The *E. coli* RNA degradosome: analysis of molecular chaperones and enolase

A thesis submitted in fulfilment of the requirements for the degree

of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

in the

Department of Biochemistry, Microbiology and Biotechnology Faculty of Science

ADÉLLE BURGER

July 2010

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science in Rhodes University. It has not been submitted before for any degree or examination in any other university.

____

____

This ____ day of _______________ 2010

Abstract

Normal mRNA turnover is essential for genetic regulation within cells. The *E. coli* RNA degradosome, a large multi-component protein complex which originates through specific protein interactions, has been referred to as the "RNA decay machine" and is responsible for mRNA turnover. The degradosome functions to process RNA and its key components have been identified. The scaffold protein is RNase E and it tethers the degradosome to the cytoplasmic membrane. Polynucleotide phosphorylase (PNPase), ATP-dependent RNA helicase (RhlB helicase) and the glycolytic enzyme enolase associate with RNase E to form the degradosome. Polyphosphate kinase associates with the degradosome in substoichiometric amounts, as do the molecular chaperones DnaK and GroEL. The role of DnaK as well as that of enolase in the RNA degradosome is unknown. Very limited research has been conducted on the components of the RNA degradosome under conditions of stress. The aim of this study was to understand the role played by enolase in the assembly of the degradosome under conditions of stress, as well as investigating the protein levels of molecular chaperones under these conditions. The RNA degradosome was successfully purified through its scaffold protein using nickel-affinity chromatography. In vivo studies were performed to investigate the protein levels of DnaK and GroEL present in the degradosome under conditions of heat stress, and whether GroEL could functionally replace DnaK in the degradosome. To investigate the recruitment of enolase to the degradosome under heat stress, a subcellular fractionation was performed to determine the localization of enolase upon heat shock *in vivo*. The elevated temperature resulted in an increased concentration of enolase in the membrane fraction. To determine whether there is an interaction between enolase and DnaK, enolase activity assays were conducted *in vitro*. The effect of DnaK on enolase activity was measured upon quantifying DnaK and adding it to the enolase assays. For the first time it was observed that the activity of enolase increased with the addition of substoichiometric amounts of DnaK. This indicates that DnaK may be interacting with the RNA degradosome via enolase.

Acknowledgements

A special acknowledgment and thanks to my supervisor, Dr Aileen Boshoff, who gave me this opportunity, and through highs and lows was always interested and supportive of the project. An absolute pleasure to be her student!

I would like to acknowledge my co-supervisor, Prof Chris Whiteley, for his support

I would also like to thank:

- Prof Greg Blatch for opening his lab for the use of equipment without which I could not have completed my thesis, and the members of his research group, BioBRU, for their support
- Prof Sue Lin-Chao for plasmids pRE196 and pRE296, encoding for FLAG- and His-tagged RNase E, respectively; pFLAG-PNP, pFLAG-ENO and pFLAG-RrhlB encoding for FLAG-tagged PNPase, enolase and RhlB helicase, respectively
- Prof Sonomoto for the *E. coli dnaK* minus strain BM271 (DE3)
- Dr Singh for advice regarding ribosomal protein L4

On a personal level I would like to thank:

- Natasha Beukes and Cassandra Louw for friendship and expertise
- Members of the AB research group for good times in the lab
- My father and my brother for their constant guidance, patience and tireless support
- Judy van der Westehuizen for her guidance and support

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Symbol	Interpretation
%	Percent
α	alpha
β	beta
λ	lambda
μl	microlitre
A360	Absorbance at 360 nanometers
A600	Absorbance at 600 nanometres
bp	base pair
kDa	kiloDalton
M	molar
mM	millimolar
ml	milliliter
μmol/min/ml	micromolar per minute per millilitre
mg/ml	milligrams per millilitre
°C	degree Celsius
w/v	weight per volume
v/v	volume per volume

Outputs

Publications

Review in preparation: Burger, A., Whiteley, C. & Boshoff, A. (2010). The *E. coli* RNA degradosome: role of molecular chaperones and enolase.

Local Conference Proceedings

Poster presentation:

Burger, A., Willmer, T., Whiteley, C. & Boshoff, A. *Escherichia coli* RNA degradosome: The analysis of heat shock proteins and enolase. 22nd Congress of the SASBMB, Bloemfontein, South Africa, 2010

Chapter 1
Literature Review

1.1 Introduction

Many *in vivo* cellular functions are performed by large multiprotein complexes as opposed to individual scattered proteins (Alberts, 1998). Similarly, prokaryotes such as *Bacteroides* and *Clostridium* contain multiprotein complexes that digest and degrade cellulose, called cellulosomes (Carvalho *et al.*, 2003). Degradosomes are complexes that degrade RNA, contributing to the posttranscriptional control of gene expression. These and other comparable complexes have been identified in *Escherichia coli* (*E. coli*) and other prokaryotes as well as in eukaryotes. Some of the prokaryotic organisms that contain these complexes include *Rhodobacter capsulatus, Pseudoalteroomonas haloplanktis* and *Bacillus subtilis* (Jäger *et al*., 2001; Bechhofer *et al.*, 2008; Carpousis *et al.*, 2008). Eukaryotes that contain multiprotein complexes that degrade RNA are called the exosome, and include *Sulfolobus solfataricus, Saccharomyces cerevisiae* (including the mitochondrial degradosome) as well as humans, to mention but a few (Oddis *et al.*, 1992; Evguenieva-Hachenberg *et al.*, 2008). Another multiprotein complex involved in degradation of a different sort is the cellulosome present in anaerobic cellulolytic bacteria. It hydrolyzes cellulosic and hemicellulosic substrates of plant cell walls (Shoham *et al.*, 1999).

The bacterial *E. coli* RNA degradosome is a large multicomponent protein complex that participates in the regulation of gene expression by affecting transcript turnover (Marcaida *et al.*, 2006). It does so by degrading mRNA and processing other RNA species (Carpousis, 2007). Through its function, the degradosome has been called the RNA decay "machine" (Carpousis *et al.*, 1999). The four major components of this "machine" include Ribonuclease E (RNase E), polynucleotide phosphorylase (PNPase), RhlB helicase and enolase. Polyphosphate kinase as well as molecular chaperones DnaK and GroEL also associates with the degradosome, in substoichiometric amounts. Minor components of the degradosome include CsdA (cold shock helicase), RraA, RraB (regulation of ribonuclease activity A and B) and r-protein L4 (RNase E protein inhibitors) (Kaberdin and Lin-Chao, 2009).

RNase E serves as the scaffold protein to which the other degradosome proteins attach and also serves to tether the degradosome through its N-terminus to the cytoplasmic membrane (Liou *et al.,* 2001). In essence, the degradosome combines a helicase (RhlB helicase), which separates nucleic acid strands, with a ribonuclease (RNase E), that cuts mRNA into pieces, and a ribonuclease (PNPase), that degrades the pieces into nucleotides (Liou *et al.,* 2002). A specific function of enolase as a component of the RNA degradosome has been elucidated as the degradosome-mediated breakdown of *ptsG* mRNA. Enolase directs compartmentalization of the degradosome to the plasma membrane for the *ptsG* mRNA to then be degraded (Morita *et al.,* 2004). Bernstein and colleagues (2003) determined that the workings of all four of these degradosome proteins are necessary for normal RNA processing in *E. coli*. RNase E controls its expression levels by cleaving its own mRNA (Mudd and Higgens, 1993; Jain and Belasco, 1995). RraA prevents the endonucleolytic cleavage of RNase E (Lee *et al.,* 2003). RraA thus allows restored growth to *E. coli* cells with an over production of RNase E (Yeom and Lee, 2006). This discovery led to the finding that overproducing the *E. coli* RNase E protein results in a decrease of cellular growth (Claverie-Martin *et al.,* 1991; Jain *et al.,* 2002).

Molecular chaperones DnaK and GroEL are present in the degradosome in substoichiometric amounts. The term “molecular chaperone” is used to define proteins that facilitate folding and assembly reactions (Ellis, 1987). Chaperones bind to hydrophobic residues of unfolded proteins functioning to prevent unsuitable molecular interactions. Chaperones have been divided into “holder” and “folder” categories depending on their functioning either to inhibit protein aggregation or promote correct protein folding (Lund, 2001). Protein misfolding and aggregation are consequences of cellular stress such as heat shock. Genes encoding for heat shock proteins (hsps) are expressed after cellular stress through the attaching of heat shock transcriptional factor (HSF) to heat shock elements (HSE) which are located upstream of the gene (De Maio, 1999). The large and varied heat shock protein family is classified into major classes as defined according to molecular weight. The role of molecular chaperones in the degradosome has not been elucidated yet, even though it is known that DnaK is present in an intermediate degradosome under conditions of stress (Regonesi *et al.*, 2006).

1.2 Ribonucleic acids

Processes, such as translation initiation and mRNA degradation, which affect mRNA turnover, can be a way of controlling gene expression (Brown *et al.*, 2002; Marcaida *et al.*, 2006). It is likely that these processes function at various levels of a regulatory hierarchy and can therefore link different aspects of metabolism and genetic control (Marcaida *et al.*, 2006). The control of mRNA levels can theoretically provide a mechanism of regulation of gene expression (Marcaida *et al.*, 2006). Research involving the study of mRNA stability has suggested that most mRNA half-lives are influenced by the 3' untranslated region (3'-UTR) (Ross, 1995). For instance, the presence of an adenine, uracil-rich element (AURE) and/or an oligo (U) region in the 3'-UTR tends to result in unstable mRNA (Caput *et al.*, 1986).

There are many different types of ribonucleic acids (RNAs) in cells. Other than the three major types of RNAs, namely messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), additional RNA classes include microRNAs (miRNA), transfer-messenger RNAs (tmRNA), small interfering RNAs (siRNA), Piwi-interacting RNAs (piRNA) and antisense RNAs. tmRNA rescue stalled bacterial ribosomes (Gillet and Felden, 2001), siRNA silences gene expression (Blevins T *et al*., 2006), piRNA are believed necessary in gametogenesis (Horwich *et al*., 2007), and antisense RNAs down-regulate bacterial genes (Wagner *et al.*, 2002). RNAs are classified by their stability, with tRNAs and rRNAs being the most stable RNAs in the cell (Carpousis, 2002). Unstable *E. coli* mRNAs have half-lives ranging from 30 seconds to 20 minutes whereas in eukaryotic cells the half-lives are shorter, but the mRNA turnover is slower (Carpousis, 2002). mRNA instability is a property that allows adjustment to a changing environment (Carpousis, 2002). Thus a normal mRNA turn-over is a necessary component of normal genetic regulation within cells (Bernstein *et al.*, 2003).

The cellular equipment responsible for RNA degradation often forms a part of multi-component assemblies, which can either target particular gene products or through differing decay rates, cause changes in the composition of various transcripts (Carpousis, 2002). In *E. coli,* a nucleolytic protein complex known as the RNA degradosome can maintain a normal rate of mRNA turnover and process RNA species into active forms (Carpousis, 2002).

1.3 RNA decay machines in other organisms

The exosome is considered to be one of the most critical complexes that regulates RNA turnover in eukaryotic cells (Raijmakers *et al.*, 2004). This large multiprotein complex has been well studied in a number of organisms, including *Saccharomyces cerevisiae, Sulfolobus solfataricus, Trypanosoma brucei, Arabidopsis thaliana, Caenorhabditis elegans* and humans (Mitchell *et al.,* 1997; van Hoof and Parker, 1999; Chekanova *et al.,* 2000; Estévez *et al.,* 2001). Even though there are conserved features and components of the exosomes between different organisms, the exosomes are not identical. This eukaryotic complex is mainly involved in the removal of RNA molecules that are processed incorrectly and ensures the maintenance of mRNA and rRNA turnover (Raijmakers *et al.*, 2004). It exists as two forms – a cytoplasmic and nuclear form that degrades mRNA and processes rRNA, respectively. Unlike the degradosome, the exosome contains only exoribonucleolytic proteins which seem to assemble in the nucleolus (Raijmakers *et al.*, 2004). The primary auto-antigenic proteins of the exosome are PM/Scl-100 and PM/Scl-75 (Raijmakers *et al.*, 2004). These proteins were shown to both be homologous to the bacterial proteins RNase D and RNase PH, respectively (Mian, 1997). Both 3' exonucleases and phosphorolytic enzymes are contained within the core. Six of the nine core components within the exosome are homologous to RNase PH (Mitchell *et al.*, 1997). The human equivalent of the exosome is the PM/Scl complex (Allmang *et al.*, 1999; Brouwer *et al.*, 2001). Another protein complex is found in the chloroplast and contains a PNPase-like exonuclease called RNP100 but it is not known if there is any interaction with an endonuclease (Carpousis, 2002). Unlike the chloroplast, the yeast mitochondrial exonuclease complex (mtEXO) has an RNA helicase 'Suv3p'. In order for Dss1p, the hydrolytic exonuclease, to degrade introns the helicase needs to associate with Dss1p (Dziembowski and Stepien, 2001).

Rhodobacter capsulatus contains the degradosome organized by RNase E. However, unlike the degradosome of *E. coli*, RNase E associates with 2 helicases, transcription factor Rho and minor amounts of PNPase (Jäger *et al*., 2001). Psychrotolerant *Pseudoalteroomonas haloplanktis* also contains the degradosome but, in this bacterium isolated from Antarctica, the RNA degradosome is efficient at low temperatures (Carpousis *et al.,* 2008). Gram-positive *Bacillus subtilis* degrades RNA through use of the exoribonucleases PNPase, RNase PH (involved in maturation of tRNA and scRNA) and RNase R (a key Mg^{2+} -dependent exonuclease that degrades 23S rRNA) (Oussenko and Bechhofer, 2000; Wen *et al.*, 2005).

1.4 Composition and structure of the *E. coli* RNA degradosome

The *E. coli* RNA degradosome is formed through the interaction of multiple proteins with the C-terminus region of the endoribonuclease, RNase E. These proteins include polynucleotide phosphorylase (PNPase) which is a phosphorolytic exoribonuclease, RhlB helicase, which forms a part of the DEAD-box family of ATP-dependent RNA helicases, and enolase (2-phosho-D-glycerate hydrolase) (Carpousis *et al.*, 1994; Miczak *et al.*, 1996; Py *et al.*,1996; Kaberdin *et al.*, 1998). GroEL and DnaK, which are heat shock proteins and polyphosphate kinase (Ppk) form part of the degradosome in substoichiometric amounts (Blum *et al.*, 1997). The degradosome complex has also been found to associate with *E. coli* poly-adenosine (A) polymerase and ribosomal protein S1 (Raynal and Carpousis, 1999; Feng *et al.*, 2001).

Only about 5-10% cellular enolase and 10-20% cellular PNPase co-purifies with RNase E to form the degradosome (Py *et al.*, 1996; Liou *et al.*, 2001). This finding suggests that both enolase and PNPase have cellular functions unrelated to the degradosome, for instance the glycolytic functioning of enolase, and the interaction of RhlB helicase with PNPase independent of RNase E (Liou *et al.*, 2002). All four of these proteins are necessary for normal genetic regulation within *E. coli* cells by ensuring normal mRNA turnover (Bernstein *et al.*, 2003).

1.4.1 Ribonuclease E

E. coli ribonuclease E (RNase E) was first discovered and characterized over 3 decades ago as being required to generate a 5S ribosomal RNA from the 9S precursor (Ghora and Apirion, 1978; Misra and Apirion, 1979). The RNase E protein has been shown to have a major role in mRNA turnover, the processing and decay of noncoding RNAs (Lundberg and Altman, 1995; Li and Deutscher, 2002; Kim and Lee, 2004). RNase E is essential for normal degradation of transcripts and processes the precursors of 16S and 5S ribosomal RNA, transfer RNA, the transfer-messenger RNA and the RNA component of RNase P (Coburn and Mackie, 1999). The purification and characterization of RNase E and work done on factors affecting the activity of polynucleotide phosphorylase (PNPase) lead to the first discovery of the *E. coli* RNA degradosome (Miczak *et al.*, 1996; Py *et al.*, 1996).

RNase E offers the backbone of the degradosome, providing the organizational scaffold on which the other proteins can assemble (Marcaida *et al.*, 2006). This protein has 1061 amino acid residues and consists of three domains: the N-terminus catalytic region (amino acid residues 0 – 604), the arginine-rich RNA-binding domain (RBD) (residues 605 – 679) and the flexible C-terminus region (residues 680 – 1061) (Figure 1.1). The N-terminus is folded and compact and is well conserved amongst bacteria (Kaberdin *et al.*, 1998). The C-terminus, in contrast, is unstructured and very divergent (Callaghan *et al.*, 2004). The RNase E scaffold domain extends over both the RNA-binding domain and the C-terminus region (residues 605 – 1061). The microdomains on RNase E where protein-protein interactions occur are at 698 – 762 base pairs (bp) for RhlB helicase, 833 – 850 bp for enolase and at 1021 – 1061 bp for PNPase. The protein-protein interaction sites were determined using yeast two-hybrid analysis (Vanzo *et al.*, 1998). An arginine-rich microdomain, other than the RNA-binding domain where protein-RNA interactions occur, is found at 798 – 819 bp which is close to the enolase binding site. These arginine-rich regions are believed to be independently involved in RNA binding (Marcaida *et al.*, 2006). The microdomains for the chaperones and Ppk have not yet been determined.

