σ^{32}, binds to E to transcribe heat shock genes.

Every three bases in DNA correspond to one of 20 amino acids (aa) that synthesize proteins. These triplets are called codons. These codons transcribe into mRNA, which transports the information to the ribosomes. Ribosomes are structures composed of RNA molecules and ribosomal proteins. Mediated by the ribosomes, RNA undergoes translation from nucleotides to amino acids. The ribosome attaches to a site near the 5' end of the mRNA called the ribosomal binding site (RBS). The amino acids are brought to the ribosomes linked to a transfer RNA (tRNA), with a corresponding anticodon that also recognizes each specific codon. There is at least one tRNA species specific for each amino acid. The aa is added to the tRNA by a special enzyme, aminoacyl-tRNA sythetase. The mRNA and tRNA lock onto the ribosome so that the codon and anticodon can join via complementary base pairing rules. Amino acids continue to be added this way moving along the mRNA, forming a peptide bond to the previous amino acid (the tRNA is released after the peptide bond is formed) until a stop signal is encountered. The messenger RNA falls of the ribosome and the new protein is released.

1.7 Antisense Technology

Antisense RNA was one of the first methods of RNA interference (RNAi), in which an RNA molecule specifically interferes with gene expression. Antisense RNA (asRNA) has been shown to effectively be utilized in Escherichia coli as a biological regulatory control factor (Mirochnitchenko and Inouye, 1999; Murray, 1992). Antisense RNAs are small (usually less than 200nt), diffusible transcripts that pair to specific complementary target RNAs and control their function and expression (Inouye, 1988; Simons, 1988). Antisense is based on basic Watson-Crick base pairing hybridization rules, binding the asRNA to its target RNA. Control includes, but is not limited to, plasmid replication, phage development, and transposition. These asRNAs can be transcribed from the same loci (cis-coded regulators) as the target genes or from a different loci (trans-coded regulators). Examples of naturally occurring antisense RNAs in prokaryotes include plasmid R1, which is controlled by an antisense RNA, CopA that binds to its target CopT, inhibiting the synthesis of the replication intiator protein RepA (Kolb et al., 2000; Malmgren et al., 1997) and sok. Sok is an antisense molecule that regulates mok mRNA in F plasmids. Mok is required for hok expression, which if expressed, kills bacterial cells without plasmids (Nellen and Lichtenstein, 1993).

Naturally occurring antisense RNAs have served as models for creating artificial antisense systems. Most of the natural regulatory asRNAs are complementary to the 5'-untranslated region including the Shine-Delgarno sequence and/or coding regions of the target mRNA, resulting in translation inhibition or mRNA destabilization (Ellison et al., 1985; Mirochnitchenko and Inouye, 1999). Artificial antisense in prokaryotic systems function by taking the desired gene and inserting it into an expression vector (e.g. plasmid) in the reverse or opposite orientation. The resulting mRNA transcript will be the complement to the target gene as shown in Figure 4. Investigations have revealed that extensive complementary of the antisense transcript with only the coding portion of the target RNA is sufficient for substantial inhibition of protein synthesis (though maximal inhibition can also be achieved including the RBS) and that asRNAs must be synthesized in a ratio of at least 100:1 to target RNA for maximal inhibition (Ellison et al., 1985; Daugherty et al., 1989). The efficiency of antisense control depends on the binding between the antisense and target RNA which is subsequently dependent on the loop-loop/loop-stem interactions of the mRNA secondary structures. These interactions determine the formation of the transient kissing complex (which alone appears to be sufficient for control) that serves as the initial step for the ultimate full RNA-RNA duplex (Franch et al., 1999; Deconge et al., 2000; Hjalt and

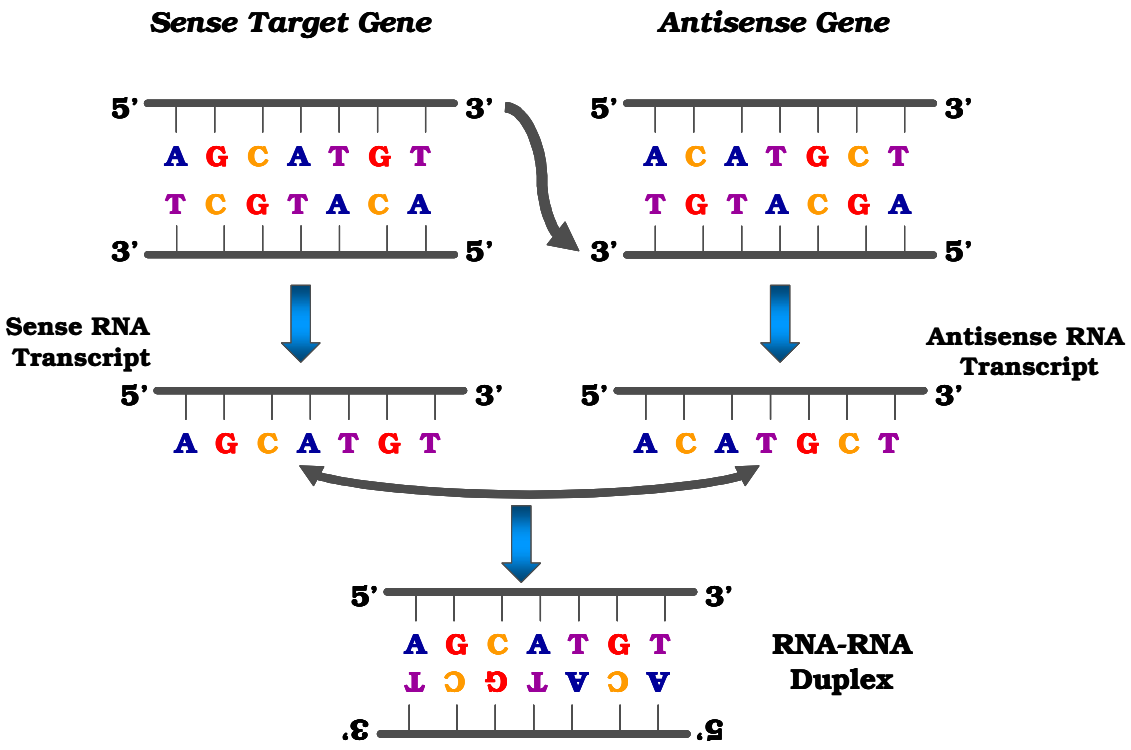


Figure 4: Antisense Mechanism

Antisense systems require taking the desired target gene and inserting the gene in the antisense orientation. The endogenous sense gene will produce a sense RNA transcript and the antisense gene will produce an antisense RNA transcript. The sense and antisense transcripts will interact to form a kissing complex that will ultimately result in a complete RNA-RNA duplex.

Wagner, 1992; Kolb et al., 2001a; Kolb et al., 2001b; Patzel and Sczakiel, 1999). These structural requirements continue to be investigated for insights to create more effective antisense systems. Antisense is even being applied to regulate heat shock response (Chistyakova and Antonov, 1990; Srivastava et al., 2000). The advantage of the antisense system is that you can specifically target any gene. This is especially usesful in prokaryotic systems where genes exists in operons and share promoters and other upstream genes. The disadvantage is that the a gene can only be targeted if its sequence is available.

1.8 Orgnophosphorus Hydrolase (OPH)

There are a variety of useful model proteins found in the literature. The model protein chosen for this study needed to have an antibody available, as well as, a well-defined activity assay. The protein chosen was organophosphorus hydrolase (OPH). OPH is a zinc metalloenzyme with zinc ions in the active site obtained from Pseudomonas diminuta or Flavobacterium. Application of OPH for bioremediation is currently of great interest due to its ability to catalyze the hydrolysis of a broad range of organophosphorus (OP) pesticides (Parathion, Paraoxon, Coumaphos, and Diazinon), as well as, chemical warfare agents (Soman, Sarin and VX [O-ethyl s- (2-

diisopropylaminoethyl) methylphosphonothiolate]) (Dumas et al., 1989; Grimsley et al., 1988). The mechanism of hydrolysis involves the breaking of various phosphorus-ester bonds (P-O, P-F, P-CN, and P-S bonds) via the addition of an activated water molecule at the phosphorus center (Lai et al., 1995; Lewis et al., 1988). In addition, many products of OPH hydrolysis can be easily monitored with enzymatic assay methods utilizing a simple spectrophotometer (Dumas et al., 1989).

1.9 Project Scope

The scope of this project is to investigate the use of antisense as a tool to downregulate the stress response in E. coli due to heterologous protein induction, namely inhibiting proteolytic degradation by intracellular proteases. Proteolysis has been shown to have a significant impact of the accumulation and final yields of recombinant proteins. Inhibition of lon proteolytic activity may result in an increase in recombinant protein production and activity. The lon (la) protease will be targeted due to its prominent role in the degradation of abnormal proteins. The objectives for this study were to:

1. Create a vector, which expresses lon antisense that effectively binds to the endogenous lon mRNA produced during stress

conditions in Escherichia coli, thus inhibiting its proteolytic activity.

2. Monitor the effects of the lon antisense on endogenous lon sense messenger RNA (mRNA) and the production yield and activity of a recombinant protein. The model recombinant protein expressed was organophosphorus hydrolase (OPH).

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains

For construction of the antisense plasmids E. coli TOP10 (F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG) (Invitrogen, Carlsbad) was used. E. coli host strain JM105 (F' [traD36 $proAB^+$ $lacI^q$ lacZ?M15] supE endA sbcB15 hsdR4 rpsL thi Δ(lac-proAB)) was used for expression in all experiments.

2.2 Antisense Plasmid Construction

The entire lon gene was amplified from the Escherichia coli K-12 genome using the polymerase chain reaction (PCR) and the following primers: 5' primer ATG AAT CCT GAG CGT TCT GAA CGC ATT GAA and the 3' primer CTA TTT TGC AGT CAC AAC CTG CAT ACC AG. This PCR product was cloned into the pCR®-Blunt vector (Invitrogen, Carlsbad, CA). Primers were then designed to amplify a 300 base pair segment of the 5' coding region of the lon gene from the pCR®-Blunt vector, incorporating an AflII restriction site on the 5' end and an NcoI restriction site on the 3' end. The 5' primer sequence used was CCC

CTT AAG ATG AAT CCT GAG CGT TCT GAA CGC ATT GAA and the 3' primer sequence used was CAT GCC ATG GAA TAC GCG CGC GCT GTA ACC. The PCR segment was then isolated and purified using a Gel Extraction Kit (Qiagen, Valencia, CA). The AflII and NcoI flanking restriction sites allowed the lon segment to be inserted in the antisense orientation between the corresponding sites in the pSE420 plasmid (Invitrogen, Carlsbad, CA) behind the trc promoter. This plasmid was called pSE420lonαs as shown in Figure 5A. New primers were then designed to amplify the promoter, antisense, and termination sequences from the pSE420lonas vector, and incorporate an NdeI restriction site on the 5' end of the promoter-antisense-termination sequence and an SphI restriction site on the 3' end. The 5' primer sequence used was TTC ATT CAT ATG CGA CAT CAT AAC GGT TCT GGC AAA TAT TC and the 3' primer sequence used was TTA TAT GCA TGC GCG GAT TTG TCC TAC TCA GGA GAG CG. The resulting segment was purified as described previously and then inserted into the corresponding restriction sites on the pTO vector. The pTO vector overexpresses the recombinant protein organophosphorus hydrolase (OPH) and was created as described by Wu et al. (2000). The opd gene, which expresses OPH, is attached to a histidined tag and is also under the control of a trc promoter. This design allowed each protein to be

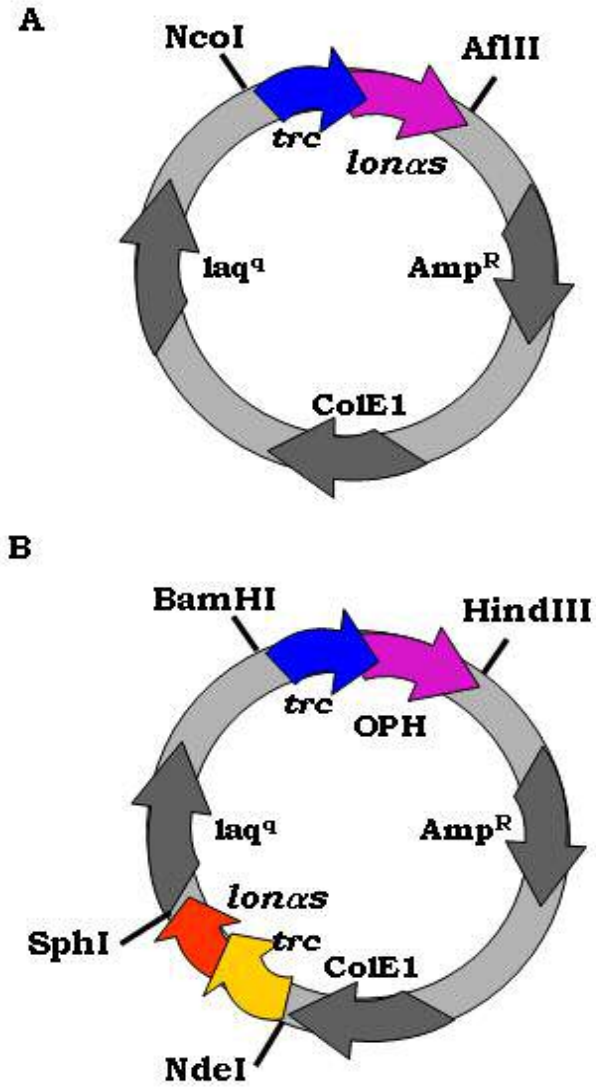


Figure 5: Antisense Vector Constructs

Construction maps of lon antisense expression plasmid pSE420αs (A) and lonαs-OPH expression plasmid pTOlonαs (B). translated independently. The resulting vector was called

translated independently. The resulting vector was called pTOlonαs as shown in Figure 5B.

2.3 Media and Culture Preparations

All shaker flask experiments were carried out in minimal M9 media (Table 2) and prepared according to Rodriguez and Tait (1983). Media was supplemented with 50 μg/mL ampicillin and 0.17 μg/mL thiamine. Overnight cultures were prepared from a 1 mL vial of -80°C E. coli freezer stock grown in 50 mL of media in a 250 mL Erlenmeyer flask at 37°C in a New Brunswick Scientific Series 25 air incubator (Edison, NJ) shaking at 250 rpm. Experimental cultures were prepared from a 5% (v/v) inoculum of overnight culture for a final working volume of 210 mL in a 500 mL Erlenmeyer flask. All experimental cultures were grown at 37°C in a New Brunswick Scientific Gyrotory® water bath shaker (Edison, NJ) shaking at 250 rpm. Cultures were grown to an OD_{600} of ~0.3, at which time they were stressed with ethanol (4% v/v) and/or induced with 1mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, MO).

2.3 RNA Extraction and Dot Blot Analysis

10mL cell culture samples were collected at selected time points, frozen with liquid nitrogen and stored at -80°C. Total RNA was isolated

Table 2. M9 Minimal Media Recipe

Components	
Sterile 10X Salt*	100.0 mL
20% Glucose	20.0 mL
0.01 M $CaCl_2$	10.0 mL
0.1 M $MgSO_4$	10.0 mL
H_2O	860.0 mL
*Salt Mix	10X
Na_2HPO_4	70 gm
KH_2PO_4	30 gm
NaCl	5 gm
NH_4Cl	10 gm
H_20	1000 mL

** E. coli JM105 requires supplemental thiamine (0.166 µg/mL).

following the Ultraspec™ RNA Isolation Sytem (Biotecx Laboratories, Inc., Houston, TX). The RNA was resuspended in 50 µL of 1 mM Tris buffer and total RNA concentration was determined by measuring the RNA resuspension at OD_{260}. The presence of RNA was confirmed by running briefly on a 1% agarose gel. Five µg of total RNA was added to 10 µL 10XMESA (Sigma Chemical Co., St. Louis, MO), 20 µL formaldehyde (37% aqueous solution; Sigma Chemical Co., St. Louis, MO) and 50 µL formamide (Sigma Chemical Co., St. Louis, MO), to a total volume of 100 µL. Samples were denatured at 67°C for 10 minutes and chilled on ice. While the samples were on ice 200 µL of 10 X SSC was used to spot rinse the nitrocellulose membrane (Roche Diagnostics Corporation, Indianapolis, IN). 150 µl of 20 X SSC (Sigma) was added to the denatured sample and the RNA was blotted on the nitrocellulose membrane using a dot blot manifold (Schleicher & Schuell, Inc., Keene, NH). The spots were rinsed with 400 µL DEPC-treated water and the membrane was baked at 80°C for 30 minutes. The probes were made prior to hybridization using the DIG Oligonucleotide End Tailing Kit (Roche Diagnostics Corporation, Indianapolis, IN) and frozen at -20°C. Both the lon sense and antisense probes were 40 base pairs in length and labeled with digoxigenin at the 3' end. The oligonucleotide sequence for the lon sense probe was TAC TTA GGA CTC GCA AGA CTT GCG TAA CTT TAG GGG CAT A and the

lon antisense probe was ATG AAT CCT GAG CGT TCT GAA CGC ATT GAA ATC CCC GTA T. The baked membranes were prehybridized in prewarmed (50°C) hybridization buffer (DIG hybridization buffer, Roche Diagnostics, Indianapolis, IN) for 30 minutes at 50°C. The prehybridization buffer was poured off of the membrane and the probe prepared in hybridization buffer was added. Two µl of probe was added to each mL of prewarmed (50°C) hybridization buffer. The membrane was incubated with this hybridization buffer for 16 hours at 50°C. After the 16 hour incubation, the hybridization buffer was poured off the membrane, it was washed 2X 5 minutes in 2X SSC+0.1% SDS (20% (w/v) sodium dodecyl sulfate (Quality Biological, Inc., Gaithersburg, MD) at room temperature and then 2X 15 min in 0.5X SSC+0.1% SDS at 50°C with slow shaking. The membrane was rinsed in 1X washing buffer (DIG Washing Buffer Kit, Roche Diagnostics, Indianapolis, IN) and then incubated 30 minutes in blocking solution (DIG Blocking Solution in 1X Maleic acid buffer) and poured off. . The anti-DIG-antibody conjugate was prepared in 1:5000 dilution in 20 mL of fresh blocking solution, added to the membrane and incubated for 30 minutes. The membrane was then washed 2X15 minutes in 1X washing buffer and equilibrated in 1X detection buffer (DIG Detection Buffer) for 5 minutes. The colored substrate solution was prepared (200 µl of NBT/BCIP in 10 ml of 1X detection solution) and added to the

membrane. The color was allowed to develop for 30 minutes to 16 hours and the reaction was stopped with distilled water. The dried membranes were scanned using a Hewlett Packard ScanJet 4c Scanner and accompanying software and analyzed using NIH Imaging Software.

2.4 Northern Blots

Total RNA for Northern Blots was harvested and isolated as described above. Sample were prepared by adding 10 µg of total RNA to 1.5 µL 10XMESA (Sigma Chemical Co., St. Louis, MO), 2.5 µL formaldehyde (37% aquaeous solution; Sigma Chemical Co., St. Louis, MO) and 6.5 µL formamide (Sigma Chemical Co., St. Louis, MO), to a total volume of 18 µL. Samples were denatured at 67°C for 10 minutes and chilled on ice. 2 µL of RNA Loading Buffer (Quality Biological, Inc., Gaithersburg, MD) was added to the sample and it was loaded into the wells of a 2% (m/v) agarose denaturing gel prepared in 1X MOPS buffer (Quality Biological, Inc., Gaithersburg, MD) containing 2% formaldehyde. The gel was run at 3-4 V/cm until the RNAs were well separated. The gel was soaked 2X 30 minutes in 0.5X TBE buffer to remove the formaldehyde and electroblotted to a nylon membrane using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The membrane was rinsed briefly in 2X SSC and processed in the same manner as the dot blots described in 2.3.