Morita *et al.* (2005) proposed that the homohexameric chaperone Hfq is also recruited to the degradosome, to assist in the processing of transcripts.

Loss of the C-terminus would result in substrates of the degradosome no longer being degraded (Kido *et al.*, 1996). Even just the loss of RhlB and enolase would cause a decrease in activity of the degradosome (Leroy *et al.*, 2002).

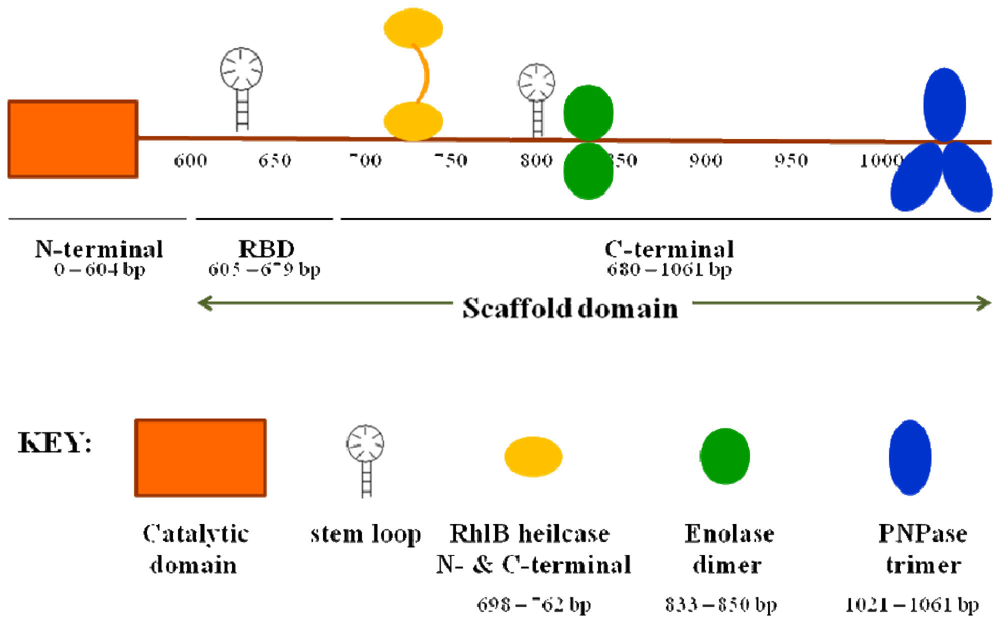

Figure 1.1 Representation of RNase E and its assembled components to form the RNA degradosome. The N- and C-terminus of RNase E are separated by a short region called the RNA-binding domain (RBD). Enolase, RhlB helicase and PNPase assemble at the scaffold domain of the C-terminus at 833 – 850 base-pairs (bp), 698 – 762 bp and 1021 – 1061 bp respectively. There are two arginine-rich regions, one close to the enolase binding site and the other within the RNA binding domain. They are suspected to independently facilitate RNA binding (adapted from Marcaida *et al.*, 2006).

Localization studies have indicated that RNase E is primarily found in the region of the cytoplasmic membrane in *E. coli* cells (Liou *et al.* 2000). The degradosome is associated with the cytoplasmic membrane through the N-terminus of RNase E (Liou *et al.* 2001). Unlike the C-terminus region, the N-terminus region is not essential for the formation of the degradosome (Kaberdin *et al.*, 1998). The N-terminus region of RNase E holds sequences engaged in oligomerization (Vanzo *et al.,* 1998).

The degradation of *E. coli* RNA transcripts, as well as the processing thereof, has been proven to be greatly influenced by the role played by ribonucleolytic RNase E (Deutscher and Li, 2001; Condon and Putzer, 2002). These roles include degradation of numerous mRNAs, processing of an assortment of RNAs, and regulation of the production of particular plasmid DNAs (Deutscher and Li, 2001; Kushner, 2002).

Endonuclease RNase E is vital for cellular viability. Other than providing the organizational scaffold for the constituents of the degradosome, RNase E is involved in processing of RNAs, and in plasmid and phage stability. It processes the 9S ribosomal precursor producing the mature 5S ribosomal RNA (Apirion, 1978; Ghora and Apirion, 1978; Roy *et al.,* 1983). Similarly the 17S ribosomal precursor is processed yielding mature 16S rRNA (Bessarab *et al.,* 1998; Li *et al.,* 1999). The endonuclease is also capable of cleaving 16S and 23S rRNAs single-stranded regions (Bessarab *et al.*, 1998; Li *et al.,* 1999).

RNase E controls its own production levels by cleaving its mRNA (Mudd and Higgens, 1993; Jain and Belasco, 1995). The discovery that a minor component of the degradosome, RraA, (regulator of ribonuclease activity A) prevents the endonucleolytic cleavage of RNase E (Lee et al., 2003) and therefore in its presence allows restored growth to *E. coli* cells with an over- production of RNase E (Yeom and Lee, 2006) led to the finding that over-producing the E. coli RNase E protein results in a decrease of cellular growth (Claverie-Martin *et al.,* 1991; Jain *et al.,* 2002). *E. coli* growth needs a concentration of 10 – 20 % cellular RNase E whereas growth is compromised when *E. coli* cells contain multiple copies of RNase E plasmids (Claverie-martin *et al.,* 1991; Jain *et al.*, 2002). Yeom and Lee (2006) showed that the over-expressed RNase E-induced stop in cell growth occurs as a result of a higher level of cellular ribonucleolytic activity.

1.4.2 Polynucleotide phosphorylase

The first bacterial ribonuclease to be discovered was PNPase in *Azotobacter vinelandii* in 1955, albeit that it was identified to synthesize RNA as opposed to degrading it (Grunberg-Manago et al., 1955). Since, the ribonuclease activity of PNPase has been found in eubacteria, Archaea, eukaryotic microbes, plants and animal cells (Kessler and Chen, 1964; Fit and See, 1970; Peterkin and Fitt, 1971; Wood and Hutchinson, 1976; Estévez *et al.,* 2001). PNPase is considered to be one of the key proteins in mRNA turnover in *Bacillus subtilis*. Approximately 80% of RNA degradation is phosphorylytic as opposed that of *E. coli*, which amounts to only 30% (Chaney and Boyer, 1972; Duffy *et al.,* 1972; Deutscher and Reuven, 1991).

PNPase exists as a 85 kDa trimer, containing three monomeric units of polynucleotide phosphate (Portier, 1975; Soreq and Littauer, 1977; Carpousis *et al.,* 1994). The RNA binding sites are found within each monomer and although not essential for the enzymes catalytic function, are necessary for polynucleotide phosphate autoregulation, growth during cold shock, and for the production of oligonucleotides (Guissani and Portier, 1976; Jarrige *et al.,* 2002; Matus-Ortega *et al.,* 2007).

PNPase has the dual purpose of 3´ to 5´ exoribonuclease activity and oligonucleotide polymerase activity at the 3´terminus (Yehudai-Resheff *et al.,* 2001). In addition to being a component of the RNA degradosome and facilitating the degradation of a range of mRNAs, this trimer has functions including being involved in the process of degrading and processing tRNA, attaching poly(A) tails to particular RNAs (Carpousis *et al.,* 1999; Coburn and Mackie, 1999; Mohanty and Kushner, 2000). mRNA degradation is dependent on the presence of PNPase, its absence results in a boost above the normal levels of mRNA (Kinscherf and Apirion, 1975). PNPase further contributes to mRNA degradation by degrading the rRNA fragments degraded by other RNases during carbon starvation (Kaplan and Apirion, 1974; Kaplan and Apirion, 1975).

1.4.3 ATP-dependent helicase RhlB

E. coli DEAD box proteins have been found to be involved in essential cellular functions. Jagessar and Jain (2010) found DEAD-box helicase to facilitate the biogenesis of the 50S ribosome at decreased temperatures, as well as at *E. coli's* optimal growth temperature, 37°C. Strains lacking DeaD displays flawed rRNA processing and ribosomal maturation at both 37°C as well as at low temperatures, and because 50% of the overall cellular RNA can be accounted for by the weight of 23S rRNA (Nomura *et al.*, 1984), DeaD is engaged in an important metabolic process (Jagessar and Jain, 2010).

RhlB, found in both prokaryotes and eukaryotes, belongs to the DEAD-box helicase family, which has a consensus sequence of aspartic acid - glutamic acid - alanine - aspartic acid (DEAD). The 50 kDa RhlB is a monomeric helicase that associates to the degradosome by binding to the C-terminus of RNase E, forming homodimers, and is also capable of binding PNPase when RNase E is not present (Liou *et al.*, 2002).

The association of RhlB helicase to the degradosome increases its ATPase activity fifteen fold, but it has not been determined how the interaction of RNase E activates RhlB (Vanzo, 1998). The helicase class of enzymes functions to unwind double stranded polynucleotide by using energy obtained from ATP hydrolysis. RhlB helicase as a constituent of the degradosome is thought to coerce the energy-dependent dislocation of proteins from RNA (Fairman *et al.*, 2004). RhlB facilitates the degrading activity of PNPase within the degradosome through the structuring of RNA, and is absolutely necessary for the full functioning of the degradosome (Py *et al.*, 1996; Coburn and Mackie, 1999).

The "inchworm" stepping model has been used to describe the mechanism of helicases. There is a consecutive transfer of nucleic acid substrate between two binding sites during the ATP-consuming cycle (Velankar *et al.*, 1999). The helicase monomer has two RNA binding sites, a translocator and a duplex-opening site (Dumont *et al.*, 2006). The RNA substrate binds to a single-stranded 3' RNA binding site. The duplex-opening site moves 2-5 bp to unwind the substrate after the translocator has moved in 11 bp to recognize the

RNA substrate. The movement between the two sites is associated with the ATP-binding cycle, hydrolysis and unwinding of RNA or translocation (Dumont *et al.*, 2006).

1.4.4 Enolase

The enolase superfamily is categorized into three subgroups according to their catalytic mechanism (Gulick et al., 2000). Mandelate racemase, muconate cycloisomerase and enolase share common features which include structural homology, functional similarity and a C-terminus active site (Gulick *et al.*, 2000).

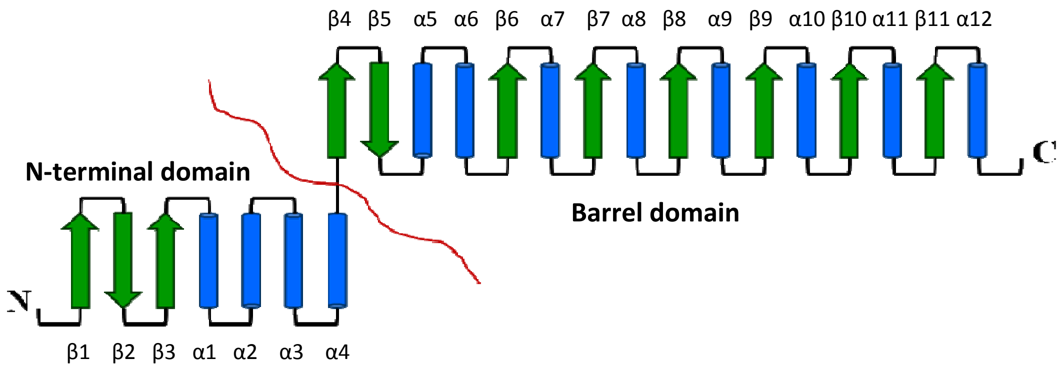

Figure 1.2 Representation of the C- and N-terminus of enolase
The arrows indicate β-strands (S) and the barrels α-helices (H). The boundary between the two domains is indicated. The $\beta\beta\alpha\alpha(\beta\alpha)_6$ topology of the barrel domain (C-terminus) as well as the N-terminus α/β-domain is clearly represented (adapted from Kuhnel and Luisi, 2001).

Enolase comprises two domains - the smaller N-terminus domain and the C-terminus domain (Figure 1.2) (Kuhnel and Luisis, 2001). The C-terminus is an eight-stranded β/α barrel which consists of eight β-strands surrounded by eight α-helices (Kuhnel and Luisis, 2001). The barrel domain has a $\beta\beta\alpha\alpha(\beta\alpha)_6$ topology which is shown in Figure 1.2. The N-terminus α/β-domain is made up of three-stranded anti-parallel β-sheets and four α-helices (Kuhnel and Luisis, 2001). The native protein dimer was shown to have a molecular weight of 92 kDa through the use of high pressure liquid chromatography (HPLC), and protein separation through sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) indicated the enolase subunit to separate at a molecular weight of 46 kDa

(Dannelly et al., 1989). The dimer contains two magnesium ions. Enolase was shown to lose activity and to start to denature when magnesium was absent (Spring and Wold, 1971). The crystal structure originally showed the dimer interface of enolase to have an abundance of charged residues (Kuhnel and Luisi, 2001). However, five years later, a lower resolution indicated the RNase E recognition site binding enolase (Chandran and Luisi, 2006). Enolase shares common features across organisms, including its need for magnesium, its catalytic restrictions and being restrained by the fluoride ion when phosphate is present (Spring and Wold, 1971).

Enolase is commonly known as a glycolytic enzyme catalyzing an inter-conversional reaction in glycolysis and gluconeogenesis. The reaction of 2-phosphoglycerate to phosphoenol pyruvate leads to the production of pyruvate via pyruvate kinase, which is the energy-producing step (Figure 1.3).

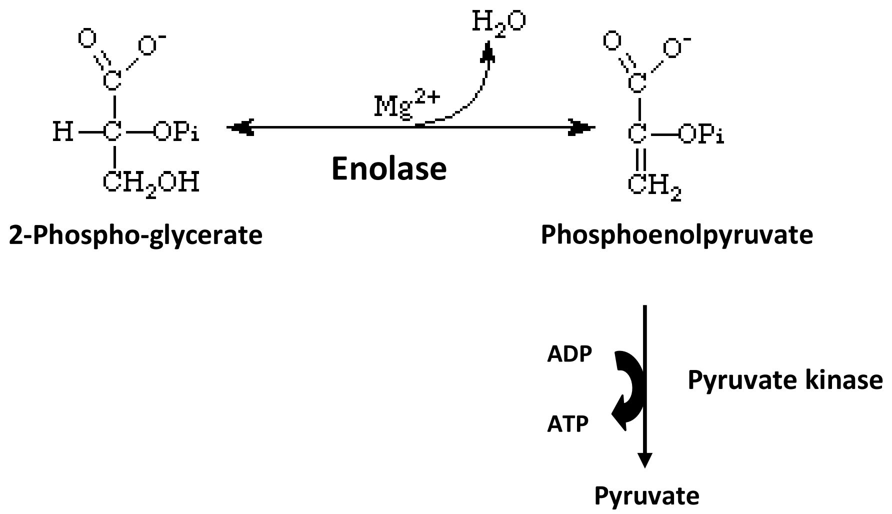

Figure 1.3 The reaction catalysed by enolase in the glycolytic metabolic pathway.
Enolase catalyses the inter-conversion of 2-phospho-glycerate to phosphoenolpyruvate with the release of a water molecule. The next reaction releases ATP in the conversion to pyruvate via pyruvate kinase.

Dephosphorylation of glucose-grown enolase treated with acid phosphatase resulted in a 70% decrease of activity (Danelly *et al.,* 1989). It was proposed that there could be an explosion of cellular gluconeogenic activity when required (Danelly *et al.,* 1989). The occurance was explained by suggesting that enolase has two phosphorylation sites, the forward reaction activity being under the control of one site and the reverse activity under the control of both sites (Dannelly *et al.*, 1989). The majority of enzymes concerned with degradative pathways have been noted to be active in the phosphorylated state and inactive in the dephosphorylated state (Ranjeva and Boudet, 1987; Randall and Blevins, 1990; Bennet, 1991; Creighton, 1993).

A specific function of enolase in *E. coli* is controlling the stability of *ptsG* mRNA which encodes for $IICB^{GLC}$, the main glucose transporter, in response to metabolic stress (Morita et al., 2004). Enolase and RNase E play a role in the response to stress caused by an overabundance of phosphosugar. By demonstrating that the rapid degradation of *ptsG* mRNA was inhibited by the removal of the scaffold under metabolic stress, and that a reduction of enolase (and not PNPase or RhlB) stops the rapid degradation of *ptsG* mRNA, Morita and colleagues (2004) came to the conclusion that enolase within the degradosome modulates RNase E action on *ptsG* mRNA under metabolic stress. RNase E, and thus the degradosome, was shown to chiefly localize close to the cytoplasmic membrane (Liou *et al*., 2001). *ptsG* mRNA located at the inner membrane along with $IICB^{Glc}$ that targets the membrane co-translationally is vital for weakening *ptsG* mRNA under metabolic stress (Morita *et al.*, 2004). It was therefore hypothesized that enolase directs compartmentalization of the degradosome to the plasma membrane for *ptsG* mRNA to then be degraded (Morita *et al*., 2004).