2.5 SDS-PAGE and Western Blots

Cell culture volumes equivalent to 1 mL at an OD_{600} of two were collected at selected time points, frozen with liquid nitrogen and stored at -80°C. The samples were thawed and centrifuged for 5 minutes at 4°C and 7,500 x g. The pellets were resuspended in SDS-PAGE sample buffer (0.5 M Tris-HCL (pH 6.8), 10% glycerol, 5% sodium dodecyl sulfate (SDS), 5% B-mercaptoethanol, 0.25% bromophenol blue). The samples in buffer were heated to 100°C for five minutes and vortexed. 10 µL samples were loaded into a 12.5% running and 4% stacking gel and run by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run at 120 V until the dye front ran off the gel. Each gel was soaked for 30 minutes in Bjerrum and Schafer-Nielson transfer buffer (48 mM Tris, 39 mM glycine, and 20% methanol; pH 9.2) and then blotted to a nitrocellulose membrane (BioRad) using a BioRad Trans-Blot SD Semi-Dry Transfer Cell for 20 minutes at 10 V and an additional 20 minutes at 20 V. The membranes were washed 2X 15 minutes in Tris-Buffered Saline (TBS; 20 mM Tris-HCl and 500 mM NaCl; pH 7.5) after blotting and incubated overnight in blocking solution (5% non-fat dry milk in TBS) at 4°C. The membranes were then washed 2X 15 minutes in TTBS wash solution (TBS plus 0.5mL Tween 20). Anti-histidine monoclonal antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:3000 was used to probe the gels for the N-

terminal hexahistadine tag on OPH for 1.5 hours. After the 1.5-hour incubation, the primary antibody was removed and the membranes were washed again 2X 15 minutes in TTBS. The membranes were incubated for 1.5 hours in goat-anti-mouse antibody diluted 1:4000 (Sigma Chemical Co., St. Louis, MO). The membranes were washed 2X 15 minute in TTBS and then 2X 15 minutes in TBS to remove the Tween 20. The membranes were finally incubated in SigmaFast color development reagent (Sigma Chemical Co., St. Louis, MO) containing 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/ NBT) tablets until the desired color development was achieved (usually 5–30 minutes). The membranes were rinsed 5 minutes in water to stop the development and then allowed to dry. The dried membranes were scanned using a Hewlett Packard ScanJet 4c Scanner and accompanying software and analyzed using NIH Imaging Software.

2.6 OPH Activity Assay

Two-milliliter cell culture samples were collected at selected time points, frozen with liquid nitrogen and stored at -80°C. The samples were thawed and centrifuged for 5 minutes at 4°C and 7,500 x g. The pellets were resuspended in one mL of PBS (20 mM sodium phosphate and 500 mM sodium chloride, pH 8.5). Each sample was sonicated for 30 seconds (half second on and 5 seconds off) with a Fisher Scientific

550 Sonic Dismembrator. The samples were spun down for 5 minutes at 4°C and 10,000 x g. Room temperature supernatant samples (75 µL) were added to 25 µL of 1 mM paraoxon (Sigma) and 900 µL of PBS. The absorbance of each sample was measure at 400 nm, for which the extinction coefficient is 17, 000 $M^{-1}cm^{-1}$. Activities were expressed in µmoles of paraoxon hydrolyzed to diethyl phosphate and p-nitrophenol per minute per OD_{600} of whole cells.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 pSE420 Vector lonαs Gene Expression

Production of abnormal proteins by heat stress, chemical addition, or foreign proteins can induce a heat shock response and the production of heat shock proteins (Bukua, 1993; Georgopoulos et al., 1996; Goff et al., 1985; Yura et al., 1993). Many heat shock proteins are molecular chaperones, chaperonins, or ATP-dependent proteases (Gottesman, 1996; Gottesman and Maurizi, 1992; Maurizi, 1992). One such protease is the lon gene encoded protease Lon/La. Along with Clp proteases, lon plays a major role in cell proteolysis and the degradation of various abnormal polypetides, including cloned foreign proteins (Chin et al., 1988; Goff and Goldberg, 1987; Maurizi, 1992).

A 300 bp segment of the E. coli lon gene, including the start codon, was cloned in the antisense (reverse) orientation in the pSE420 vector (Invitrogen) under the control of the IPTG inducible trc promoter. This plasmid was then named pSE420lonαs as shown in Figure 5 in Chapter 2. To investigate whether this construct abundantly overexpresses lon antisense , E. coli JM105 was transformed with

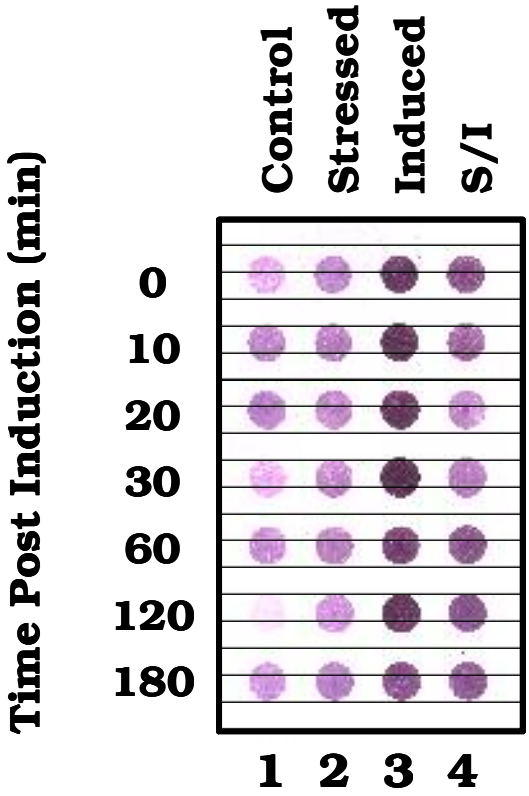


Figure 6: Dot Blot of JM105 pSE420 lonαs Expressing Cultures

pSE420lonαs. Cultures were stressed with 4% (v/v) ethanol, induced with 1mM IPTG, or stressed/induced. Dot blots were performed and the membranes were probed for lon antisense mRNA. Figure 6 verifies the abundant overexpression of lon antisense mRNA in the induced cultures shown in lanes 3 and 4 as compared to the control and ethanol stressed only cultures in lanes 1 and 2. The induced only cultures, lane 3, showed significant expression immediately upon induction (time zero is immediately after IPTG addition) and remained maximal throughout the entire experiment. Lane 4 shows the stressed/induced cultures, which from 0 to 60 minutes expressed only slightly more than the control and stressed only cultures, but from 60 to 120 minutes exhibited expression close to the induced cultures alone. The presence of detectable signals in the control and stressed cultures indicates that lon antisense is being expressed constitutively. This is most likely due to the use of the trc promoter, a lac derivative, which is sometimes prone to leakiness.

3.2 pTOlonαs Growth Profile

The second objective in creating the pSE420lonαs vector was to serve as a preliminary plasmid to facilitate the cloning of the pTOlonαs vector. In addition to using this vector to assure that lon antisense could effectively be expressed, pSE420lonαs also provided the lon

antisense insertion with its own promoter and termination sequences, for subsequent cloning into the pTO vector. The resulting pTOlonαs vector served to explore the effectiveness of lon antisense in inhibiting lon protease degradation of the recombinant protein OPH, also under the control of the trc promoter.

E. coli JM105 was transformed with pTOlonαs. As stated previously in Chapter 2 cultures were stressed with 4% (v/v) ethanol, induced with 1mM IPTG, or stressed/induced. One-milliliter samples were taken of each of the four cultures immediately after ethanol stress and/or IPTG induction and at every ten minutes until 30 minutes post stress/induction. After the 30-minute time point, the next samples were taken at the 60-minute time point and every 60 minutes thereafter until 6 hours after stress/induction. The optical density at a wavelength of 600nm was taken for each sample.

Figure 7 shows the growth time course of the pTOlonαs cultures post stress/induction. The plotted time points correspond to the average of triplicate experiments. The OD_{600} readings for each of the four cultures – control (neither stressed nor induced), stressed, induced, stressed/induced followed very closely for the first 30 minutes, after which the curves separate into two groups. The first group is the control and induced cultures, which continued to steadily increase in value. The second, the stressed and stressed/induced cultures, began