Enolases have been described as having “moonlighting” functions (Pal-Bhowmick et al., 2007) which refers to unsuspected or unappreciated “second jobs”, or in the case of enolase, non-glycolytic functions. Many proteins have been found to have “moonlighting” functions and often it is as a result of switching cellular localizations, oligomeric states or varying ligand concentrations (Jeffery, 1999). Iida and Yahara (1985) discovered enolase and HSP48 (heat shock protein in yeast) to be identical, thereby suggesting enolase to be significant in the tolerance of various temperatures and control of growth in yeast. Enolase has been found to associate with the yeast cell wall (Edwards *et al.,* 1999). In the inner layers of the cell wall in *Caenorhabditis albicans,* enolase binds with glucan and is an important antigen in general infections of candidiasis (Angilella *et* *al.*, 1996). Enolase was shown to have weak *in vivo* RNA-binding properties in *E. col*i and yeast (Entelis *et al.*, 2006). It lowers the dissociation constant (Kd) of an RNA complex in yeast, tRK1–preMsk1p. The fact that the affinity of tRK1 to preMsk1p is higher than that of tRK1 to enolase (showing an apparent Kd decrease of fourteen-fold) supports this finding (Entelis *et al.*, 2006). The enzyme was also shown in the generation of phosphoenolpyruvate for an activating phospho-transfer reaction (Entelis *et al.*, 2006). Enolase in the degradosome may serve to link cellular metabolic status with post-transcriptional gene regulation (Chandran and Luisi, 2006). Kuhnel and Luisi (2001) suggested that enolase could potentially couple RNA degradation to *in vivo* glycolytic processes. As of yet (2010), very little is known about the exact role enolase plays within the RNA degradosome.

1.4.5 <u>Polyphosphate kinase</u>

The transferase polyphosphate kinase (Ppk) is a tetramer of 69 kDa. Polyphosphate is synthesized by the reversible reaction of Ppk transferring a terminus phosphoryl group of adenosine triphosphate (ATP) to the acceptor, a polyphosphate polymer (Hoffmann-Ostenhof *et al.*, 1954). The role of Ppk in *E. coli* has not been determined but it is thought that this enzyme could be the source of energy, could replace ATP in kinase reactions or could be a pool of phosphate with osmotic benefits, thereby upholding a suitable micro-environment for the degradosome (Kornberg, 1957; Dirheimer and Ebel, 1965). Ppk can also take on the role of a nucleoside diphosphate kinase, converting diphosphates to triphosphates, the reverse of the above reaction of the synthesis of polyphosphate. Ppk probably functions to autophosphorylate when it is a part of the degradosome because of the form it is in as a tetramer – Ppk exists as a trimer when it generates linear guanosine tetraphosphate, and as a dimer when it autophosphorylates (Tzeng and Kornberg, 2000). The functions of Ppk appear to include upholding a suitable micro-environment and regenerating ATP, which is required by the degradosome, as well as eliminating polyphosphates and nucleotide diphosphates, which are inhibitory (Blum *et al.*, 1997).

1.4.6 Functions and assembly of the *E. coli* RNA Degradosome

The *E. coli* RNA degradosome is a conglomeration of proteins interacting to serve the general purpose of mRNA degradation. The joined endo- and exoribonucleolytic activities of RNase E and PNPase are directed by the complex. Both RNase E and PNPase are hindered by RNA secondary structure which renders the interaction of RhlB with the degradosome important (Coburn *et al.*, 1999). PNPase will frequently be forced to pause due to regions of RNAs that have internal structures; RhlB accommodates PNPase by unwinding these structures (Carpousis, 2002). PNPase degrades transcripts in a 3'-5' pathway, and through the use of inorganic phosphates produces nucleotide diphosphates such as adenosine diphosphate (ADP) (Carpousis, 2002). Even though PNPase is a phosphate-dependent exonuclease, it is important to note that exonucleases are defined as hydrolytic enzymes (Donovan and Kushner, 1986). *E. coli* strains that do not contain poly(A) polymerase show retarded degradation. This is due to poly(A) polymerase adding poly(A) tails to the 3' ends thereby accelerating mRNA decay (Xu *et al.*, 1993).

Many bacterial mRNAs as well as nascent transcripts have 3' ends sequestered in stem loops (Carpousis, 2002), which protect the mRNAs from degradation. Endonucleases serve to produce single-stranded substrates for the exonucleases by removing the 3' end stem loops and freeing the nascent transcripts (Carpousis, 2002). Single-strand-specific RNase E plays an important part in the degradation of mRNAs by initiating endonucleolytic cleavage. Covalently closed circular mRNA is immune to the action of RNase E (Bouvet and Belasco, 1992). The cleavage of 5' monophosphate ends of linear templates occurs at a much faster rate than that of 5' triphosphate ends, which could explain why stem-loops hinder degradation mediated by RNase E (Bouvet and Belasco, 1992). This, as well as oligonucleotides that hybridize to the 5' ends, serve as protective mechanisms for nascent transcripts (Bouvet and Belasco, 1992). RNase E activity produces 5' monophosphates and since these are the favoured substrates of RNase E, the initial cleavage which occurs more slowly is predicted to lead to a later rapid cleavage (Carpousis, 2002). The resulting mRNA pieces are cut by PNPase and RNase II (3' to 5' exonucleases), and by poly(A) polymerase (Morita *et al.*, 2004).

Sequence analysis has revealed that the RNase E scaffold region is not likely to have a distinct structure, but that it is inclined to condense (Marcaida *et al.*, 2006). *E. coli* RNase E was shown to exist in the form of a homotetramer after the crystal structure of the N-terminus half of RNase E had been determined (Callaghan *et al.*, 2005). The RNA-binding domain forms a homodimer. The RNase E tetramer consists of a coupling of dimers to form a dimer with a divalent magnesium ion (Callaghan *et al.*, 2005). This dimer of dimers is kept by cysteine-zinc-cysteine associations between adjacent RNase E monomeric units (Callaghan *et al.*, 2005). Marcaida *et al.* (2006) suggested that "unstructured tails" extend from the catalytic domain, each of which can associate with four enolase dimers and four helicase monomers (Figure 1.4). PNPase is capable of associating with three of the RNase E tails. If all the protein-protein interaction sites were filled there would be a ratio of three RNase E tetramers to four PNPase trimers. However, it has not been determined if all the constituents can associate with the degradosome simultaneously (Morita *et al.*, 2005). RNase E must have its RNA-binding regions facing outward and so needs to be in the form of a tetramer in order to function catalytically (Callaghan *et al.*, 2003).

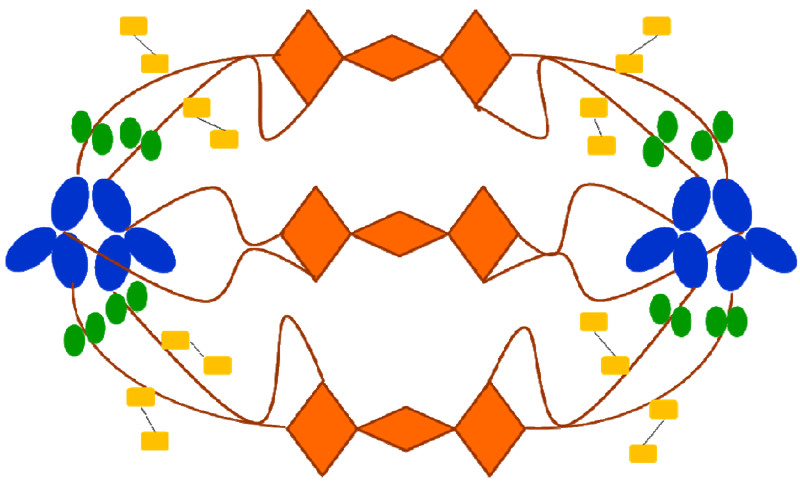

Figure 1.4 Schematic of the ratio and assembly of the components of the *E. coli* RNA degradosome
There is a ratio of 4 PNPase trimers (blue) to 3 RNase E tetramers (orange). The enolase dimers (green) and the RhlB helicase domains (yellow) bind to RNase E through its C-terminus (adapted from Marcaida *et al.*, 2006).

1.5 Molecular chaperones

The term molecular chaperone was first used by Laskey in 1978 to define a nuclear protein that resolved misassembly of nucleosomes in the process of formation in amphibian eggs (Laskey *et al.,* 1978). Later the term was broadened to include the protein rubisco located in the chloroplast (Barraclough and Ellis, 1980; Musgrove and Ellis, 1986). Eventually "molecular chaperone" was defined as proteins that facilitate folding and assembly reactions (Ellis, 1987). Chaperones bind to hydrophobic residues of unfolded proteins functioning to prevent unsuitable molecular interactions (Lund, 2001). However the final structure of the native polypeptide excludes that of the chaperone because the chaperone releases the substrates (Lund, 2001). Chaperones have been divided into "holder" and "folder" categories depending on their functioning either to inhibit protein aggregation or promote correct protein folding (Lund, 2001). Molecular chaperones have a wide range of functions, including degradation of proteins, translocation, folding of co-translational products, protein function regulation and protein complex assembly (Eggers *et al.,* 1997; Hamman *et al.,* 1998; Brodsky and McCracken, 1999; Lund, 2001). Chaperones in essence prevent the association of hydrophobic surfaces with each other by holding non-native intermediates that are unfolded and in so doing prevent the build-up of proteins that are misfolded when the cell is under stress (Schroder *et al.,* 1993; Parsell *et al.,* 1994). To make sure the proteins don't keep aggregating the intermediates are then either degraded or refolded once the cell is no longer under stress (Schroder *et al.,* 1993; Parsell *et al.,* 1994).

The large and varied heat shock protein family is classified into major classes as defined according to molecular weight (Table 1.1). The term DnaK refers to the *E. coli* member of the eukaryotic 70 kDa heat shock protein (Hsp70) family of proteins (Bardell and Craig, 1984). HscA and Hsc62 are also members of this family with cochaperones HscB and HscC, respectively (Silberg *et al*., 1998; Hoff *et al*., 2000; Arifuzzaman *et al*., 2002). DnaK's cochaperone, DnaJ, is the *E. coli* member of the Hsp40 eukaryotic family (Feldheim *et al.,* 1992). The ATP-dependent GroEL/GroES system belongs to the Hsp60 family (Fenton and Horwich, 1997). The prokaryotic HtpG belongs to the protein family Hsp90 and is also an ATP-dependent chaperone (Jakob *et al.,* 1995). ClpA, ClpB, ClpX and ClpY, amongst others, all form part of the Hsp100 family and are involved in ATP-dependent proteolysis and reversing protein aggregation (Mhammedi-Alaoui *et al,.* 1994; Squires *et* *al.,* 1991;Wang *et al.,* 1997; Gottesman *et al.,* 1997; Bochtler *et al.,* 2000). ClpA/B/X share co-chaperone ClpP and ClpY has co-chaperone ClpQ. Prokaryotic Hsp33 is a member of the Hsp30 family and is involved in prevention of protein aggregation; and small hsp's form a class of their own and include *E. coli* proteins IbpA and IbpB (Kaldis *et al.,* 2004).

Table 1.1 Major molecular chaperone families and corresponding *E. coli* members

Chaperone Family	*E. coli* member	Co-chaperone	Function	References
Hsp100	ClpA	ClpP	ATP-dependent proteolysis	Gottesman *et al.* (1997); Wang *et al.* (1997)
	ClbB		Reversing protein aggregation	Squires *et al.* (1991)
	ClpX	ClpP	ATP-dependent proteolysis	Mhammedi-Alaoui *et al.* (1994)
	ClpY(HslU)	ClpQ (HslV)	ATP-dependent proteolysis	Bochtler *et al.* (2000)
	FtsH		ATP-dependent proteolysis	Neuwald *et al.* (1999)
	Lon		ATP-dependent proteolysis	Neuwald *et al.* (1999)
Hsp90	HtpG		ATP-dependent chaperone	Jakob *et al.* (1995)
Hsp70	DnaK	DnaJ, CbpA, DjlA	Refolds nascent peptides	Hartl and Hayer-Hartl (2002)
	HscA	HscB	Reversing protein aggregation	Silberg *et al*. (1998); Hoff *et al*. (2000)
	Hsc62	HscC	Regulates transcription	Arifuzzaman *et al*. (2002)
Hsp60	GroEL	GroES	ATP-dependent chaperone	Fenton and Horwich (1997)
Hsp40	DnaJ		Protein translocation	Feldheim *et al.* (1992)
Hsp30	Hsp33		Prevents aggregation	Kaldis *et al.* (2004)
Small Hsp	IbpA, IbpB		Prevents aggregation	Kaldis *et al.* (2004)

Synthesis of molecular chaperones increases when the cell is under stress such as that of heat shock (Eggers *et al.,* 1997). The heat shock response results in an increase of heat shock protein (Hsp) gene transcription which leads to increased synthesis of Hsp proteins (Finkelstein *et al*., 1982). DnaK is induced by heat shock and is essential for the cell at temperatures above 37°C (Vorderwulbecke *et al.*, 2004). This response is controlled by the σ^{32} subunit of RNA polymerase, which is specific to the heat shock promoter, at the level of transcription (Gross, 1996; Arsene *et al*., 2000). DnaK, along with co-chaperones DnaJ and GrpE, are key factors of the heat shock regulon via σ^{32} (Bukau, 1993). GrpE mRNA expression, being under the control of the promoters σ^{70} and σ^{32}, is induced quickly at increased temperatures (Lipanska *et al.*, 1988). In the presence of ATP, a complex is formed between σ^{32} transcription factor, and purified DnaK and DnaJ. This association shuts off the heat-shock-mediated gene expression by preventing the binding of σ^{32} to RNA polymerase (Liberek and Georgopoulos, 1993**).** It was proposed that if DnaK were to fail in the promotion of protein folding, a protein would specifically be marked for rapid degradation (Sherman and Goldberg, 1992).

1.5.1 *E. coli* DnaK

Interestingly Hsp70 members are found in all eukaryotic organelles (cytosol, chloroplasts, endoplasmic reticulum and mitochondria) but not in all prokaryotic organelles (Ungewickell 1985; Munro and Pelham, 1986; Chirico et al., 1988; Murakami et al., 1988). The two classes of Hsp70s include those that are constitutively expressed termed heat shock cognate (Hsc) proteins (Ingolia and Craig, 1982) and the stress inducible proteins (Hsp70). The primary difference between the two forms is the cognate protein will function as a molecular chaperone during standard environmental conditions whereas the heat shock protein will act under conditions of stress.

Hsp70 proteins are highly expressed under normal cellular conditions functioning to fold nascent polypeptides by binding and stabilizing them, and thus allowing the hsps time to fold the polypeptides correctly; assemble and disassemble large protein complexes and translocate newly synthesized polypeptides across membranes (Ungewickell 1985; Munro and Pelham, 1986; Chirico *et al.*, 1988). When the cell is placed under stress conditions such as heat shock, *E. coli* DnaK along with its co-chaperones DnaJ and GrpE, functions to bind to nascent polypeptides and proteins that are partially folded to prevent aggregation and facilitate refolding, or otherwise guide the intermediate folded polypeptides towards degradative machinery (Freeman *et al.*, 1995; Minami *et al.*, 1996; Bercovich *et al.*, 1997; Eggers *et al.*, 1997). DnaJ and GrpE regulate DnaK through ATP hydrolysis and exchange of ADP/ATP, respectively, and are thus involved in facilitating the nascent polypeptide folding (Cyr *et al.*, 1994).

Three Hsp70 proteins are present in *E. coli*: DnaK, HscA and Hsc62 (Ingolia and Craig, 1982; Bardwell and Craig, 1984). DnaK is the primary prokaryotic homologue of Hsp70. HscA is the second homologue of Hsp70 and is approximately five times less plentiful than DnaK (Hesterkamp and Bukau, 1998). Co-chaperone HscB interacting with HscA cannot replace the functioning of DnaK along with its co-chaperone, DnaJ, and nucleotide exchange factor, GrpE (Hesterkamp and Bukau, 1998). HscA/HscB has been determined to have a role in the assembly of iron sulfur clusters in iron sulfur proteins (Zeng *et al.,* 1998). The cognate genes (*hscA* and *hscB*) form part of a cluster of genes containing *isc* (iron sulfur cluster assembly) genes. The gene products are able to repair the bacterial iron sulfur protein equipment, thus possessing a "housekeeping" function (Zeng *et al.*, 1998). The third Hsp70 homologue, Hsc62, also is not capable of replacing DnaK functioning (Yoshimune *et al.*, 2002). Yoshimune *et al.* (2002) determined Hsc56 (a gene product of ybeV with a J-domain like sequence) and GrpE influence the ATPase activity of Hsc62. *E. coli* thus contains three Hsp70 chaperone systems: Dnak/DnaJ/GrpE, HscA/HscB and Hsc62/Hsc56/GrpE.

E. coli DnaK with a molecular mass of 70 kDa has three domains spanning 638 nucleotide residues. The primary sequence consists of a 45 kDa adenosine triphosphatase (ATPase) domain (residues 1-385) at the N-terminus region, which contains binding sites for the co-chaperone GrpE, an 18 kDa substrate-binding domain (residues 385-561) and a 10 kDa C-terminus domain (residues 561-638) (Zhu *et al.,* 1996) (Figure 1.5). The substrate-binding domain appears to be specific for substrates rich in phenylalanine, isoleucine and valine (Richarme and Kohiyama, 1993; Gragerov *et al.*, 1994).

The ATPase domain of 45 kDa, spanning residues 1 – 385, is a common structural feature across proteins possessing different biological function, including bovine Hsc70, *E. coli* DnaK and human Hsp70 (Figure 1.5) (Flaherty *et al.*, 1990; Harrison *et al.*, 1997; Osipiuk *et al.*, 1999). It consists of two similar lobes on either side of a cleft which contains the ATP binding site at the bottom. The ATPase domain contains the binding site for GrpE (residues 20 – 60, 129 – 132, 257 and 285). The substrate binding domain of 18 kDa, spanning residues 386 – 561, is comprised of a subdomain containing a sandwich of β-sheets and a domain of α-helix segments termed the lid which serves to stabilize the complex (Zhu *et al.*, 1996). The substrate binding portion binds the peptide through a canal formed by the loops of the β-sandwich (Zhu *et al.*, 1996).