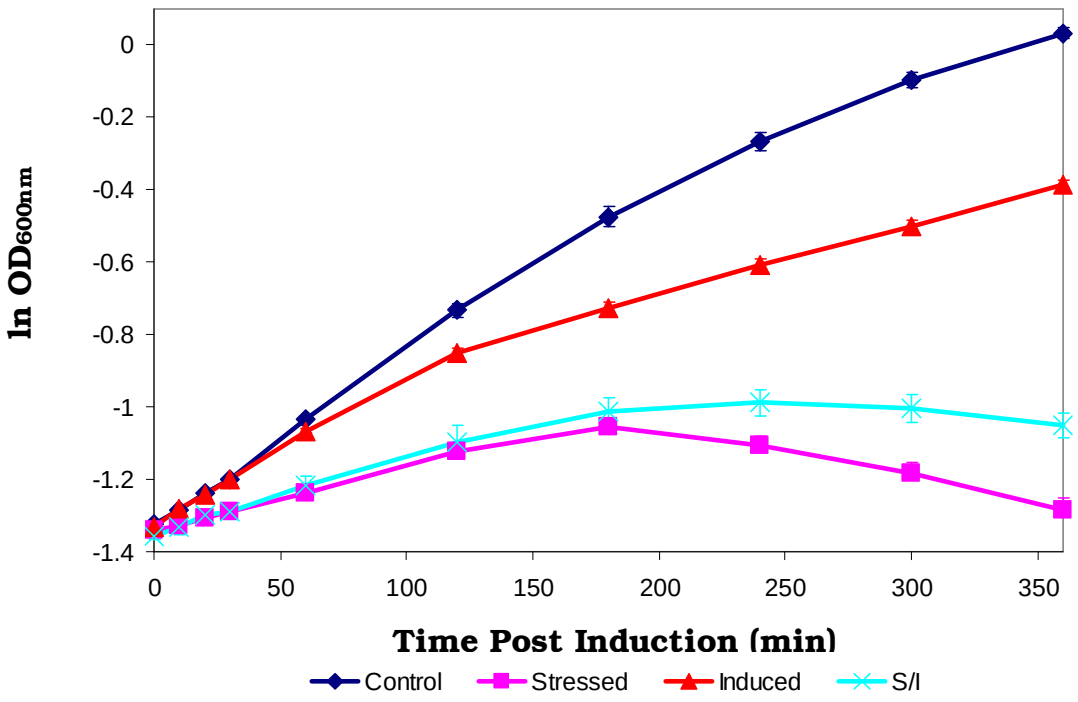


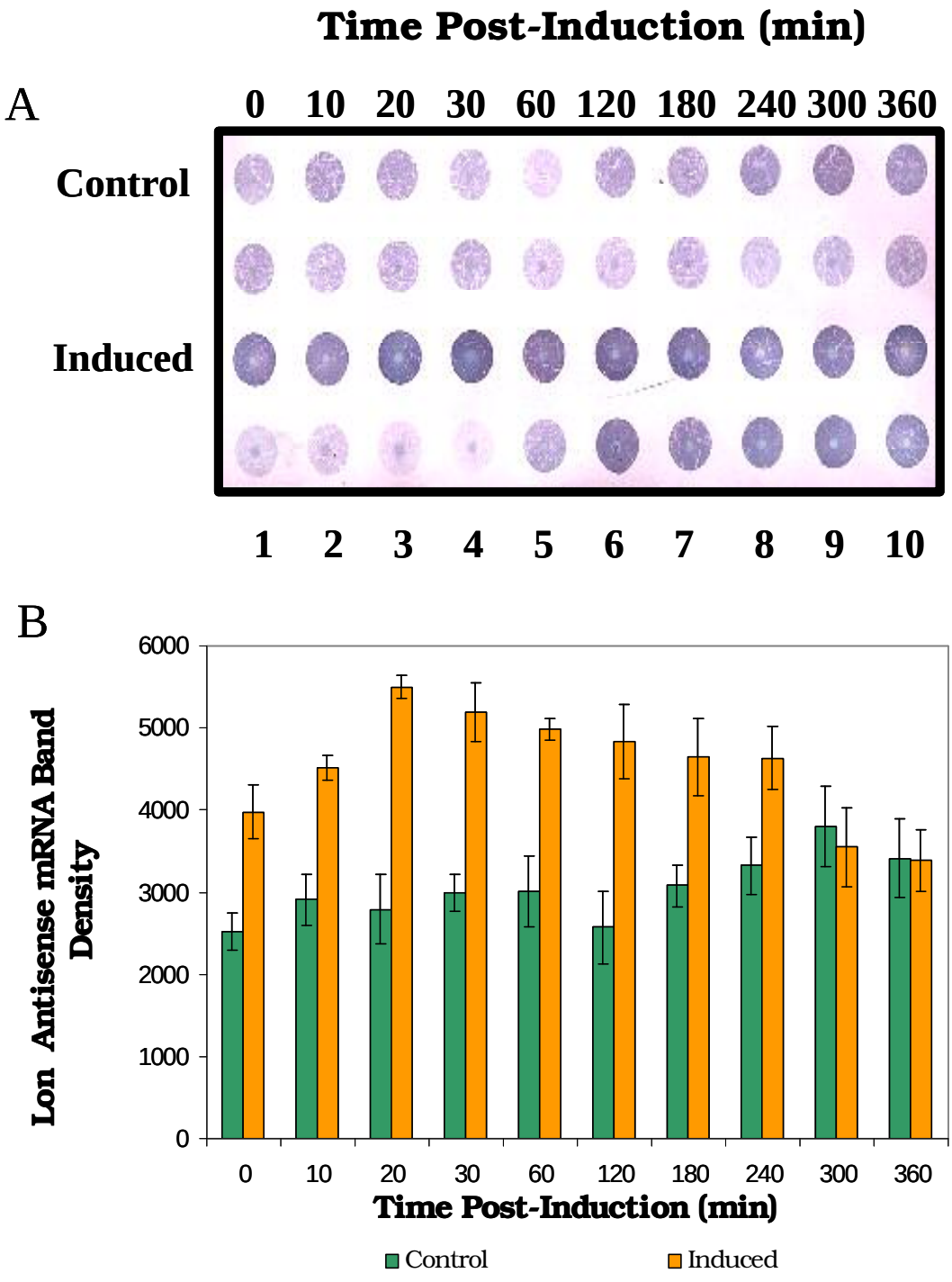
Figure 7: Growth Profile JM105 pTOlonαs Expressing Cultures

The plotted time points indicate the average of triplicate experiments. Control points refer to cultures that were neither stressed nor induced. Stressed cultures were stressed with 4% (v/v) ethanol. Induced cultures were induced with 1mM IPTG. Stressed/Induced cultures were stressed with 4% ethanol and induced with 1mM IPTG. The error bars represent the standard error of the mean.

to increase only slightly as expected due to the 4% (v/v) ethanol addition, resulting in decreased cell proliferation and viability. By the 180-minute time point the control cultures actually began to increase more than the induced cultures that were producing the lon antisense, indicating that the induction of pTOlonαs does affect cell growth rate. At this same point the stressed cultures appeared to reach their peak value and actually started to steadily decrease until the end of the experiment. By the 240-minute time point, the control cultures were actually 1.5 fold higher than the induced cultures and continued this increase until the end of the experiment. By this time there was quite a noticeable change in growth rate. At this time point the stressed/induced cultures also reached their peak, after which they steadily decreased as the stressed cultures did. However, the stressed/induced cultures decreased at a lower rate and actually end with slightly higher optical density readings than the stressed cultures.

3.3 pTOlonαs Vector Gene Expression

In addition to the 1 mL samples collected for OD_{600} readings, a 10 mL sample was also collected for each of the cultures at the previously stated time points. Each sample was purified for total RNA and quantitative analysis as described in Chapter 2. Again, as explained for pSE420lonαs cultures, dot blots were performed to establish the


A
Time Post-Induction (min)
0 10 20 30 60 120 180 240 300 360
Control
Induced
1 2 3 4 5 6 7 8 9 10
B
Lon Antisense mRNA Band Density
6000
5000
4000
3000
2000
1000
0
0 10 20 30 60 120 180 240 300 360
Time Post-Induction (min)
Control
Induced

C

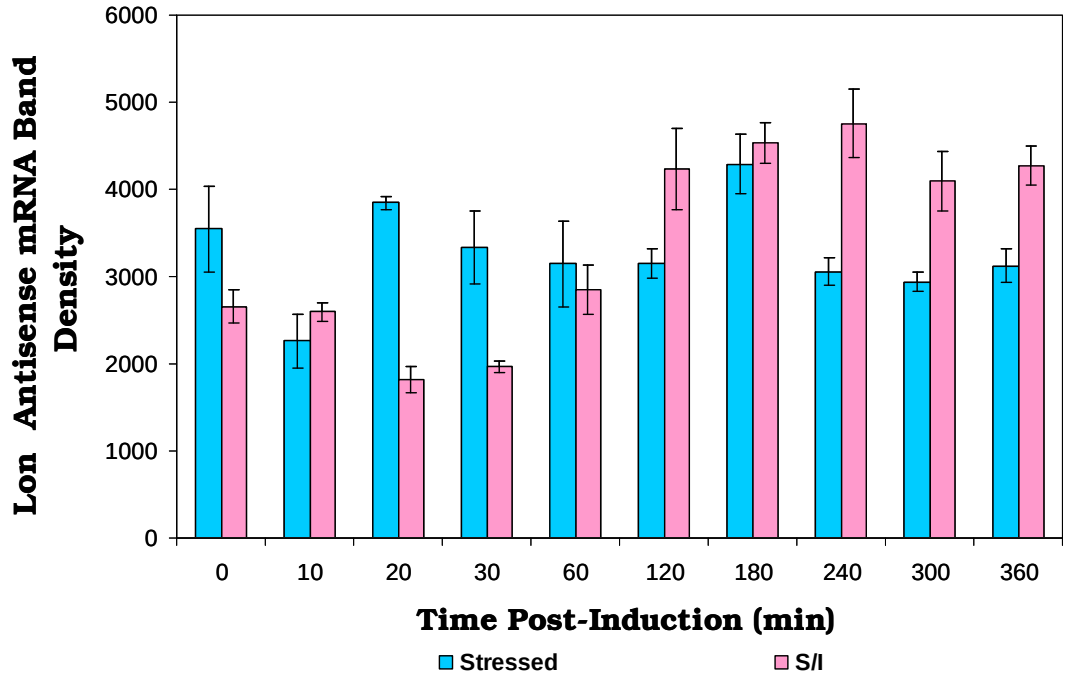


Figure 8: Antisense Dot Blot of pTOlonαs Cultures

(A) The control (no IPTG) and induced culture dot blot membrane (B) Dot blot densitometry reading from NIH Imaging software for control and induced cultures. (C) Dot blot densitometry reading from NIH Imaging software for stressed and S/I cultures. The plotted time points indicate the average of triplicate experiments. The error bars represent the standard error of the mean.

presence and levels of lon antisense mRNA mediated by the pTOlonαs vector in each of the growing cultures. Figure 8A shows a representative example of the dot blot results. Each row on the dot blot depicts the mRNA time course for each of the four cultures (control, ethanol stressed, IPTG induced, and stressed/induced) respectively. Each lane represents the same sample time for each culture. In the case of the unstressed cultures (control and induced), the quantity of lon antisense mRNA produced in the induced cultures far exceeded the amount produced in the control cultures (no IPTG). The induced cultures immediately began to over express lon antisense mRNA upon induction and continue throughout the duration of the experiment. However, the control cultures do reveal an increase in lon antisense production. Starting at the 120-minute time point and continuing until the end of the experiment, this level increased slightly. Again this result, as in the case with the pSE420lonas vector, indicates that lon antisense is being produced constitutively.

Figure 8B depicts the quantitative values of the dots shown in A. Each value is the average of three separate blots obtained from triplicate experiments. Just as can qualitatively be seen in the dot blots, the induced cultures produced high levels of lon antisense mRNA, peaking in lane 2, at 20 minutes, and only slightly decreasing for the remainder of the time course. The amount of lon antisense produced in

the induced cultures was about 2 fold higher than that made in the control cultures at its peak. The amount of mRNA in the control cultures stayed relatively constant throughout and by 5 hours (lane 9) it had the same amount as the induced cultures. Examining the dot blots in lanes 9 and 10 reveal that both the control and induced cultures had similar dot intensities. This observation corresponds nicely to the quantities values at the same time points, for which the reading for the control and induced cultures are approximately the same. This is consistent with other studies showing that recombinant protein induction is transient.

Figure 8A also depicts a dot blot probed for lon antisense with the focus on the ethanol (4% v/v) stressed and the stressed/induced cultures. Here fainter blots are shown for the stressed cultures as compared to the induced cultures throughout the experiment, and results were more similar to the control dot blots. The stressed cultures did not experience any qualitative increase in lon antisense production as the control cultures. The stressed/induced cultures revealed small amounts of lon antisense mRNA being made up until lane 6, at 120 minutes, when it looked to be producing as much mRNA as the induced only cultures.

Quantitative values for the stressed and stressed/induced dot blots are shown in Figure 8C. Here we again see that the stressed

cultures, like the control cultures, never go beyond 4000. It is interesting to note the 0-minute (immediately after induction), 20-minute and 180-minute time points when the quantitative values noticeably increased as compared to the other time points., higher even than the control values at the same sample point. From 0 to 60 minutes post induction the stressed/induced cultures again were similar to the control and stressed only cultures, experiencing evidently lower values at the 20 and 30 minute time points. However, at the 60-minute time point (just as observed from the dot blot), the amount of lon antisense produced in the stressed/induced cultures closely mirrored the induced only cultures, even slightly exceeding it in the last two hours.

Figure 9 displays the trend of lon antisense production in each of the four cultures. Each quantitative dot blot value was normalized to its corresponding OD_{600} value at that same time. The resulting values for three individual dot blots were averaged and plotted along the experimental time course. From this plot the general quantity of lon antisense mRNA yielded with respect to the amount of cell culture growth can be distinguished. It shows that for the control cultures the mRNA production initially very slightly increased to a peak (10 minutes), remains fairly constant until 60 minutes when transcription clearly decreased, and then continued a slight decrease throughout the

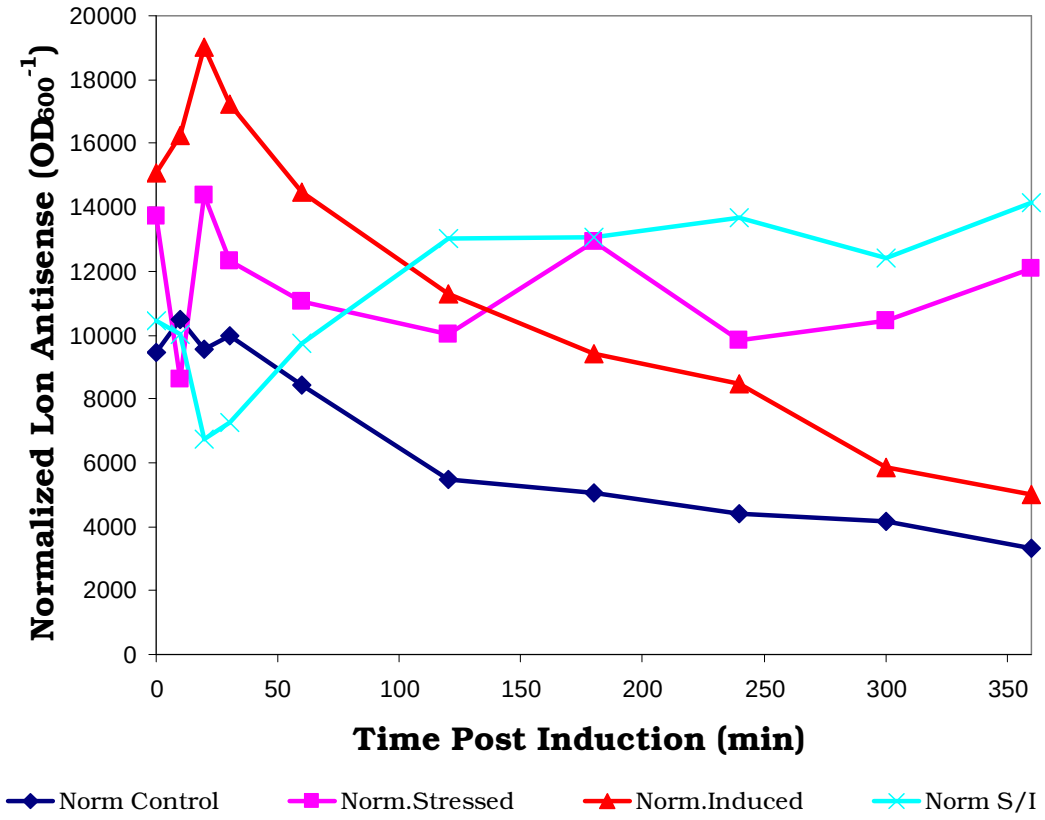


Figure 9: Normalized Lon Antisense Band Density

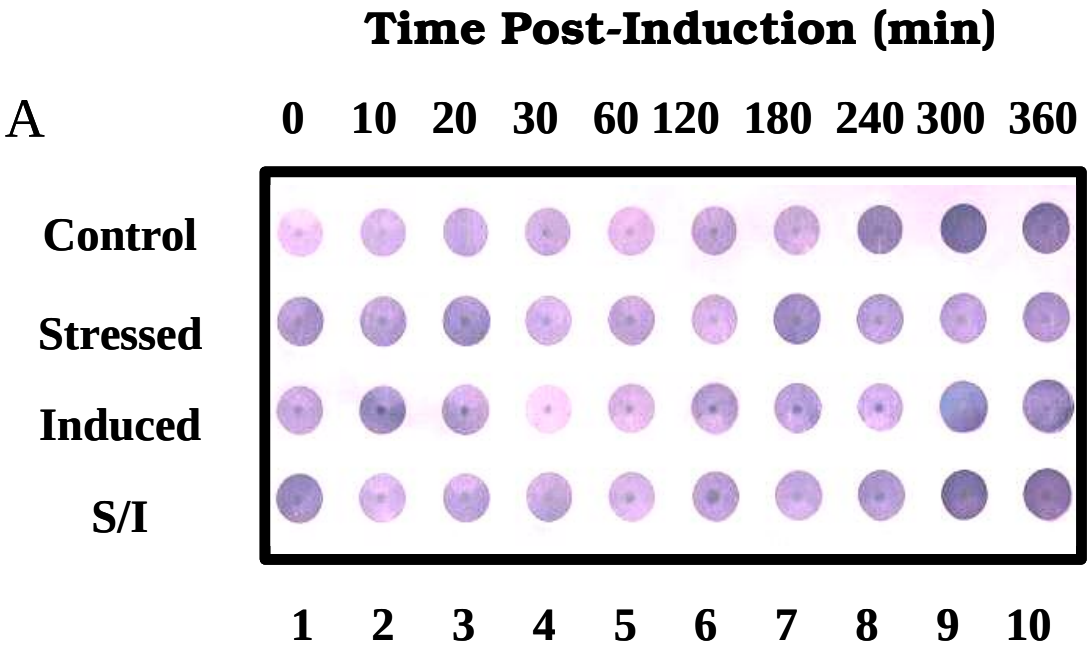
Each quantitative dot blot band density value was normalized to its corresponding OD_{600} value at that same time. The resulting values for three individual dot blots were averaged and plotted along the experimental time course.

remainder of the time course. The induced cultures began higher than the other cultures and undergo an initial increase for 20 minutes, at which point a marked decrease occurred for the rest of the time experiment. There was also ~2- fold increase in the amount of lon antisense mRNA in the induced cultures as compared to the control cultures. The stressed cultures experienced one notable decrease at the 10-minute time point, otherwise maintaining relatively constant throughout the time course. The stressed/induced cultures experienced a decrease in production for 20 minutes, at which point it significantly increased and remained relatively steady throughout the rest of the experiment.

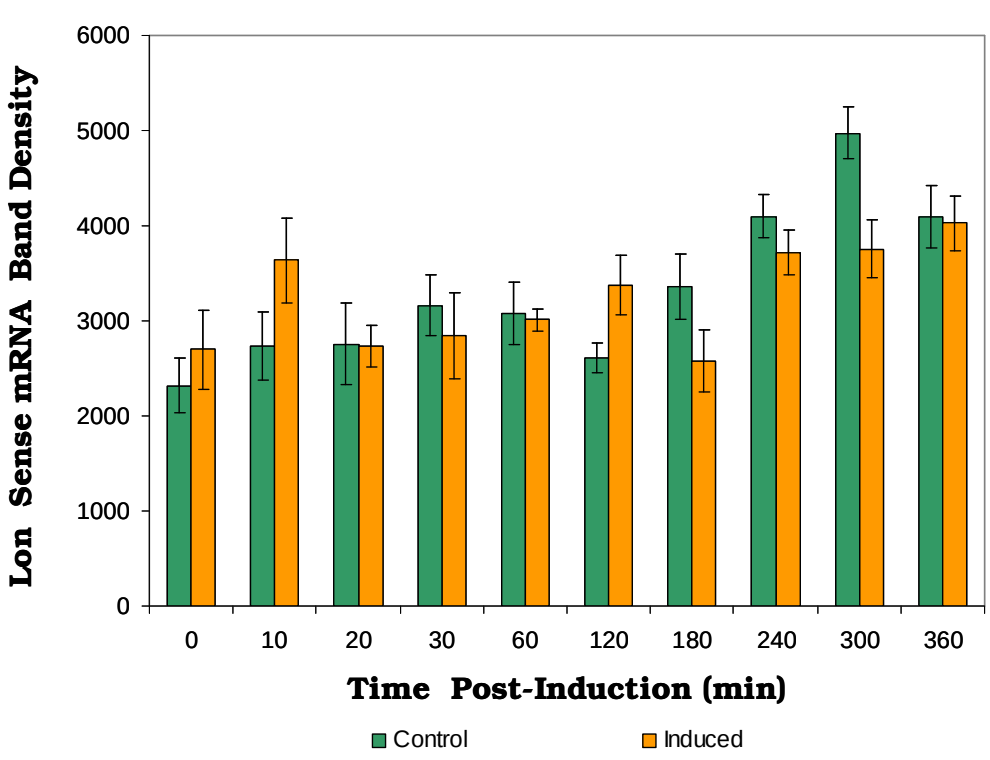
3.4 pTOlonαs Effect on Lon Sense mRNA

After establishing that lon antisense was adequately overexpressed in induced cultures, it was also important to evaluate the effect of the overexpression on the regulation of lon mRNA. To do this dot blots were again performed and this time probed for lon sense mRNA. Figure 9A shows a representative dot blot for lon sense mRNA. Each row on the dot blot depicts the lon sense mRNA time course for each of the four cultures (control, ethanol stressed, IPTG induced, and stressed/induced) respectively. Each lane represents the same sample