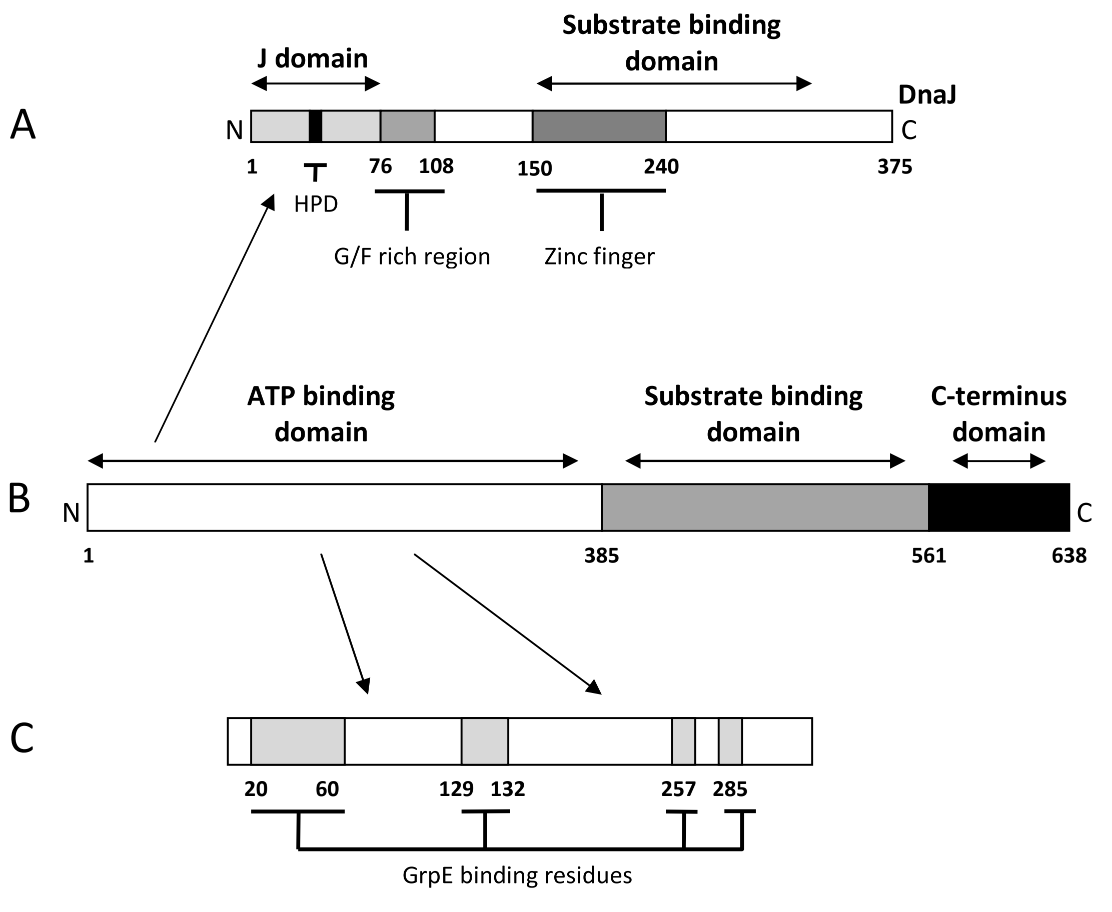

Figure 1.5 Schematic representation of the structure of *E. coli* DnaK.
(A) *E. coli* DnaJ contains the J-domain (residues 1 – 75) which possesses a highly conserved tripeptide (HPD), the G/F rich region (residues 76 – 108), a zinc-finger like domain (residues 150 – 240) and the C-terminus region (residues 240 – 375). (B) *E. coli* DnaK consists of 638 nucleotide residues grouped into three domains. The ATP binding domain at the amino terminus covers residues 1-385, the substrate binding domain covers residues 385-561 and the regulatory domain at the carboxy terminus covers residues 561-638. (C) Co-chaperone GrpE associates with DnaK at the GrpE binding sites located in the ATPase binding domain (adapted from Lund, 2001).

The C-terminus domain of 10 kDa, spanning residues 562 – 638, is less well characterized. Both the ATPase and substrate binding domains are necessary for complete chaperone activity (Freeman *et al.* 1995). DnaJ contains the J-domain that is needed for stimulation of ATPase activity of DnaK (Figure 1.5) (Langer *et al.*, 1992). The J-domain has 4 helices that contain a highly conserved tripeptide (HPD), necessary for association with DnaK (Pellecchia *et al.,* 1996). The G/F rich region of DnaJ facilitates packing of the J-domain and is thus needed along with the J-domain to stimulate ATPase activity, or ATP-dependent substrate binding (Wall *et al.,* 1995). The zinc-finger like domain and the C-terminus region also form part of DnaJ. DnaJ is able to function freely from DnaK, maintaining unfolded proteins in a folded competent state (Szabo *et al.,* 1996).

The DnaK folding cycle begins with DnaK binding a nascent peptide; the allosteric coupling of the ATPase domain allows binding and releasing of substrates (Figure 1.6). DnaK has a low affinity for unfolded proteins when adenosine triphosphate (ATP) is bound. DnaJ binds DnaK and stimulates ATPase activity by stimulating hydrolysis. Hydrolysis of ATP to ADP changes the low affinity to a high affinity for unfolded proteins at the substrate-binding domain (Liberek *et al.,* 1991). A higher affinity increases the time factor for protein stabilization resulting in more proteins being folded more accurately (Liberek *et al*., 1991). DnaJ bound to DnaK facilitates the association of the substrate with DnaK (Mayer *et al.,* 2000). DnaJ is also capable of recruiting DnaK to unfolded polypeptides by directly interacting with them (Young *et al.,* 2003). In the next step of the cycle nucleotide exchange factors, such as GrpE, binds to the ATPase domain to catalyze the ADP-ATP exchange (Figure 1.6). GrpE binds to ADP-bound DnaK, stabilizing the nucleotide-binding cleft of the DnaK ATPase domain into an "open" conformation (Harrison *et al.,* 1997). The presence of ATP however results in an immediate release of DnaK from GrpE (Brehmer *et al.,* 2001). The binding ability of DnaK for ADP is decreased by 200-fold by GrpE, and ADP decreases the affinity of GrpE for DnaK by 200-fold (Packschies *et al.,* 1997). This allows the release of the substrate. The substrate can go in one of several directions: it can be folded, refolded by re-entering the folding cycle, degraded or taken up by another chaperone for further processing (Figure 1.6).

DnaK associates with the RNA degradosome under conditions of stress such as over-production of RNase E, when cells are grown in the cold, at temperatures below 15˚C (Lelivelt and Kawula, 1995) or at elevated temperatures such as heat shock (Regonesi et al., 2006).

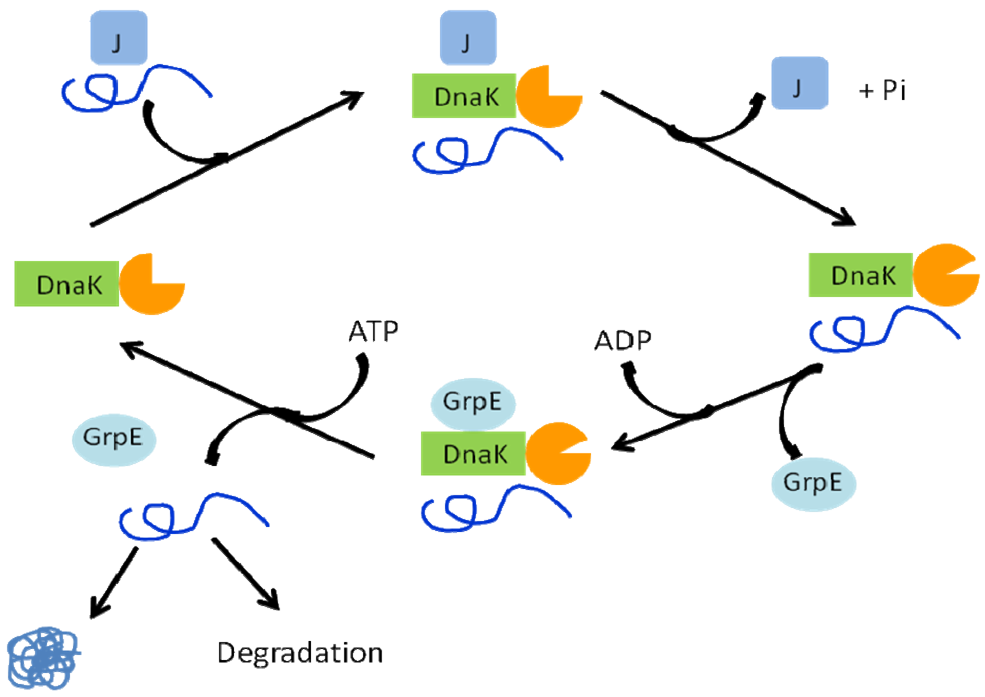

Figure 1.6 Schematic of the DnaK folding cycle
DnaJ mediates the passage of unfolded peptide to ATP-bound DnaK. Bound DnaJ causes ATP to be hydrolysed to ADP resulting in DnaK binding the peptide tightly. DnaJ is released from DnaK along with inorganic phosphate. GrpE catalyzes the release of ADP from the DnaK complex. ATP binds allowing the release of the peptide by inducing the opening of the αhelical lid of DnaK. The dissociated peptide folds into its native form or is degraded (adapted from Hartl and Hayer-Hartl, 2009)

Proteins aggregate under elevated temperatures. It appears that DnaK needs to be present in order for the degradosome to assemble and that it is required in immature degradosome intermediates that build up under conditions of stress (Regonesi et al., 2006). It is not known what the role of DnaK is when it associates to the RNA degradosome or even if its presence is required constantly.

1.5.2 *E. coli* GroEL

The GroEL system has been described as a nano-machine found in chloroplasts, mitochondria and bacteria, and is required by *E. coli* for growth (Fenton and Horwich, 1997; Sigler *et al.,* 1998; Ellis, 2001; Vorderwulbecke *et al.,* 2004).

GroEL has a complex arrangement of fourteen 57-kDa subunits in a double layer of seven-membered rings (Figure 1.7) (Fenton and Horwich, 1997; Sigler *et al.,* 1998; Ellis, 2001). Non-native proteins bind to the two binding sites found in the hollow cylinder formed by the 14 subunits (Figure 1.7) (Lund, 2001). The 10 kDa co-chaperone GroES is a seven-membered single ring that is dome-shaped. The conformation of GroEL changes upon the binding of GroES.

It is only the chaperone GroEL, along with its co-chaperone GroES that are crucial for *E. coli* growth under any cellular conditions (Fayet *et al.,* 1989). GroEL/GroES partakes in many biological cellular processes including folding newly produced polypeptides, maintenanace of protein structure, proteolysis, secretion and morphogenesis of λ and T4 (Friedman *et al.,* 1984; Kusukawa *et al.,* 1989; Laminet *et al.,* 1990; Horwich *et al.,* 1993; Kandror *et al.,* 1994; Houry *et al.,* 1999).

Through hydrophobic interactions, GroEL interacts selectively with nascent peptide chains to minimize off-pathway interactions (Fenton and Horwich, 1997; Sigler *et al.,* 1998; Ellis, 2001). The binding of ATP and GroES to GroEL results in a cyclic change of its conformation – a cis ternary complex is formed whereby the polypeptide is completely surrounded by the GroEL-GroES structure, which controls the chaperone-substrate interaction (Fenton and Horwich, 1997; Sigler *et al.,* 1998; Ellis, 2001). The conformational change increases the span between the two polypeptide binding regions which can stimulate the unfolding of the substrates due to the stretching forces placed on it (Fenton and Horwich 1997; Xu and Sigler 1998; Coyle *et al.,* 1999; Horwich *et al.,* 1999; Shtilerman *et al.,* 1999). Nucleotide binding thus changes the high-affinity state of GroEL to a low-affinity state for substrate binding and so controls the binding and dissociation of substrates.

Folding chaperones DnaK and GroEL differ in the type of substrates they bind. DnaK binds native polypeptides; GroEL binds highly unstructured non-native polypeptides. Research concerning non-native polypeptide is difficult because of their tendency to aggregate or refold (Lin *et al.,* 1994). Unfolded proteins have a high degree of hydrophobic surface that is exposed, motivating the finding that the association of non-native polypeptide chains with GroEL is hydrophobically driven (Lin *et al.,* 1994). GroEL thus recognizes substrates through their exposed hydrophobicity, binding the segments through hydrophobic interactions (Lin *et al.,* 1994). The hydrophobic amino acids stimulate the ATPase activity of GroEL (Richarme and Kohiyama, 1994).

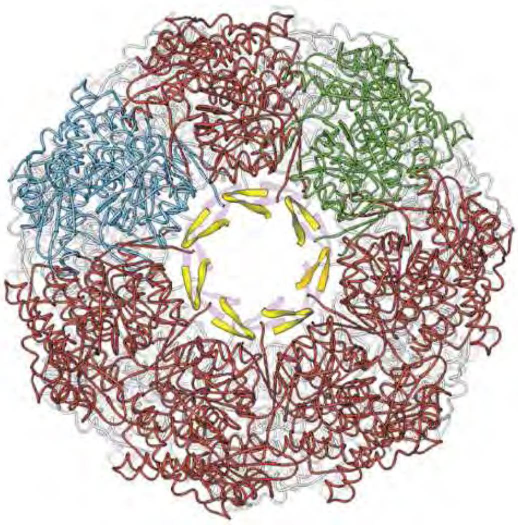

Figure 1.7 Crystal structure of GroEL in complex with bound peptide
The structure shows the view down the seven-membered ring. The GroEL lower ring (silver) and upper ring (yellow) binds the peptide (magenta). Two of the GroEL upper subunits are represented with blue and green (Wang and Cheng, 2003).

1.6 Problem statement and motivation

The degradosome functions as an RNA-processing and decay machine and its key components have been identified. It is however not known how molecular chaperones associate with the RNA degradosome, where they bind the scaffold protein, or even what their specific functions are within the protein complex. Molecular chaperones are present in immature RNA degradosome intermediates at times of cellular stress. Molecular chaperones have been shown to play roles in assembly and disassembly of other multi-protein complexes. Studies investigating molecular chaperones would determine if they have a similar role in the RNA degradosome under conditions of heat shock. *In vivo* analysis will determine which molecular chaperones are present in the RNA degradosome at elevated temperatures and if co-chaperones affect the functioning of the chaperones in this protein complex. Further analysis could show where and how the chaperones associate with the scaffold protein RNase E. It is possible that the chaperones associate with RNase E via enolase. Enolase is very diverse in its functioning across species and has a wide variety of moonlighting functions. It has previously been shown to act as a heat shock protein in yeast; studies will determine whether enolase has a role in the heat shock response in the RNA degradosome

1.7 Objective of the project

1.7.1 Broad objective

The main objective of this project is to study the role of molecular chaperones in the *E. coli* RNA degradosome under conditions of stress and to determine the localization of enolase and molecular chaperones in the degradosome under heat stress.

1.7.2 Specific objectives

1. Verification of plasmids by restriction analysis. Plasmids were obtained encoding FLAG-tagged and His-tagged RNase E; FLAG-tagged polynucleotide phosphorylase, helicase and enolase as well as His-tagged *E. coli* DnaK.
2. Competent cells need to be prepared. Plasmids encoding FLAG-tagged proteins and His-tagged RNase E will be transformed into *E. coli* BL21, while the plasmid

encoding His-tagged *E. coli* DnaK will be transformed into *E. coli* XL1-Blue cells.

3. Protein production studies will be performed on all the protein used in this study. Western analysis, through the use of specific antibodies (anti-His and anti-FLAG) will verify the presence of the proteins.
4. The RNA degradosome will be purified using both His-tagged RNase E and FLAG-tagged RNase E as the scaffold protein.
5. The plasmid encoding His-tagged RNase E will be transformed into the *E. coli dnaK* deletion strain, in an attempt to purify the RNA degradosome from cells lacking functional DnaK. The ability of GroEL to functionally replace DnaK will be investigated using anti-GroEL antibodies. The strain will be studied under normal and heat-shock conditions.
6. The localization of enolase and GroEL will be studied under normal conditions as well as under heat stress by a subcellular fractionation.
7. An enolase assay will be used to monitor enolase activity in the presence and absence of DnaK. *E. coli* DnaK will be purified and quantified for the assays, and commercial enolase will be obtained for the assays.

Chapter 2

Purification of the *E. coli* RNA Degradosome

2.1 Introduction

Endoribonucleolytic RNase E is the scaffold protein to which the components of the *E. coli* RNA degradosome complex associate. This protein is divided into three regions – the catalytic N- terminus, which tethers the degradosome to the cytoplasmic membrane, the arginine-rich RNA binding region and the C- terminus which acts as the scaffold to which the components of the degradosome, PNPase, RhlB helicase and enolase, associate (McDowall and Cohen, 1996; Miczak *et.al.,* 1996; Liou *et. al.,* 2001). DnaK, GroEL and polyphosphate kinase also associate with the multi-enzyme complex, but in substoichiometric amounts (Miczak *et.al.,* 1996; Blum *et. al.,* 1997).