A


Time Post-Induction (min)
0 10 20 30 60 120 180 240 300 360
Control
Stressed
Induced
S/I
1 2 3 4 5 6 7 8 9 10

B


Lon Sense mRNA Band Density
6000
5000
4000
3000
2000
1000
0
0 10 20 30 60 120 180 240 300 360
Time Post-Induction (min)
Control
Induced

C

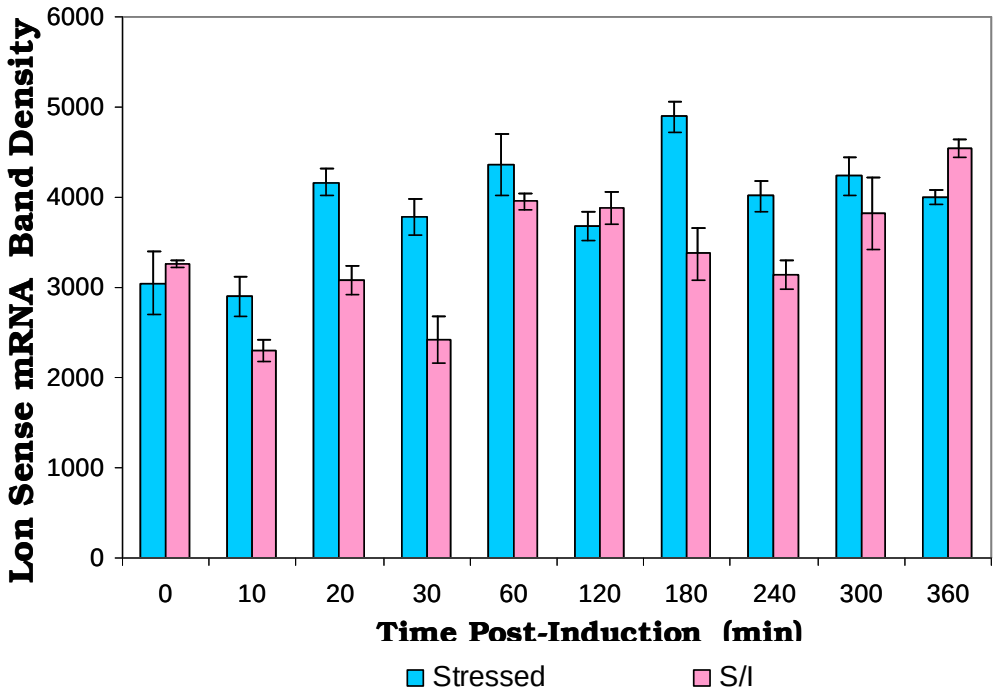


Figure 10: Sense Dot Blot of pTOlonαs Cultures

(A) The control (no IPTG), stressed, induced and stressed/induced cultures dot blot membrane (B) Dot blot densitometry reading from NIH Imaging software for control and induced cultures. (C) Dot blot densitometry reading from NIH Imaging software for stressed and S/I cultures. The plotted time points indicate the average of triplicate experiments. The error bars represent the standard error of the mean.

time for each culture. In this case the control cultures produce more lon sense mRNA than the induced cultures. Qualitatively the control cultures immediately expressed detectable lon sense mRNA, but while the control seemed to stay rather consistent, the induced cultures appeared to slightly drop off in lane 3 (20-minute time point), with the dot becoming darker once more in lane 4 (30-minute time point), lasting until the 60-minute time point. At 120 minutes the dot intensities again suggested a decrease in sense mRNA transcription until the end of the time course in lane 9 (300-minute time point) where it again slightly increased. This result suggests that in the cultures producing lon antisense, lon sense is successfully being inhibited.

Quantitative values for the control and induced dot blots are shown in Figure 10B. The control values were pretty consistent with the dot blot, remaining pretty consistent throughout the time course, slightly increasing towards the end. The induced values suggested that the amount of lon sense RNA being produced is similar to that of the control cultures. The dot blot is only a representative dot blot while the quantitative values are the mean of three separate dot blots from triplicate experiments.

Figure 10A also depicts the dot blot probed for lon sense mRNA with the focus on the ethanol (4% v/v) stressed and the stressed/induced cultures. Here darker blots are shown for the

stressed culture as compared to the induced culture throughout the experiment. Both the stressed and stressed/induced cultures had values consistently above 3000 as opposed to the unstressed cultures, which usually stayed below 3000. The stressed cultures experienced a slight decrease at 10 minutes and then increased at 20 minutes. At this point transcription seemed to remain mostly steady, experiencing another slight short-lived increase at 180 minutes. The stressed/induced cultures, on the other hand, revealed lower amounts of sense mRNA transcript (below 3000) until the 60-minute time point, when it began to parallel the stressed cultures for the remainder of the experiment, except at the 180-minute time point.

Figure 11 displays the trend of lon sense mRNA production in each of the four cultures. Again, each quantitative dot blot value was normalized to its corresponding OD_{600} value at that same time. The resulting values for three individual dot blots were averaged and plotted along the experimental time course. From this plot, the general quantity of lon sense mRNA yielded with respect to the amount of cell culture growth can be distinguished. In the case of lon sense mRNA it appeared that all of the four cultures roughly started out with comparable amounts of sense mRNA. In the control cultures the sense

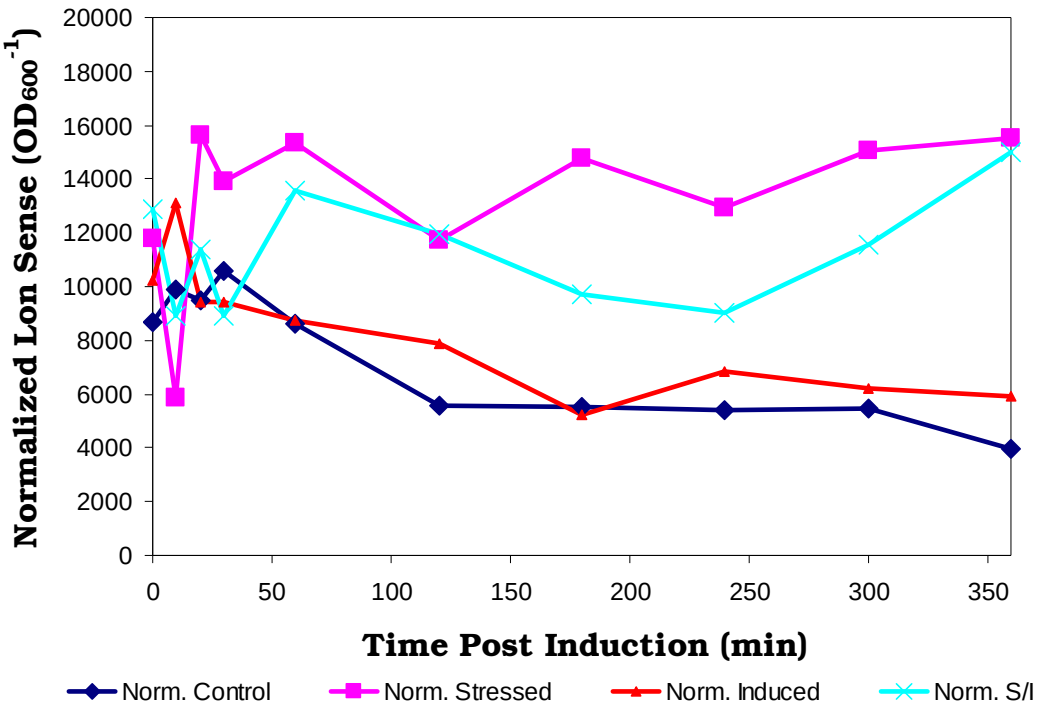


Figure 11: Normalized Lon Sense Band Density

Each quantitative dot blot band density value was normalized to its corresponding OD_{600} value at that same time. The resulting values for three individual dot blots were averaged and plotted along the experimental time course.

mRNA production initially very slightly increased to a peak (30 minutes), when transcription appreciably decreased up until about 120 minutes, and then remains relatively constant throughout the remainder of the time course. The induced cultures underwent an initial increase for 10 minutes, at which point it began to steadily decrease for the next two hours and 50 minutes (180-minute time point). Transcription stayed about the same for the remainder of the time course. Again, showing that lon antisense was effectively inhibiting lon sense mRNA transcription. Further proof can be inferred by the behavior of lon sense mRNA transcription in the stressed cultures. The addition of the ethanol elicited a stress response, which ordinarily results in the increase of σ^{32}, and subsequently lon transcription. As observed in Figure 11, lon sense mRNA production dropped very early and though it immediately recovered, the amount of lon sense being produced was steady, not increasing. The stressed/induced cultures fluctuated up and down for the first 60 minutes, when it started to steadily decrease until the 240-minute time point. Here it experienced a steady increase until the end of the experiment.

3.5 Northern Blot Verification of pTOlonαs Gene Expression

Dot blots offer rapid qualitative mRNA results. However, further proof was needed to verify that the mRNA being overexpressed was in fact lon antisense mediated by the pTOlonαs vector. Northern blots were used to accomplish this task. Northern blots provide not only a qualitative look at mRNA, but also quantitative because the different RNAs are run on an agarose gel and separated. This being different from dot blots in that all mRNAs present are concentrated in a single dot. The Northern Blot allows the probe to bind only to the desired mRNA. Figure 12 reveals the results from the Northern Blot for the first 30 minutes of the experiment for the control and induced cultures. This time we can see the stark contrast between the amount of lon antisense mRNA transcript between the control and induced cultures. These bands were also quantified by NIH Imaging software for densitometry readings. These results were then plotted against the time course sample points. As with the dot blots, we can see that the lon antisense mRNA in the induced cultures was maximal at 20 minutes. All other values were normalized to this 20-minute value. According to the quantitative values, the amount of antisense mRNA produced during the first 30 minutes was about the same, except a notable difference at the 10-minute time point when transcription