Previous researchers had successfully purified the degradosome utilizing the FLAG-tagged RNase E as scaffold protein (Miczak *et al.,* 1996). The immunoaffinity purification of a FLAG epitope fusion protein is a non-denaturing procedure that allows the elution of an epitope-tagged protein (Einhauer and Jungbauer, 2001). The principle follows that of immunoaffinity protein purification. The FLAG fusion protein (RNase E) has a peptide of eight amino acids fused to its N- terminus to which the anti-FLAG monoclonal antibody binds (anti-FLAG M2) (Miczak *et al.*,1996). This union is not calcium-dependent and therefore antigens bound to the resin would not be eluted through the addition of chelating agents (Brizzard *et al.,* 1994). The tagged protein is either eluted through the addition of excess synthetic FLAG-peptide (Chiang and Roeder, 1993), or through lowering the pH (LeClair *et al.,* 1992).

The nature of the FLAG-fusion protein system would allow purification of the degradosome and its constituents using FLAG-tagged RNase E. The advantage of the anti-FLAG M2 antibody is that it is not specific to only the N- terminus Met-FLAG sequence, but can also be used against the FLAG sequence fused to the C- terminus of the target protein (Einhauer and Jungbauer, 2001). The fusion of the N- terminus to the

FLAG peptide has the advantage of being stable and not being broken down by *E. coli* proteases, and leads to a protein that is correctly processed (Plückthun, 1992). Another advantage of the purification is that the tag won't interfere with the production of the fusion protein, proteolytic activity or maturation; the system is non-denaturing thereby allowing purification of the fusion protein by anti-FLAG affinity chromatography and allows immunological western blot analysis using anti-FLAG antibodies (Guimaraes *et al.,* 1996; Muzamdar *et al.,* 1996; Robeva *et al.,* 1996). The FLAG purification also has the advantage of isolated protein purity in the range of 90 % (Schuster, et al., 2000).

The hexahistidine tag is the most frequently used affinity tag for protein purification. It has the advantage of being a small tag that can associate to a chromatographic matrix. The His_6-tag of the fusion protein has an affinity for the metals copper, nickel, cobalt and zinc thereby allowing the purification of the fusion protein by its interaction with a metal-containing matrix such as nickel beads when a protein extract is passed over the matrix (Hochuli *et al.,* 1988). Metal chelate affinity chromatography can allow up to 95 % purity of the fusion protein expressed in a prokaryotic system (Hochuli *et al.,* 1988). The matrix (Ni-beads) has the advantage of enduring numerous regenerations and has a high binding capacity; the elutions can be carried out under native as well as denaturing conditions (Waugh, 2005). The mild and flexible conditions should therefore allow successful purification of His-tagged RNase E along with the constituents of the degradosome.

The aim of the study was to successfully purify the *E. coli* RNA degradosome along with its components. The first objective would be to use bioinformatics tools to perform BLAST searches and sequence alignments to investigate the degree of similarity between homologues. The FLAG-tagged RNase E plasmid encodes for the scaffold protein of the *E. coli* RNA degradosome. The FLAG-tagged and His-tagged purification of RNase E can therefore be used to purify all the components of the degradosome. The study was focused on constructing plasmid maps and conducting restriction analysis for plasmid verification. The third objective was to study the protein production levels of the constituents of the *E. coli* RNA degradosome, including RNase E, PNPase, RhlB helicase and enolase. The FLAG-tagged PNPase, RhlB helicase and enolase plasmids encoding for the respective proteins were obtained to express the PNPase, RhlB helicase and enolase proteins. A solubility study would determine which detergent will detach RNase E from the cytoplasmic membrane. FLAG-tagged and His-tagged RNase E will be used for purification of the *E. coli* RNA degradosome using FLAG immunoaffinity chromatography and metal affinity chromatography, respectively. The presence of the RNA degradosome components associated to the purified RNase E protein will be verified through the protein production of FLAG-tagged PNPase, RhlB helicase and enolase and western blot analysis using anti-FLAG antibodies.

2.2 Materials and Methods

2.2.1 Materials

Reagents used were purchased from Sigma Chemicals Co. (St. Louis, Mo U.S.A.), Merck Chemicals (Darmstadt, Germany), BioRad (U.S.A.) or Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). peqGOLD Plasmid Miniprep Kit I was purchased from peQLab Biotechnologie GmbH (Germany). peqGOLD Protein Marker II was purchased from peqlab (Germany). Nickel beads were purchased from Pharmacia Biotech, (Uppsala, Sweden).

Table 2.1 Description of *E. coli* strains and plasmids

Plasmid	Description	Reference
pRE196	pGM102 encoding *E. coli* FLAG-tagged RNase E, Amp^R	Miczak *et al.* (1996)
pRE296	pGM102 encoding *E. coli* His-tagged RNase E, Amp^R	Miczak *et al.* (1996)
pFLAG-ENO	pGP1–2 encoding *E. coli* enolase, Amp^R	Liou *et al.* (2002)
pFLAG-PNP	pGP1–2 encoding *E. coli* PNPase, Amp^R	Liou *et al.* (2002)
pFLAG-RhlB	pGP1–2 encoding *E. coli* RhlB helicase, Amp^R	Liou *et al.* (2002)
pEKJ30	pQE30 encoding *E. coli dnaK*, Amp^R	Boshoff *et al.* (2008)

Strains	**Description**	**Reference**
E. coli BL21 (DE3)	F- ompT gal [dcm] [lon] hsdSB λDEs	Studier *et al* (1990)
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r)].	Bullock *et al.*(1987)

Restriction enzymes *Hind* III and *Bam* HI were purchased from Amersham, (USA) and anti-His antibody from Amersham Pharmacia Biotech (UK). Plasmids pRE196, pRE296, pFLAG-PNP, pFLAG-RhlB and pFLAG-Eno were generous donations from Prof. S. Lin-Chao *(Institute of Molecular Biology, Academia Sinica, Taiwan)* (Table 2.1). Plasmid pEKJ30 was constructed by Dr A. Boshoff.

2.2.2 Methods

2.2.2.1 Bioinformatic analysis of RNase E

A comparative investigation of RNase E from five different organisms was conducted using sequence analysis tools. The comparison was made using amino acid sequences of the protein from each organism. The *E. coli, Methylococcus capsulatus, Pseudoalteromonas haloplanktis, Salmonella typhimurium* and *Shigella flexneri* RNase E sequences were downloaded from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The sequences were aligned using the CLUSTALW multiple sequence alignment tool from GenomeNet (http://align.genome.jp/). The alignment was shaded using the Mobyl@pasteur Boxshade v.3.0 (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=boxshade).

2.2.2.2 Preparation of plasmid DNA

Each plasmid was prepared using peqGOLD Plasmid Miniprep Kit I (peQLab Biotechnologie GmbH, Germany) by transformation into *E. coli* BL21 cells after which overnight culture was harvested by centrifugation at 10 000 g for 1 min and the pellet resuspended in 250 µl Solution I/RNase A. Upon having added 250 µl Solution II and inverting, the mixture was incubated for 2 min at room temperature and 350 µl Solution

III then added. The solution was centrifuged for 10 min at 10 000 g after which the lysate was transferred to a HiBind® miniprep column provided by the kit in a collection tube and again centrifuged for 1 min at 10 000 g. The flow-through was discarded and 500 µl HB Buffer was added and centrifuged at 10 000 g for 1 min. The flow-through was again discarded, the column washed with 750 µl Wash Buffer and then centrifuged for 1 min at 10 000 g. The wash was repeated and the column dried by centrifuging for an additional minute. The DNA was eluted by the addition of 50 µl Elution Buffer and centrifuged for a minute at 10 000 g and stored at -20°C.

2.2.2.3 <u>Plasmid verification through restriction endonuclease and agarose gel analysis</u>

Plasmids were verified by restriction analysis. Plasmids pRE196 and pRE296 were digested with 2 U *Hind*III and 2 U *Nde*I. The appropriate 10 x restriction buffer (2 µl) was added and the reaction volume brought to 20 µl. Similarly plasmids 2 U pFLAG-PNP, pFLAG-RhlB and pFLAG-ENO (2µl) were digested with 2 U *Bam*HI and 2 U *Bgl*I with the appropriate 10 x restriction buffer (2 µl). The reactions were incubated at 37ºC for 4 hours.

An agarose gel (0.8 %) was prepared using TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Prior to casting the gel 20 µg/ml ethidium bromide was added. The restricted DNA samples were prepared for loading with the addition of loading dye (30 % glycerol, 0.25 % Bromophenol Blue). The gel electrophoresed for an hour at 100 volts and was viewed under UV light.

2.2.2.4 <u>Competent cell preparation</u>

A colony of *E. coli* BL21 (DE3) cells was inoculated into 25 ml 2 x yeast tryptone (YT) broth (1.6 % tryptone, 1 % yeast, 0.5 % NaCl) and grown with shaking overnight at 37°C. The overnight culture was inoculated into 2 x YT broth in a 1 : 25 dilution and grown with shaking to an optical density of A_{600nm} = 0.6. The cells were harvested at 5 000 g for 5 minutes at 4°C. The pellet was resuspended in cold, sterile 0.1 M $MgCl_2$ and left to stand on ice for 20 minutes. A pellet, formed by centrifuging at 5 000 g for 5 minutes at 4°C, was resuspended in cold, sterile 0.1 M $CaCl_2$ and left on ice for 4 hours.

The cells were centrifuged again at 5 000 g for 5 minutes at 4°C. The pellet was resuspended in cold 0.1 M $CaCl_2$ and in a 1 to 1 ratio, 30 % glycerol was added and stored at -80°C.

2.2.2.5 Transformation of competent cells

Plasmid DNA (2 µl) was added to 100 µl thawed competent *E. coli* cells. The cells were incubated on ice for 20 minutes after which they were heat shocked in a water bath at 42°C for 45 seconds. The cells were incubated on ice for 2 minutes before 900 µl 2x YT broth was added. The cell suspension was allowed to incubate at 30°C for 1 hour. The cells were harvested by centrifugation at 12 000 g for 1 minute and the pellet was resuspended in 100 µl of the supernatant. The cells were grown on 2 x YT agar (YT broth with 1.5 % agar) with ampicillin (100 µg/ml). The cells were incubated overnight at 30°C to allow the formation of colonies. Competent E. coli BM271 (DE3) cells transformed with plasmid DNA were grown on 2 x YT agar with antibiotics ampicillin (100 µg/ml) and chloramphenicol (20 µg/ml).

2.2.2.6 Determination of optimal protein production

An overnight culture was prepared by inoculating a single colony of *E. coli* BL21 (DE3) cells transformed with plasmids pRE196 or pRE296 into 25 ml 2 x YT broth with 100 µg/ml ampicillin at 30ºC. The overnight culture was inoculated into 225 ml 2 x YT with 100 µg/ml ampicillin and grown to an optical density of A_{600nm} = 0.6. Protein production was then induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 1 mM. Previous experiments indicated that RNase E degraded quickly and that samples should be taken within 2 hours of induction as protein levels decreased substantially beyond this point (data not shown). Samples were therefore taken 30, 40, 50, 60, 90, 150 and 180 minutes after induction. The samples were centrifuged for 1 minute at 12 000 g and the pellet resuspended in a volume of PBS as determined by the absorbance reading where 30 µl of PBS was added for each 0.5 OD_{600nm} absorbance unit. The samples were analyzed on a 10 % SDS-PAGE gel. The protein production study of PNPase, enolase and RhlB helicase differed in that protein samples were taken over hourly periods for duration of 5 hours. The enolase overnight culture was grown to an optical density of A_{600nm} = 0.4. Western blot analysis confirmed protein production. Monoclonal mouse anti-His antibodies were used to detect His-tagged RNase E with ECL anti-mouse IgG HRP-linked species-specific whole antibody. M2 monoclonal anti-FLAG antibody allowed for the detection of the FLAG-tagged proteins with the above mentioned anti-mouse antibody.

2.2.2.7 <u>Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)</u>

SDS-PAGE was performed using the standard procedure (Laemmli, 1970). Samples were prepared for analysis by adding 5 x SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10 % SDS, 0.5 % w/v bromophenol blue, 5 % β-mercaptoethanol) and boiling for 5 minutes. A 10 % resolving gel (1.5 M Tris-HCl pH 8.8, 10 % SDS, 40 % Acrylamide/Bis) was prepared, 10 % ammonium persulphate and N,N,N',N'-tetramethylenediamine were added to initiate the polymerization of the acrylamide. Once the acrylamide had polymerized a 4 % stacking gel (1.0 M Tris-HCl, pH 6.8, 10 % SDS, 40 % Acrylamide/Bis) was prepared. The gels in SDS-PAGE running buffer (25 mM Tris base, 192 mM Glycine, 1 % SDS) were left to electrophorese at 120 volts for 90 minutes. To view the protein bands, the gels were stained with a Coomassie staining solution (50 % methanol, 7.5 % glacial acetic acid, 0.24 % Coomassie Brilliant Blue) and destained with SDS-PAGE destain (20 % methanol, 7.5 % glacial acetic acid).

2.2.2.8 <u>Western blot analysis of proteins</u>

Electrophoresis was carried out as described in section 2.2.2.7. Separated proteins were transferred onto the nitrocellulose sheet in transfer buffer (25 mM Tris, 192 mM glycine, 20 % MeOH) for 1 hour at 100 volts. Transferred proteins were visualized by staining the blot in Ponceau S stain (0.5 % Ponceau S, 1 % glacial acetic acid) and destaining with water. The membrane was washed twice at room temperature with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). To block the hydrophobic binding sites from non-specific proteins, the membrane was soaked in a protein solution (5 % (w/v) non-fat dried milk in TBS) for 1 hour at 4°C. The membrane was incubated with primary antibody diluted 1 : 5 000 in 5 % block overnight at 4ºC and washed twice at 4ºC for 20 minutes in TBS-Tween 20 (0.1 % Tween 20 in TBS). The membrane was then incubated for 45 minutes in secondary antibody diluted 1 : 5 000 in 5 % block at 4°C followed by 4 x 15 minute washes in TBST at 4ºC. Antibodies were detected using the ECL Advance Blotting Detection Kit and viewed using the Chemidoc ™ EQ (BioRad, USA).

2.2.2.9 Solubility studies

An overnight culture of *E. coli* BL21 (DE3) transformed with pRE196 was diluted 1 : 10 in 2x YT broth with added ampicillin and incubated at 30ºC. The culture was grown to an optical density of A_{600nm} = 0.6. Protein production was induced by adding IPTG to a final concentration of 1 mM. The cells were harvested 40 minutes after induction by centrifuging at 4°C for 1 minute at 12 000 g. The cells were then resuspended in lysis buffer (0.1M Tris-HCl, pH 8, 0.3 M NaCl) with 1 mg/ml lysozyme and 1 mM PMSF. Various detergents were added separately to the final concentration of 0.1 % Polyethylene imine, 7.5 % N-lauroyl sarcosine, 3 % Triton and 3 % nonyl phenoxylpolyethoxylethanol (Tergitol-type NP-40). These solutions were sonicated (4 x 20 seconds at 60 Hz) after which they were centrifuged for 40 minutes at 12 000 g at 4ºC. A sample of both the supernatant and the pellet resuspended in phosphate buffered saline (PBS) (16 mM sodium phosphate, 150 mM NaCl, 4 mM potassium phosphate, pH 7.5) was taken and analyzed on a 10 % SDS-PAGE gel.

2.2.2.10 FLAG-tagged RNase E purification

This protocol was modified from Miczak *et al.,* 1996, Liou *et al.,* 2002, Klein *et al.,* 2005 and from SIGMA Product Information. ANTI-FLAG® M2 affinity gel (Sigma-Aldrich, Germany) has the monoclonal antibody IgG_1, by hydrazide linkage, bound to agarose (Sigma Product Information). The monoclonal ANTI-FLAG® M2 Antibody (Sigma-Aldrich, Germany) binds to the FLAG peptide sequence of fusion proteins (Brizzard *et al.,* 1994). FLAG® peptide (Sigma-Aldrich, Germany) was purchased for the competitive elution of the FLAG fusion protein. An overnight culture of *E. coli* BL21 (DE3) transformed with pRE196 was diluted 1:10 into 2x YT broth with added ampicillin and grown to an optical density of A_{600nm} = 0.6. Protein production was induced by adding IPTG to a final concentration of 1 mM. The cells were harvested 40 minutes after induction at 4°C for 5 minutes at 5 000 g. The resulting pellet was resuspended in 2.5 ml cold DNaseI buffer (50mM Tris-HCl pH 7.5, 100 mM NaCl, 5 % glycerol, 30 mM magnesium acetate) and frozen at – 80°C. The frozen cells were thawed in the presence of 250 µl lysozyme (10 mg/ml) and 25 µl PMSF (100 mM) and incubated on ice for 30 minutes. The samples were sonicated at 60 Hz at intervals of 4 x 20 seconds after the addition of 0.1 % polyethylene (PEI). The samples were pooled together and 1 ml DNaseI buffer with 1 mM PMSF and 20 µg/ml DNaseI was added to the sample solution. Ammonium chloride to a final concentration of 1.25 M was added slowly with stirring after the solution had been incubated on ice for 30 minutes. A supernatant was collected by centrifuging for 15 minutes at 12 000 g at 4°C. The supernatant was precipitated with 50 % saturation of ammonium sulphate, incubated on ice and centrifuged at 12 000 g for 20 minutes at 4°C. The supernatant was precipitated with 50 – 80 % saturation of ammonium sulphate, again incubated on ice and centrifuged at 12 000 g for 20 minutes at 4°C. The pellet was then washed with 50 % saturated ammonium sulphate in TBS buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl), centrifuging at 12 000 g for 20 minutes at 4°C. TBS buffer containing 20 % glycerol was used to dissolve the pellet in and to adjust the volume to 2 ml. The pH of the protein extract was adjusted to between pH 7 – 8 and 0.15 M NaCl was added after which cellular debris was removed by centrifuging at 12 000 g for 10 minutes at 4°C. The preparation of the resin and storing of the column was carried out following the SIGMA® Product Information ANTI-FLAG® M2 Affinity Gel instructions. The protein extract was added to 1 ml resin and incubated for 1 hour at 4°C with gentle rocking. In order to collect the resin the preparation was centrifuged for 5 minutes at 1 000 g. The resin was washed with TBS until the supernatant had an absorbance less than 0.05 versus the blank at 280 nm. The FLAG-tagged protein was eluted with 100 µg/ml FLAG peptide in TBS with 10 % glycerol, centrifuging at 1 000 g for 5 minutes. The supernatant was stored at -80°C.

2.2.2.11 <u>His-tagged RNase E purification</u>

An overnight culture of *E. coli* BL21 (DE3) transformed with pRE296 was diluted 1 : 10 into 2x YT broth, incubated at 30ºC and grown to an optical density of A_{600nm} = 0.6. Protein production was induced by adding IPTG to a final concentration of 1 mM. The cells were harvested 40 minutes after induction by centrifuging at 4°C for 5 minutes at 5

000 g. The resulting pellet was resuspended in lysis buffer (0.1M Tris-HC, pH 8, 0.3 M NaCl) and frozen at –80°C. The frozen cells were thawed in 1 mg/ml lysozyme and 1mM PMSF and rocked on ice for 30 minutes. After the addition of N-lauroyl sarcosine to the final concentration of 7.5 %, the samples were sonicated. Cellular debris was removed by centrifuging at 12 000 g for 40 minutes at 4°C. The supernatant was incubated with 1 ml nickel-charged Sepharose beads (50 % slurry) overnight on ice. The bead-suspension was centrifuged for 5 minutes at 5 000 g at 4°C. Three washes were performed on the beads with 5 ml wash buffer (0.1 M Tris-HCl pH 8, 0.3 M NaCl, 50 mM imidazole) and centrifuged for 1 minute at 5 000 g at 4°C. The protein was eluted by first adding 1 ml elution buffer 1 (0.1 M Tris-HCl pH 8, 0.3 M NaCl, 500 mM imidazole) and allowing the suspension to incubate on ice for 10 minutes then centrifuging for 1 minute at 5 000 g at 4°C. Two further elutions were conducted in the same manner, using elution buffer 1 for the second elution and elution buffer 2 (0.1 M Tris-HCl pH 8, 0.3 M NaCl, 1 M Imidazole) for the third elution. The protein elutions were stored at -20°C.

2.3 Results and discussion

2.3.1 Multiple sequence alignment of prokaryotic RNase E

An amino acid sequence alignment of the RNase E protein of *E. coli* and other prokaryotes was performed (Figure 2.1). *E. coli* RNase E homologues were identified using a BLAST search in which sequences from various prokaryotes were aligned to determine the degree of similarity. Using *E. coli* RNase E as a template, it was determined that *Methylococcus* RNase E shared a 66 % identity with *E. coli* RNase E; *Pseudoalteromonas* RNase E shared a 74 % identity with *E. coli* RNase E; *Salmonella* 80 % identity and *Shigella* RNase E a very high identity of 98 % with *E. coli* RNase E. Russel *et al.* (1997) indicated that two sequences could be considered close homologues if the percentage identity was above 50 %. *Methylococcus, Pseudoaltermonas, Salmonella* and *Shigella* RNase E are thus all homologous to *E. coli* RNase E. Generally there appeared to be a higher degree of conservation across the N-terminal domains of the prokaryotes than across the scaffold domain. *Pseudoaltermonas* has a sequence identity of 85 % across the catalytic domain, but there is very little conservation across the non-catalytic domain. The motifs for the degradosome-associated proteins are not present in

***Pseudo.* N-terminal domain**

***E. coli* N-terminal domain**

```
E. coli      MKRMLINATQQEELRVALVDGQRLYDLDIESPGHEQKKANIYKGKITRIEPSLEAAFVDY   60
Shigella     MKRMLINATQQEELRVALVDGQRLYDLDIESPGHEQKKANIYKGKITRIEPSLEAAFVDY   60
Salmonella   MKRMLINATQQEELRVALVDGQRLYDLDIESPGHEQKKANIYKGKITRIEPSLEAAFVDY   60
Pseudo.      MKRMLINATQQEEMRVALVDGQRLYDLDIESPGHEQKKANIYKGKITRIEPSLEAAFVEY   60
Methyloc.    MKRMLINATQpEELRVALVDGQKLYDfDIEiPArEQKKANIYKGlITRVEPSLEAAFVnF   60

E. coli      GAERHGFLPLKEIAREYFPAN-YSAHGRPNIKDVLREGQEVIVQIDKEERGNKGAALTTF  119
Shigella     GAERHGFLPLKEIAREYFPAN-YSAHGRPNIKDVLREGQEVIVQIDKEERGNKGAALTTF  119
Salmonella   GAERHGFLPLKEIAREYFPAN-YSAHGRPNIKDVLREGQEVIVQIDKEERGNKGAALTTF  119
Pseudo.      GADRHGFLPLKEIARtYFPAg-YTfHGRPNIRDVIKEGQEVIVQVDKEERGQKGAALTTF  119
Methyloc.    GAERHGFLPfKEIlpkYlgAggeesvsRreIKDVLKEGQEVVVQVEKEERGtKGAALTTY  120

E. coli      ISLAGSYLVLMPNNPRAGGISRRIEGDDRTELKEALASLELPEGMGLIVRTAGVGKSAEA  179
Shigella     ISLAGSYLVLMPNNPRAGGISRRIEGDDRTELKEALASLELPEGMGLIVRTAGVGKSAEA  179
Salmonella   ISLAGSYLVLMPNNPRAGGISRRIEGDDRTELKEALASLELPEGMGLIVRTAGVGKSAEA  179
Pseudo.      ISVAGSYLVLMPNNPRAGGISRRIEGDERTELKEsLsrLELPkGMGLIVRTAGVGKSfEe  179
Methyloc.    ISLAGSYLVLMPNNPKAGGISRRIEGDvRSDMKEtLsqLhVPEdMGVIIRTAGgGKTvEe  180

E. coli      LQWDLSFRLKHWEAIkKAAESRPAPFLIHQESNVIVRAFRDYLRQDIGEILIDNPKVLEL  239
Shigella     LQWDLSFRLKHWEAIkKAAESRPAPFLIHQESNVIVRAFRDYLRQDIGEILIDNPKVLEL  239
Salmonella   LQWDLSFRLKHWEAIqKAAESRPAPFLIHQESNVIVRAFRDYLRQDIGEILIDNPKVMEM  239
Pseudo.      LNYDLkalLvHWEAIgvAADSakAPFLIHQESNVIfRAiRDYLRrDIGEILIDkPRVfEe  239
Methyloc.    LQWDLnYlLqlWEAIeRstreKPAPFLIfQESNVIIRAlRDhLRgDIdEILVDNPstfrL  240

E. coli      ARQHIAALGRPDFSSKIKLYTGEIPLFSHYQIESQIESAFQREVRLPSGGSIVIDSTEAL  299
Shigella     ARQHIAALGRPDFSSKIKLYTGEIPLFSHYQIESQIESAFQREVRLPSGGSIVIDSTEAL  299
Salmonella   ARQHIAALGRPDFSSKIKLYTGEIPLFSHYQIESQIESAFQREVRLPSGGSIVIDSTEAL  299
Pseudo.      AKaHIerfr-PDFmSRVKLYqGDtPLFTHYQIESQIESAFQREVRLPSGGSIVIDpTEAL  298
Methyloc.    vhs-flqqvmPqFinKaRLYqdnVPLFSrYQIESQIETAYaREVpLPSGGaIVIDhTEAL  299

E. coli      TAIDINSARATRGGDIEETAFNTNLEAADEIARQLRLRDLGGLIVIDFIDMTPVRHQRAV  359
Shigella     TAIDINSARATRGGDIEETAFNTNLEAADEIARQLRLRDLGGLIVIDFIDMTPVRHQRAV  359
Salmonella   TAIDINSARATRGGDIEETAFNTNLEAADEIARQLRLRDLGGLIVIDFIDMTPVRHQRAV  359
Pseudo.      TsIDINSsKATKGGDIEETAlNTNLEAADEIARQLRLRDLGGLIVIDFIDMTPpRHQReV  358
Methyloc.    TtIDINSARATKGGDIEETAlNTNLEAADEIARQLRLRDLGGLfVIDFIDMmaaRnQRAV  359

E. coli      ENRLREAVRQDRARIQISHISRFGLLEMSRQRLSPSLGESSHHVCPRCSGTGTVRDNESL  419
Shigella     ENRLREAVRQDRARIQISHISRFGLLEMSRQRLSPSLGESSHHVCPRCSGTGTVRDNESL  419
Salmonella   ENRLREAVRQDRARIQISHISRFGLLEMSRQRLSPSLGESSHHVCPRCSGTGTVRDNESL  419
Pseudo.      ENRLKDAVRpDRARVQIgkISRFGLLEMSRQRLrPSLGEaSqgpCPRCSGqGTIRsNESI  418
Methyloc.    ENRLREAVRlDRARIQLgrISRFGLMEMSRQRLrPSLtETalltCPRCkGqGTIRsvESL  419

E. coli      SLSILRLIEEEALKENTQEVHAIVPVPIASYLLNEKRSAVNAIETRQDGVRCVIVPNDQM  479
Shigella     SLSILRLIEEEALKENTQEVHAIVPVPIASYLLNEKRSAVNAIETRQDGVRCVIVPNDQM  479
Salmonella   SLSILRLIEEEALKENTQEVHAIVPVPIASYLLNEKRTAVNAIETRQDGVRCVIVPNDQM  479
Pseudo.      aLSILRLIEEEAIKDNTaqVnAqVPVaVAaYLLNEqRrsVhrMEk-QhkcdiVIIPNqhM  477
Methyloc.    aLSILRVLEEEtMKkNTdrIiAqLPVesATYLLNEKRaAIQqIEvRhnva-itIIPNphL  478

E. coli      ETPHYhVLRVRKGEETPTLSYMLPKLHEEAMALPSEEEFAERKRPEQPALATFAMPDVPP  534
Shigella     ETPHYhVLRVRKGEETPTLSYMLPKLHEEAMALPSEEEFAERKRPEQPALATFAMPDVPP  534
Salmonella   ETPHYsVLRVRKGEETPTLSYMLPKLHEEAMALPSEEEYAERKRPEQPALATFAMPDVPP  534
Pseudo.      ETPHYeVMRLRKdEtieTVSYgqivapEpeafemSkspvApvRee-------------Pm  530
Methyloc.    ETPnYdIqRIRsGgaggeeeirkssyqliAeksP--------------------------  535

E. coli      APTPaEPAAPVvApAPkAapAtpATPAQPGLLSRFfgALKALFSGGEEtKptEqpaPKAE  593
Shigella     APTPaEPAAtVvApAPkAatAtpAaPAQPGLLSRFfgALKALFSGGEEaKptEqptPKAE  593
Salmonella   APTPvEPAvsVatAk--kdnvaaAqPAQPGLfSRFlnALKqLFSG-EEtKtvEtaaPKAE  590
Pseudo.      lkgvvmPAAPapqAAPanvvApaeTkAQsGLLdaigkwfKsLFasetvevkkEetqkqqq  579
Methyloc.    -eTPkptrtPsaAAeapAvrefipTapQPGggdqraqAqpGqpgGGlikRflniltgqri  573
```

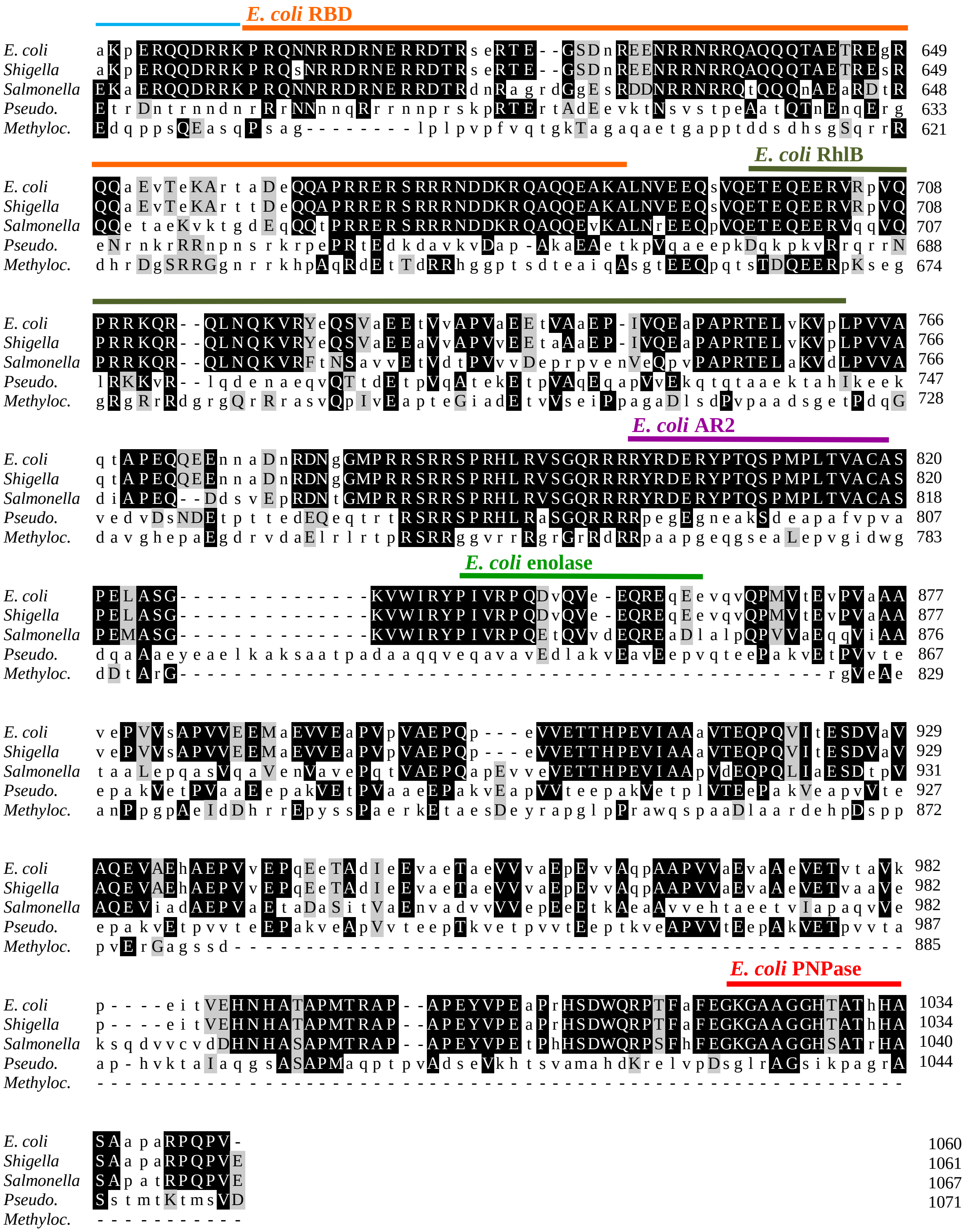

Figure 2.1 Mutiple sequence alignment of prokaryotic RNase E

E. coli: *E. coli* RNase E protein (genBank: YP003077303); *Shigella*: *Shigella felxneri* RNase E protein (GenBank: YP688620); *Salmonella: Salmonella typhymurium* RNase E protein (GenBank: NP460156); *Pseudo*: *Pseudoaltermonas haloplanktis* RNase E protein (GenBank: YP340331) and *Methyloc.*: *Methylococcus capsulatus* RNase E protein (GenBank: MCA1481). Light blue: *E. coli*, *Salmonella* and

Shigella N-terminal domain; dark blue: *Pseudoalteromonas* N-terminal domain; orange: *E. coli, Salmonella* and *Shigella* RNA binding domain (RBD); olive green: *E. coli, Salmonella* and *Shigella* RhlB helicase binding region; purple: *E. coli, Salmonella* and *Shigella* arginine-rich region 2 (AR2); bright green: *E. coli, Salmonella* and *Shigella* enolase binding region; red: *E. coli, Salmonella* and *Shigella* PNPase binding region. The scaffold domain of *E. coli, Salmonella* and *Shigella* stretches from amino acid residue 605 to 1061 (unmarked).

Pseudoalteromonas, indicating that these motifs have either diverged significantly or the RNase E protein associates and interacts with different proteins (Carpousis *et al.,* 2008).