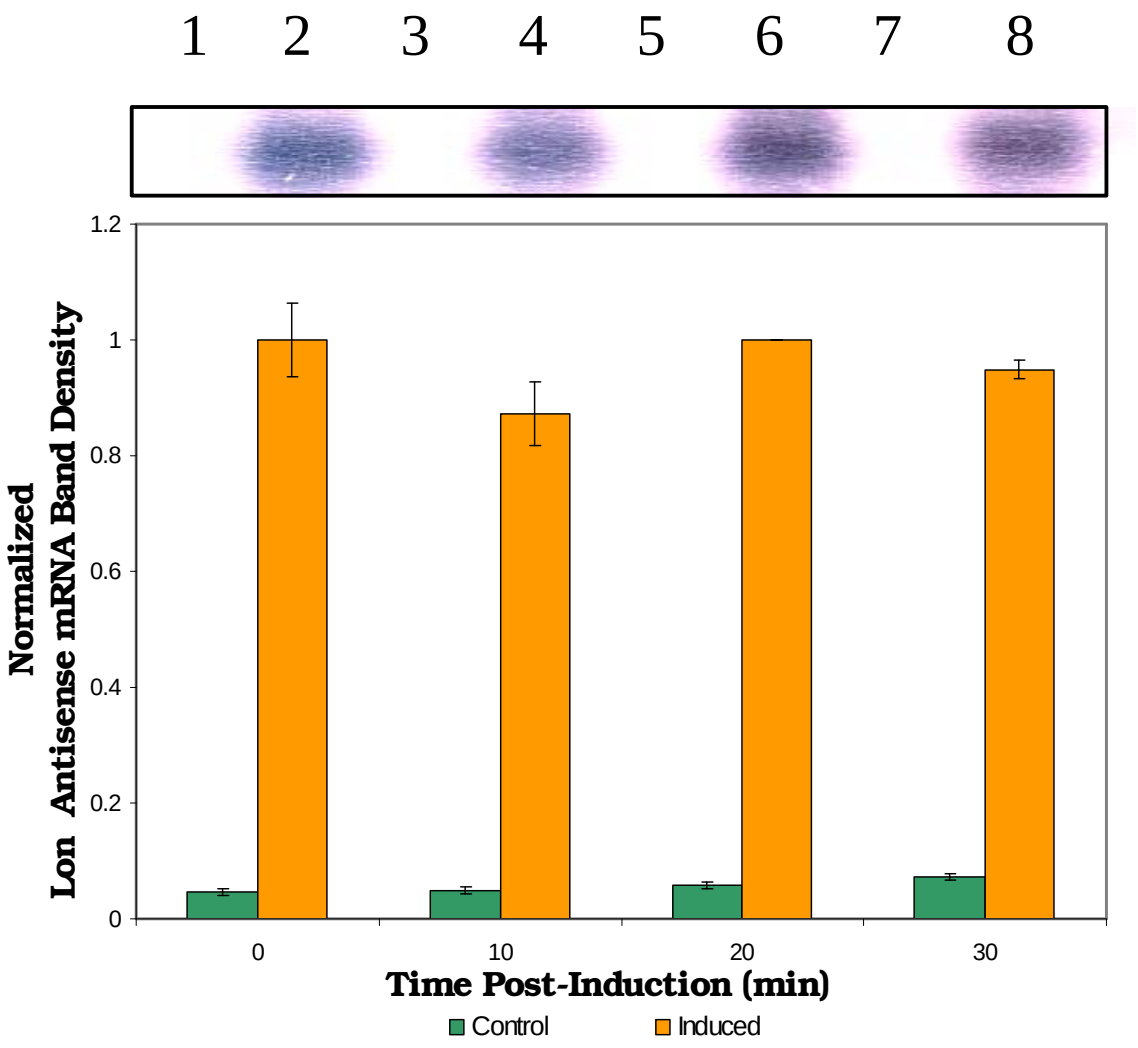


Figure 12: Normalized Lon Antisense Northern Blot for Unstressed Cultures

Lanes 1,3,5, and 7 are time course samples for control samples to which to IPTG have been added. Lanes 2,4,6, and 8 are time course samples for induced samples to which IPTG has been added. Each quantitative band density value was normalized to the 20-minute post-induction sample (lane 6). The resulting values for three individual Northern blots were averaged and plotted along the experimental time course.

briefly decreased to some extent. The control cultures produced small but detectable messages, though the cultures had not been induced with 1mM IPTG.

3.6 Effect of Lon Antisense on OPH Protein Level

To investigate the efficiency of lon antisense on inhibiting the translation of endogenous lon sense during recombinant protein/ethanol induced stress response, OPH protein levels were monitored by Western Blots. Whole cell protein from E. coli JM105 induced cultures containing the pTOlonαs (OPH and lon antisense) vector was compared to cultures containing the pTO vector (no lon antisense). Figure 13 reveals that for both vectors the control and induced cultures steadily increased along the time course (blot intensity increasing). Lanes 2, 4, 6, 8, 10, 12, 14, 16,18 and 20 designate the induced cultures. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 are control cultures. Quantitative results show that for the first 10 minutes, pTOlonαs cultures produced twice as much OPH as cells transformed with pTO. By 20 minutes, post-induction both pTOlonαs and pTO cultures were producing relatively the same amount of OPH to the end of the time course. It was also interesting to observe that the OPH protein level was higher at 10 minutes than 20 minutes, when the lon antisense mRNA transcript was at its maximum. Even pTOlonas

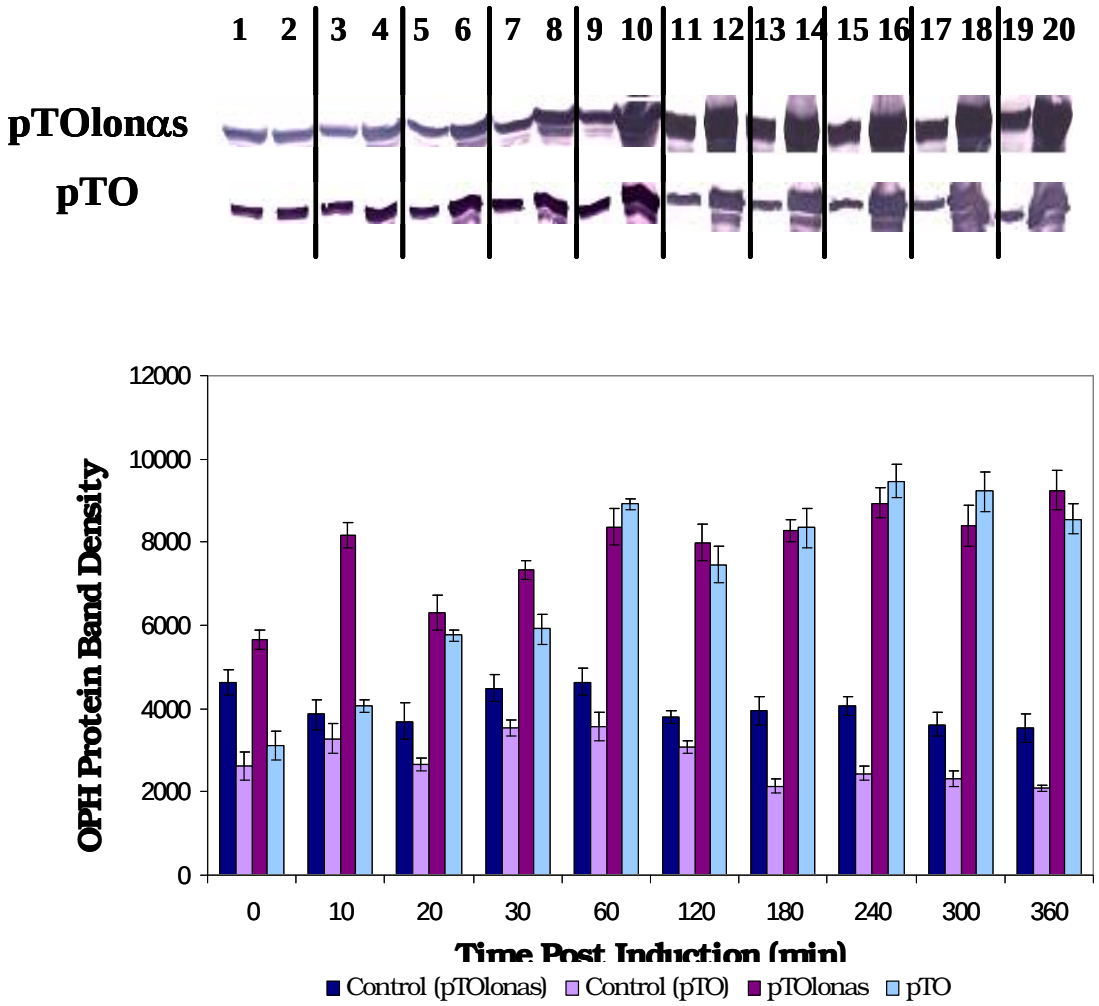


Figure 13: Western Blot Analysis of OPH Protein Levels with and without Lon Antisense

Lanes 1,3,5,7,9,11,13,15,17 and 19 are time course samples for control samples to which to no IPTG has been added. Lanes 2, 4, 6, 8,10,12,14,16,18 and 20 are time course samples for induced samples to which IPTG has been added. The reported densities are for organophosphorus hydrolase (OPH) protein levels in cultures induced to synthesize lon antisense (pTOlonαs) and not induced for lon antisense synthesis (pTO). The quantitative values for three individual Western blots were averaged and plotted along the experimental time course. Error bars represent the standard error of the mean.

control cultures that were not induced exhibited higher levels of OPH protein, probably due to constitutive antisense expression.