Pseudoaltermonas RNase E consists of 11 amino acid residues more than *E. coli. Methylococcus* shows some conservation across the catalytic domain, but none across the non-catalytic domain. RNase E has fewer amino acid residues than *E. coli; Methylococcus:* 885, *E. coli:* 1061. The RNA binding sites (RBD and AR2) of *E. coli* RNase E homologues *Shigella* and *Salmonella* are well conserved. The protein-protein motifs of RNase E, including that of RhlB helicase, enolase and PNPase are also well conserved in these two organisms, and it is possible that they would recruit these RNA degradosome components in a similar manner to *E. coli* RNase E (Marcaida *et al.*, 2006).

2.3.2 Plasmid verification through restriction analysis

Plasmid pRE196, encoding the protein for FLAG-tagged RNase E, will be used to purify the degradosome using immunoaffinity purification of the FLAG-epitope fusion protein. The epitope-tagged scaffold protein would enable the purification of all the components of the degradosome. Once purified, the components of the degradosome would need to be verified. Using proteins enolase, PNPase and RhlB helicase, western blot analysis with anti-FLAG antibodies would allow the identification of proteins of molecular mass 47 kDa, 85 kDa, and 50 kDa, respectively, of the purified degradosome components. These plasmids were used for verification because antibodies had not been produced against each of the components of the degradosome. Thus, through SDS-PAGE the protein bands at their respective sizes of 47 kDa, 85 kDa and 50 kDa could be compared to the protein bands in the RNase E elution lane.

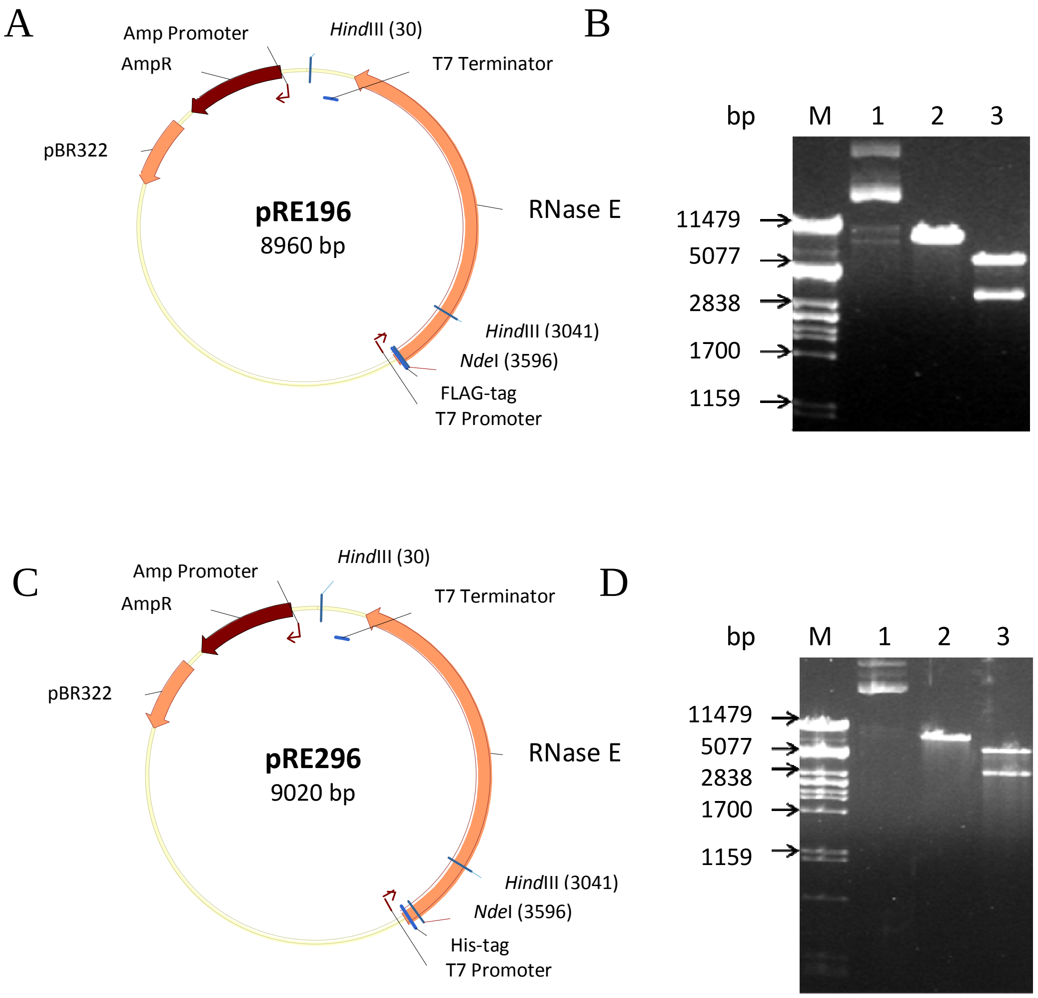

Figure 2.2 Plasmid map and restriction analysis of FLAG-tagged RNase E and His-tagged RNase E
Plasmid map of pRE196 (A), agarose gel of restriction digestion of pRE196 (B), plasmid map of pRE296 (C) and agarose gel of restriction digestion of pRE296 (D). (A) The plasmid map of 8960 base pairs shows the coding region for FLAG-tagged RNase E in orange, the ampicillin resistance gene in red, the FLAG-tag, and the sites for the restriction enzymes *Hind*III (30 bp, 3041 bp) and *Nde*I (3596 bp). (B) Restriction analysis of pRE196 with restriction enzymes *Hind*III and *Nde*I. Lane M: *Pst*I restricted lambda DNA molecular marker, lane 1: undigested pRE196, lane 2: single digest with *Nde*I (8960 bp), lane 3: double digest with *Hind* III (3011 bp, 5949 bp). (C) The plasmid map of 9020 base pairs contains the same features as that of pRE196 except for the His-tag. (D) Restriction analysis of pRE296 with restriction enzymes *Hind*III and *Nde*I. Lane M: *Pst*I restricted lambda DNA molecular marker, lane 1: undigested pRE296, lane 2: single digest with *Nde*I (9020 bp), lane 3: double digest with *Hind* III (3011 bp, 6009 bp)

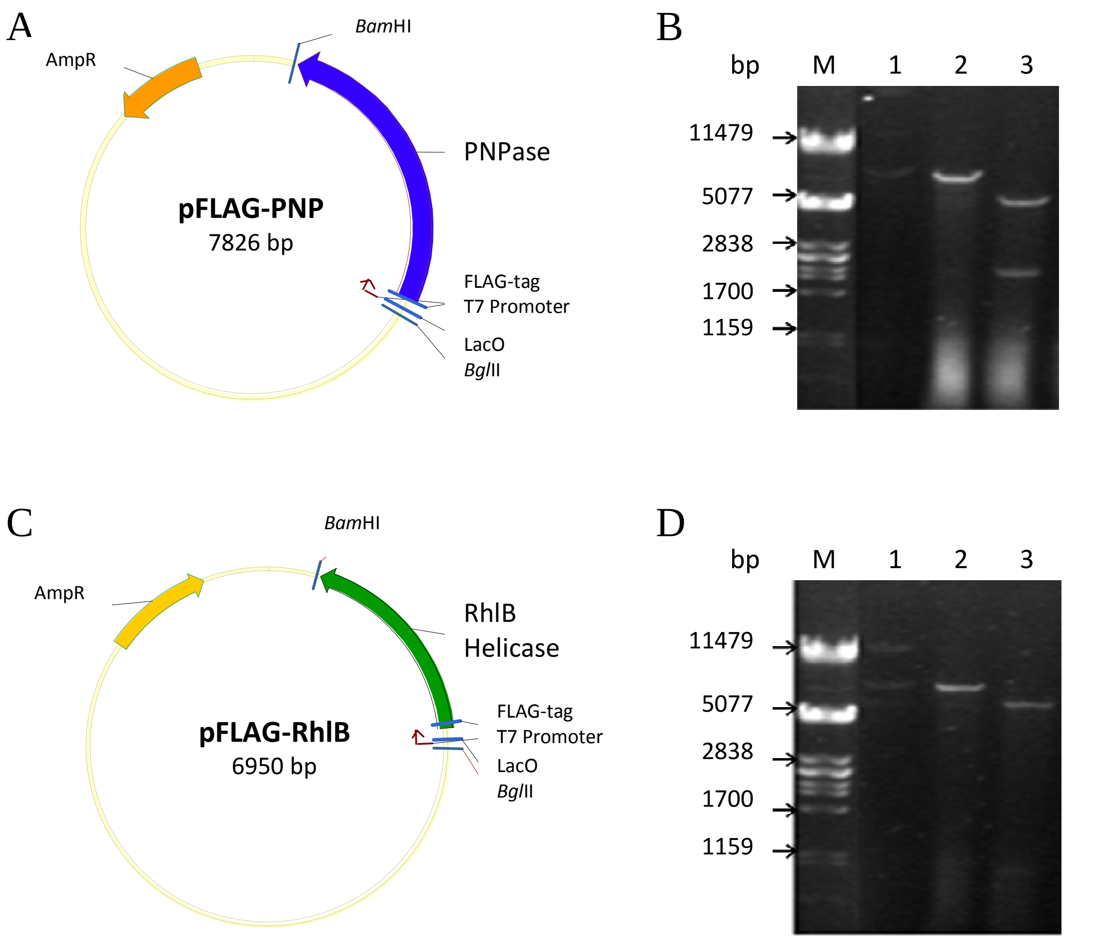

Figure 2.3 Plasmid map and restriction analysis of FLAG-tagged polynucleotide phosphorylase and helicase

Plasmid map of pFLAG-PNP (A), agarose gel of restriction digestion of pFLAG-PNP (B), plasmid map of pFLAG-RhlB (C), and agarose gel of restriction digestion of pFLAG-RhlB (D). (A) The plasmid map of 7826 base pairs shows the coding region for polynucleotide phosphorylase in blue, the ampicillin resistance gene in orange, the FLAG-tag, and the sites for the restriction enzymes *Bam*HI (293 bp) and *Bgl*II (1737 bp), both of which digest the plasmid outside the coding region of polynucleotide phosphorylase. (B) Restriction analysis of pFLAG-PNP with restriction enzymes *Bam*HI and *Bgl*II. Lane M: *Pst* I restricted lambda DNA molecular marker, lane 1: undigested pFLAG-PNP, lane 2: single digest with *Bam*HI (7826 bp), lane 3: double digest with *Bam*HI and *Bgl*II resulting in two bands of sizes 1444 bp (PNPase coding region) and 6382 bp (plasmid). (C) The plasmid map of 6950 base pairs shows the coding region for RhlB helicase in green, and has the same features as discussed in (A). (D) Restriction analysis of pFLAG-RhlB with restriction enzymes *Bam*HI and *Bgl*II. Lane M: *Pst*I restricted lambda DNA molecular marker, lane 1: undigested pFLAG-RhlB, lane 2: single digest with *Bam*HI (6950 bp), lane 3: double digest with *Bam*HI and *Bgl*II resulting in two bands of sizes 1444 bp (RhlB coding region) and 5506 bp (plasmid).

Plasmid maps were constructed from the amino acid sequences obtained from National Centre for Biotechnology Information (NCBI**),** and using the Vector NTI® Software (Informax, USA). The plasmids maps for pRE196 and pRE296 indicated the coding regions for the FLAG- and His-tagged RNase E proteins respectively (Figure 2.2, panels A and C). After restriction endonuclease digestion the resulting digests were analyzed with agarose gel electrophoresis (Figure 2.2, panels B and D). Plasmids pRE196 and pRE296 were successfully linearized with *Nde*I (8960 bp). Both plasmids were cut twice with *Hind* III, producing DNA fragments of sizes 3011 bp and 5949 bp from pRE196, and fragments of 3011 bp and 6009 bp from pRE296.

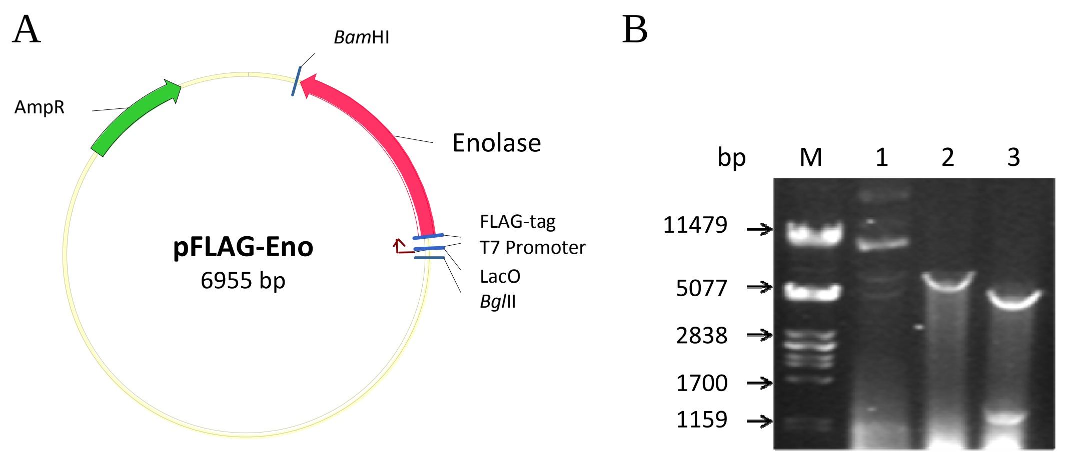

Figure 2.4 Plasmid map and restriction analysis of FLAG-tagged enolase.
Plasmid map of pFLAG-Eno (A) and agarose gel of restriction digestion of pFLAG-Eno (B). The plasmid map of 6955 base pairs shows the coding region for enolase in red, the ampicillin resistance gene in green, the FLAG-tag, and the sites for the restriction enzymes *Bam*HI (293 bp) and *Bgl*II (1742 bp). (B) Restriction analysis of pFLAG-Eno with restriction enzymes *Bam*HI and *Bgl*II. Lane M: *Pst*I restricted lambda DNA molecular marker, lane 1: undigested pFLAG-Eno, lane 2: single digest with *Bam* HI (6955 bp), lane 3: double digest with *Bam*HI and *Bgl*II resulting in two bands of sizes 1449 bp (enolase coding region) and 5506 bp (plasmid).

Agarose gel electrophoreses indicated that Pflag-PNP (Figure 2.3, panel B) was successfully linearized with *Bam*HI (7826 bp) and digested twice with *Bam*HI and *Bgl*II producing fragments of sizes 1444 bp and 6382 bp. Similarly Pflag-RhlB (Figure 2.3, panel D) and pflag-Eno (Figure 2.4, panel B) was made linear with a single digest (6950 bp and 6955 bp, respectively) and cut into fragments of sizes 1444 bp and 5506 bp from pflag-RhlB, and sizes of 1449 bp and 5506 bp from pflag-Eno with double digests. In the restriction endonuclease digestion of enolase the fragment of 5506 bp (Figure 2.4, panel B, lane 3) ran below the 5044 bp marker, likely due to a poor plasmid preparation which was evident from the contaminating material at the bottom of the gel.

2.3.3 Determination of optimal protein production

The purpose of studying the optimal protein production for each protein is to determine when and at what temperature cell cultures should be harvested so that the highest quality and quantity of protein can be purified. A protein production study was performed on each of the constituents of the degradosome (RNase E, PNPase, RhlB helicase and enolase). Previous results showed that harvested RNase E protein extracts had to be concentrated in order to be identified by it molecular mass by western blot analysis. It was also previously determined that less protein was produced at 37°C than at 30°C (results not shown). However, the remaining three of the four major proteins of the degradosome, enolase, PNPase and RhlB helicase, did not require to be concentrated to have their molecular mass identified by western blot analysis and could be overproduced at 37°C.