3.7 Lon Antisense Effect on OPH Specific Activity Levels

Western blots revealed that the inhibition of lon by lon antisense initially results in increase OPH production, however, protein aggregation and the presence of inclusion bodies upon expression of recombinant proteins is common. To explore if lon inhibition would result in the recovery of more viable protein, the specific activity of the OPH in each time course sample was determined using the paraoxon based OPH assay. The values in Figure 14 are not normalized but reflect specific activity on a per OD_{600} basis. OPH produced in pTO cultures, remained low throughout the entire time course, the maximal value (0.023U) occurred at 120 minutes at which time it continually declined for the remaining 4 hours. On the other hand, OPH produced in pTOlonαs cultures revealed an activity of 0.07U in the first 20 minutes, decreasing steadily until the 3-hour (180-minute) time point where it again rose peaking at ~0.1U at 5 hours. Even when decreasing, the activity for pTOlonαs cultures was always still more than twice that in pTO cultures. At 240 and 300 minutes, the activity even reached ~9 fold higher in pTOlonαs cultures. It appears that lon

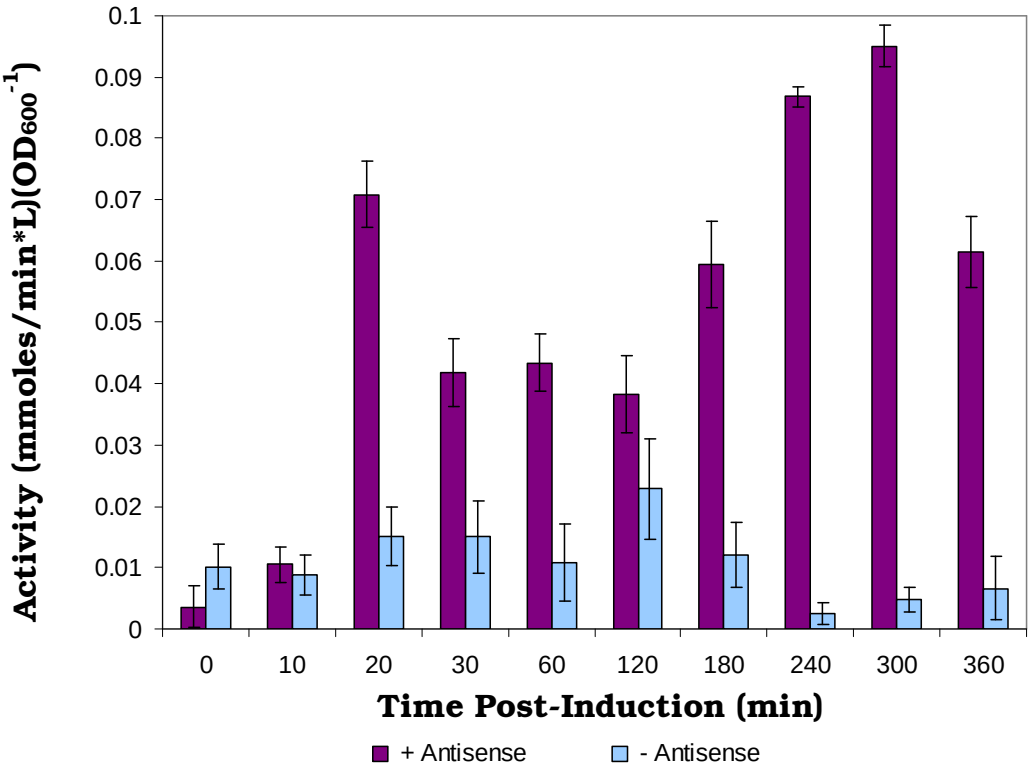


Figure 14: OPH Activity Analysis of OPH Protein Levels with and without Lon Antisense

OPH activity assay results for cultures producing lon antisense (pTOlonαs) and for cultures not producing lon antisense (pTO). The values plotted are the mean values for duplicate experiments. Each value is normalized to its individual corresponding OD_{600} value at that same time. Error bars represent the standard error of the mean.

antisense allowed more active OPH to continually be produced and survive.

3.8 Discussion

In order to evaluate antisense as an effective inhibitor of endogenous lon proteolytic activity, several dynamics were considered. Effective inhibition requires that antisense be expressed in excess of the target gene (Daugherty et al., 1989; Mirochnitchenko and Inouye, 1999). The mRNA results from dot blots and Northern blots (and their subsequent quantification) confirmed that induced pTOlonas cultures were, indeed, producing high levels of lon antisense and in the correct orientation. This higher level of antisense accumulated rapidly and expression persisted for several hours. In fact, all cultures whether stressed, induced, stressed and induced, or neither stressed nor induced exhibited evidence of some detectable lon antisense. This is likely due to the choice of the trc promoter. The need to ensure high levels of antisense requires the use of a very strong, inducible promoter, such as the trc promoter. This promoter is a derivative of the lac promoter, a strong promoter which can also be leaky, which can lead to constitutive expression. When induced the trc promoter can produce over

30% of the total protein in E. coli (Amann et al., 1983; Brosius et al., 1985; Georgiou 1988). This resulted in the control cultures slowly accumulating enough lon antisense to be detected pretty heavily in the later hours. Under heat or ethanol shock, σ^{32} synthesis is increased (Blaszczak et al., 1995; Grossman et al., 1987; Kanemori et al., 1994; Lesley et al., 1987; Straus, 1989; Tilly et al, 1989). The stressed cultures which should be producing increase levels of endogenous lon due to σ^{32} upregulation, even experience a slightly higher level on antisense by the end of the experiment. This is consistent with the fact that the stress response is transient, and in the later hours heat shock chaperones and proteases should have returned to normal levels, allowing for more stable amounts of lon antisense to exist.

pTOlonαs successfully demonstrated that cultures transformed with this vector would produce substantial levels of lon antisense. Next, we had to evaluate the effect of lon antisense on lon sense inhibition. Again, mRNA results from dot and Northern blots show that in cultures induced for lon antisense, lon sense mRNA was significantly decreased within the first 15 minutes, this decrease prevailing for the next several hours, as compared to ethanol stressed cultures. Even in stressed culture there was not significant increase in lon sense mRNA, the levels

remained relatively stable throughout the entire time course. This stability due to lon antisense inhibition is further confirmed by the fact that the upregulation and stability of σ^{32} in the cytoplasm also upregulates chaperone proteins and proteases (lon). Though the amount of sense found in the stressed cultures is more than that found in the cultures, it is still the about the same amount existing in the end for all the cultures. In the case of the other three cultures the end represent the greatest mRNA amounts. This suggests that even constitutively expressed lon antisense was possibly enough to inhibit the maximal upregulation of lon sense in the first 10 minutes to an hour when the stress response is at its strongest. The half-life of σ^{32} is still only increased to about 10 minutes in stressed conditions (Straus et al., 1987). Between 10 and 60 minutes, the amount of lon sense would ordinarily be expected to be greater. Lon sense in the stressed/induced cultures is decreased within the first 10 minutes of induction, remaining appreciably lower than the stressed only cultures for the first hour, at which point it rises to the values or the stressed culture for the remaining 5 hours. Again, lower levels of lon sense were attained due to the inhibition caused by the lon antisense.

Western blot analysis assisted in determining the effect of lon antisense on the production of OPH protein levels. Recombinant protein induction evokes a stress response in E. coli. Transcription of lon rises when cells produce large amounts of abnormal polypeptides, such as cloned foreign proteins (Goff and Goldberg, 1985).This again results in upregulation of σ^{32} and ultimately proteases like lon, which will degrade the abnormal protein and reduce the desired yield (Bentley et. al. 1990). Western blots reveal that within the first 20 minutes when lon antisense mRNA is greatest during the stress response, OPH protein levels are twice as high as levels in non-antisense producing cultures. Further confirming the inhibiting effect of lon antisense on lon mediated proteolysis. OPH activity in this same first 20 minutes also reveals superior levels of activity as compared to pTO cultures with no lon antisense synthesis. Even when inhibiting lon proteolysis it is also important to consider the possible degradation OPH, by the Clp family of proteases that also play a role in abnormal protein degradation.

From all plotted results it obviously appears to be two separate phenomena occurring, the first taking place in the first hour or two and the second in the last four to five. The first hour is actually the effects of the stress response (either by ethanol

addition or recombinant protein induction) and high levels of recombinant protein production. At this time, σ^{32} is being upregulated, as is all stress related molecular chaperones and proteases. This is when the effects of antisense inhibition are really evident. After these chaperone proteins and proteases have accumulated, σ^{32} is then more swiftly degraded by FtsH and ClpQY (Blaszczak et al., 1999; Gamer et al., 1996; Herman et al., 1995; Kanemori et al., 1999). At the same time the recombinant lon antisense transcripts are being produced at higher levels and seeking its target lon sense transcript for inhibition. This leads to the state of mRNA and protein levels and activity in the second time frame. The increased amounts are not believed to be due to increased transcription and translation, but attributed to the stability of transcripts and translated protein due to the lack of chaperones that would target the abnormal proteins and proteases that would degrade them. In addition, recombinant protein induction is not an enduring response and eventually cells will no longer optimally produce the desired protein. This means a decrease in inhibiting lon antisense and OPH productions.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

An investigation of the inhibitory effects of lon antisense mRNA on lon sense proteolytic activity was executed. An in vivo antisense system for effective delivery and downregulation of lon was clearly demonstrated, presenting a feasible option to gene mutations which can affect cell viability. This was achieved with antisense targeting only the 5' coding region of the lon gene. This is especially attractive because many bacterial genes exist in operons. The coding region of the desired target protein can be accomplished without affecting other genes in the operon, which could have similar upstream promoter sequences or ribosomal binding sites. Additionally, it was again shown that antisense inhibition is transient and causes no permanent changes to the bacterial cell system. Lastly, the investigation demonstrated that the lon antisense system can effectively result in not only increased recombinant protein yield, but also elevated levels of the specific activity of our model protein, OPH.

4.2 Recommendations and Future Works

Ultimately the goal is to increase viable recombinant proteins. Extension of the existing work can then include several possibilities. The first can expound upon the lon data collected here. The inclusion of lon protein work would be especially informative for further interpretation of the existing results. This could not be included because of difficulty in acquiring a lon antibody. As mentioned previously, the Clp family of proteases is also largely involved in abnormal protein degradation. Protein data and mRNA data could be attained to see if lon sense inhibition, upregulated the Clp proteins. Antisense targeted directly to any or all of the Clp proteins could be explored as well, monitoring the effects on degradation and comparing to the current lon data. Monitoring the effect of chaperone and chaperonin proteins as a result of lon sense inhibition can also be investigated. An increasing amount of information on the kinetics and loop-loop/loop-stem interactions of antisense mRNA and its targets in natural systems has been gathered which can be applied to vector mediated antisense systems. Optimal lengths and locations for the lon antisense system can be attained and applied. This lab, as well as others, has had success using fusion proteins. It may be possible to successfully fuse both antisense

lon and antisense clp, or the antisense of one of these proteases and a chaperone protein, such as DnaK. Overexpression of chaperone proteins, especially of DnaK which is instrumental to the stability of σ^{32}, has been shown to be successful in increasing recombinant protein yields. Lastly there is a growing wealth of information for characterizing novel small RNAs, which have come to be considered crucial to gene expression. Perhaps antisense to a small RNA will prove to be instrumental in increased protein yields.

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