Even though RNase E has been predicted to have a molecular mass of 118 kDa it has been observed that this scaffold protein emerges on SDS-PAGE as a 180 kDa band. This occurrence is thought to be due to the presence of two proline-rich regions in the central domain of RNase E (Casaregola et al., 1992, 1994; Cohen and McDowall, 1997; Marcaida *et al.,* 2006). A protein production study of FLAG-tagged RNase E (pRE196) with a decreased mobility of 180 kDa was performed (Figure 2.5, panel A). No RNase E protein was visible prior to induction (Figure 2.5, panel A, lane 1). RNase E protein was visible in all the IPTG induced samples taken at times 30, 40, 50, 60, 90, 150 and 180 minutes, and appeared to produce constant levels of protein (Figure 2.5, panel A, lanes 2 – 8). Panel B shows western blot analysis of FLAG-tagged RNase E. Similar protein levels were observed up to 40 minutes after induction, after which there was an increase in protein production (Figure 2.5, panel B, lanes 2 - 3, 5 – 7, respectively). The highest level of protein was produced at 90 minutes, after which levels of protein production decreased (Figure 2.5, panel B, lanes 6, 7 and 8, respectively). However, the amounts of protein loaded into the lanes are not constant, with the result of seemingly enhanced

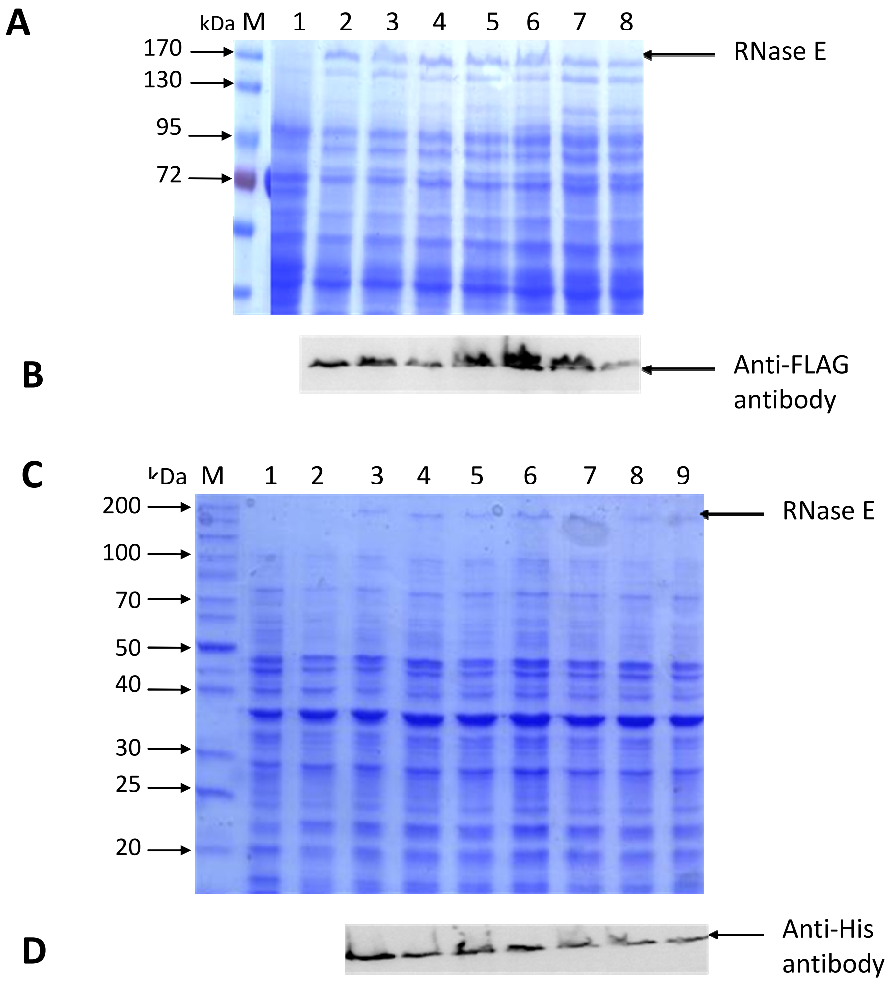

Figure 2.5 Protein production study and western blot analysis of FLAG-tagged RNase E and His-tagged RNase E.
Study of optimal protein production of *E. coli* BL21 (DE3) [pRE196] through 7% reducing SDS-PAGE analysis (A), western blot analysis (B) and study of optimal protein production of *E. coli* BL21 (DE3) [pRE296] through 10% reducing SDS-PAGE analysis (C) and western blot analysis (D). (A) lane 1: uninduced cells transformed with FLAG-tagged RNase E; lane 2: transformed cells 30 minutes after IPTG induction; lane 3: 40 minutes after induction; lane 4: 50 minutes after induction; lane 5: 60 minutes after induction; lane 6: 90 minutes after induction; lane 7: 150 minutes after induction; lane 8: 180 minutes after induction. The RNase E protein band has a mobility of 180 kDa. (B) Western blot analysis the molecular mass of 180 kDa of RNase E using anti-FLAG antibody. (C) lane 1: negative control of untransformed *E. coli* BL21 cells; lane 2: uninduced cells transformed with His-tagged RNase E; lanes 3 to 9: transformed cells 30, 40, 50, 60, 90, 150 and 180 minutes after IPTG induction. RNase E is visible at 180 kDa. (D) RNase E of 180 kDa was identified by means of an anti-FLAG antibody using western blot analysis.

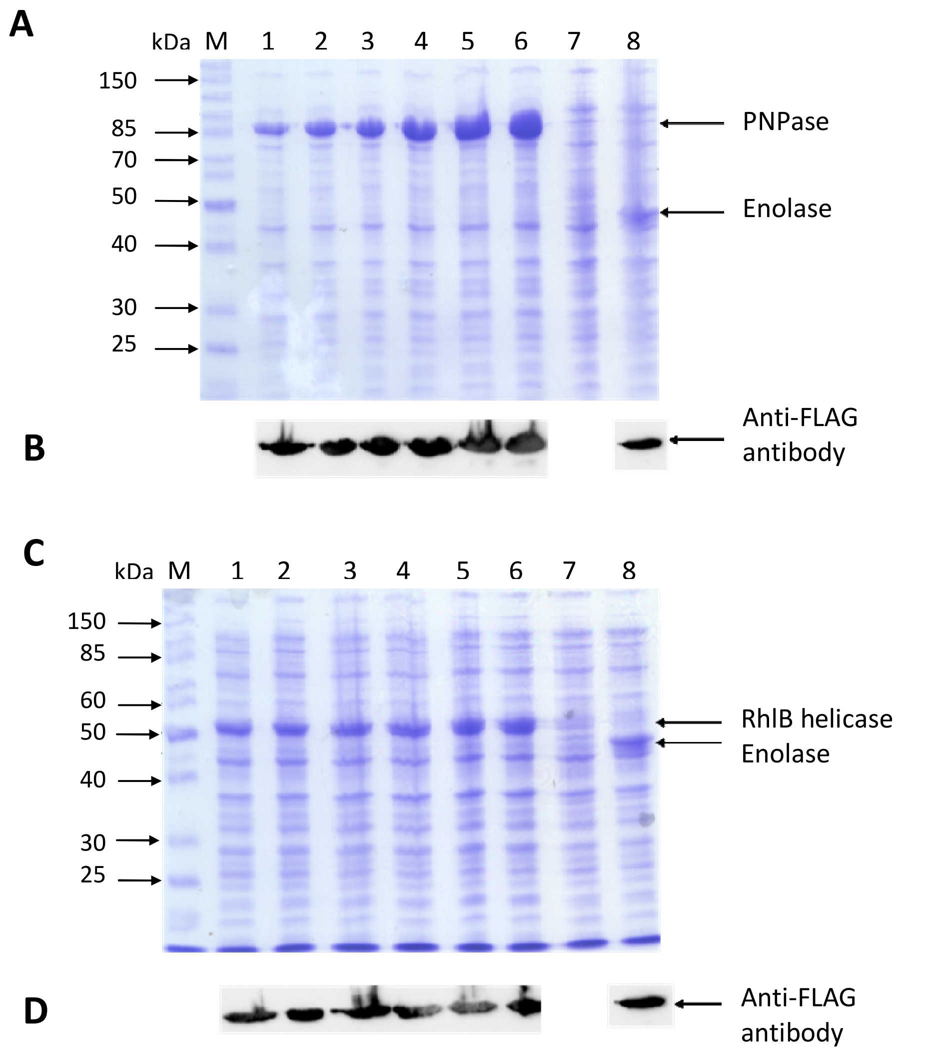

Figure 2.6 Protein production study and western blot analysis of FLAG-tagged PNPase and RhlB Helicase.
Study of optimal protein production of *E. coli* BL21 (DE3) [pflag-PNP] through 10% reducing SDS-PAGE analysis (A) and western blot analysis (B), and study of optimal protein production of *E. coli* BL21 (DE3) [pflag-RhlB] through 10% reducing SDS-PAGE analysis (C) and western blot analysis (D). (A) lane 1: uninduced cells transformed with FLAG-tagged PNPase; lane 2: transformed cells 1 hour after IPTG induction; lane 3: 2 hours after induction; lane 4: 3 hours after induction; lane 5: 4 hours after induction; lane 6: 5 hours after induction. lane 7: negative control of untransformed *E. coli* BL21 cells; lane 8: positive control with enolase 5 hours after induction. The PNPase protein band has a mobility of 79 kDa. (B) PNPase of 79 kDa was identified by means of an anti-FLAG antibody using western blot analysis. (C) lane 1: uninduced cells transformed with FLAG-tagged RhlB helicase; lane 2 to 6: transformed cells 1, 2, 3, 4 and 5 hours after IPTG induction; lane 7: negative control of untransformed *E. coli* BL21 cells; lane 8: positive control with enolase 5 hours after induction. The RhlB helicase protein band has a mobility of 50 kDa. (B) RhlB helicase of 50 kDa was identified by means of an anti-FLAG antibody using western blot analysis.

protein production (Figure 2.5, panel A, lanes 6 – 8 compared to lanes 2 - 4). It is therefore likely that there is a total protein increase as opposed to an increase in RNase E production.

The protein production study of His-tagged RNase E (pRE296) showed an overall lower level of protein production than that of FLAG-tagged RNase E (Figure 2.5, panel C). No RNase E protein was visible in both the untransformed *E. coli* BL21 cells as well as prior to induction (Figure 2.5, panel C, lanes 1 and 2). Very low protein levels were observed in the induced samples taken at times 30, 40, 50, 60, 90, 150 and 180 minutes which remained constant over the duration of the study (Figure 2.5, panel C, lanes 3 – 9). Western blot analysis the molecular mass of 180 kDa of RNase E and showed the highest level of RNase E protein to be 30 minutes after induction, after which the protein levels decreased (Figure 2.5, panel C, lanes 3 – 9).

In contrast to RNase E, the remaining major constituents (PNPase, RhlB helicase and enolase) of the degradosome are all over expressed in *E. coli* BL21 (DE3) cells. The protein production study of PNPase with size 85 kDa is shown in Figure 2.6, panel A. PNPase protein is not visible in the untransformed *E. coli* BL21 cells (Figure 2.6, panel A, lane 7). The PNPase protein band appears to be higher than the 85 kDa marker band which may very well be due to the additional presence of the FLAG-tag, making the protein slightly larger (Figure 2.6, panel A, lanes 1 - 6). A very distinct sequential increase in PNPase protein production from prior to induction to 5 hours after induction was visible, with the 5th hour sample showing the highest protein level (Figure 2.6, panel A, lanes 1 – 6). Western blot analysis along with SDS-PAGE showed: PNPase protein levels were highest 5 hours after induction (Figure 2.6, panel B, lanes 1 – 6). Protein production of enolase (47 kDa) was shown in the SDS gel 5 hours after induction, and was identified by an anti-FLAG antibody using western blot analysis (Figure 2.6, panel A and B, lane 8). FLAG-tagged enolase served as a positive control for the western blot analysis to confirm that the anti-FLAG antibody detected the FLAG-tagged proteins. The complete enolase protein production study is discussed in Section 4.3.2.

RhlB helicase protein production study is shown in Figure 2.6, panel C. Helicase protein, with a mobility of 50 kDa, was not visible in untransformed *E. coli* BL21 cells (Figure 2.6, panel C, lane 7). The study showed helicase protein production from prior to IPTG induction to 5 hours after induction (Figure 2.6, panel C, lanes 1 – 6). RhlB helicase of 50 kDa was identified by means of an anti-FLAG antibody using western blot analysis (Figure 2.6, panel D, lanes 1 – 6). The protein production of enolase was included as in Figure 2.6, panel A and B, lane 8 (Figure 2.6, panel C, lane 8). Enolase of 47 kDa was identified by means of an anti-FLAG antibody using western blot analysis (Figure 2.6, panel D, lane 8).

2.3.4 <u>Solubility study of RNase E</u>

The scaffold protein of the *E. coli* RNA degradosome, RNase E, is tethered to the cytoplasmic membrane through its N-terminal region making the isolation of the protein a challenging task (Liou *et. al.*, 2001). A membrane-bound protein needs to be removed from the membrane for it to be rendered soluble. For this reason a variety of detergents were used to determine which would remove RNase E from the membrane, thereby allowing further purification of the protein.

Four detergents were used in this study for the solubilization of RNase E. The first, polyethyleneimine, is an organic polyamine polymer that contains no aromatic rings, and has a high charge density as well as a high molecular weight and so is able to change a proteins surface charge (Simpson, 2006). The second, octylphenol ethylene oxide condensate, otherwise known as Triton X-100, is a surfactant that is nonionic and is frequently used to solubilize proteins. This non-denaturing detergent is produced from octylphenol polymerized with ethylene oxide, and has an average of 9.5 ethylene oxide units per molecule (Supplier data, Sigma-Aldrich Product Information). The third detergent N-lauroyl sarcosine (sarcosyl), is anionic and can be used to assist solubilizing and separating membrane proteins and glycoproteins (Dawson *et al.*, 1986). The last detergent used, Tergitol-type NP-40, a nonyl phenoxylpolyethoxylethanol, is a nonionic surfactant that can rupture the cytoplasmic membrane but is not strong enough to rupture the nuclear membrane (Sigma-Aldrich specification sheet, Schwartz and Nathenson, 1971).

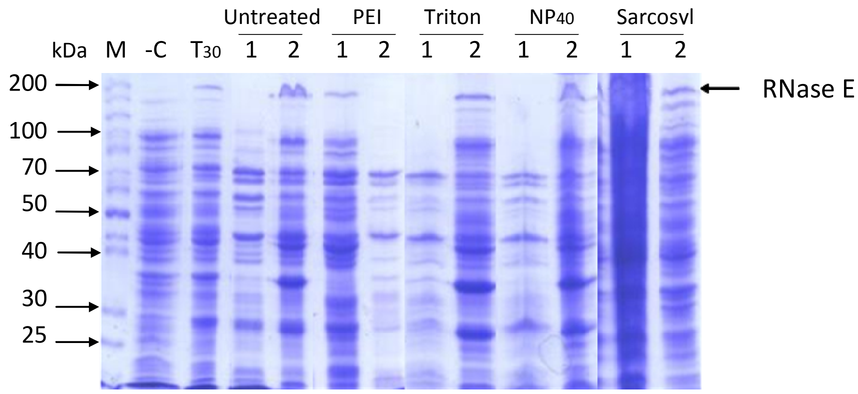

Figure 2.7 Determining the effect of detergents PEI, Triton, NP40 and Sarcosyl on the solubility of RNase E
10% reducing SDS-PAGE analysis of *E. coli* BL21 (DE3) [pRE296] harvested 30 minutes after induction, cells in lysis buffer were divided and different detergents added to solubilize RNase E. Polyethyleneimine (PEI), triton X-100, NP_4O_5 and N-lauroyl sarcosine added prior to sonication were compared with the untreated sample. Lane M: molecular weight marker, lane –C: *E. coli* BL21 cells, lane T_{40}: sample of *E. coli* BL21 (DE3) [pRE296] 30 minutes after induction, lane 1: sample of centrifuged supernatant after sonicating, lane 2: sample of pellet resuspended in PBS.

E. coli BL21 (DE3) [pRE296] cells were harvested 30 minutes after IPTG induction. The first aliquot was left untreated; the second treated with PEI, the third with Triton X-100, the fourth with NP_{40} and the last with N-lauroyl sarcosine (Figure 2.7). The cell extracts were sonicated to rupture the cells, and then centrifuged to separate the soluble supernatant (Figure 2.7, lane 1) from the cellular debris (Figure 2.7, lane 2). RNase E was found to be insoluble in the untreated samples, being present in the pellet sample and not visible in the supernatant. The cell extract treated with PEI showed the RNase E protein to be present in the supernatant, and not in the pellet. Triton-treated extracts showed RNase E to only be visible in the pellet. NP_{40} again resulted in RNase E only being visible in the pellet. Sarcosyl treated extracts resulted in sonicated and supernatant lanes that are both poorly resolved, consistently poorly resolved upon numerous sarcosyl treatments, and an RNase E protein band visible in the pellet, leading to a non-conclusive result. Triton and NP_{40} were not able to solubilize RNase E. Treatment of cell extracts with sarcosyl leads to an inconclusive result. However, PEI successfully solubilized RNase E.

2.3.5 Purification of FLAG-tagged RNase E

Purification using FLAG affinity chromatography was used because it is a non-denaturing system which would allow purification not only of FLAG-tagged RNase E, which is trapped in the membrane, but of all the other constituents that associate to this scaffold protein to form the degradosome (Einhauer and Jungbauer, 2001).

Overproducing RNase E results in a reduction of cellular growth (Claverie-Martin *et al.,* 1991; Jain *et al.,* 2002). It thus follows that induced RNase E expresses low levels of protein over a short period of time and because of that it was expected to obtain low levels of purified protein. In an attempt to obtain purified RNase E with the components of the degradosome co-purified, a FLAG epitope fusion protein was used in immunoaffinity purification. The FLAG-tagged purification of RNase E produced very low levels of RNase E protein (Figure 2.8). No RNase E protein was visible in both untransformed *E. coli* BL21 cells as well as prior to induction (Figure 2.8, panel A, lanes 1 and 3). RNase E protein yield was very low 90 minutes after IPTG induction; RNase E of 180 kDa was identified by means of an anti-FLAG antibody using western blot analysis (Figure 2.8, panels A and B, lane 2). A sample of harvested cells treated with 0.1 % PEI showed an increased RNase E protein yield, identified with anti-FLAG antiobody (Figure 2.8, panels A and B, lane 4) However, the FLAG-tagged protein eluted by the addition of excess synthetic FLAG peptide was not visible in the SDS gel, and barely detectable by western blot analysis (Figure 2.8, panels A and B, lane 5). It was expected that all the major degradosome constituents would copurifiy with RNase E, including PNPase, RhlB helicase and enolase with expected mobilities of 85 kDa, 50 kDa and 47 kDa, respectively.

Denaturants affect the antibody stability or the fusion and it is therefore not recommended for a FLAG fusion protein to be purified in the presence of a denaturant (Einhauer and Jungbauer, 2001). The failure at purifying the RNA degradosome was

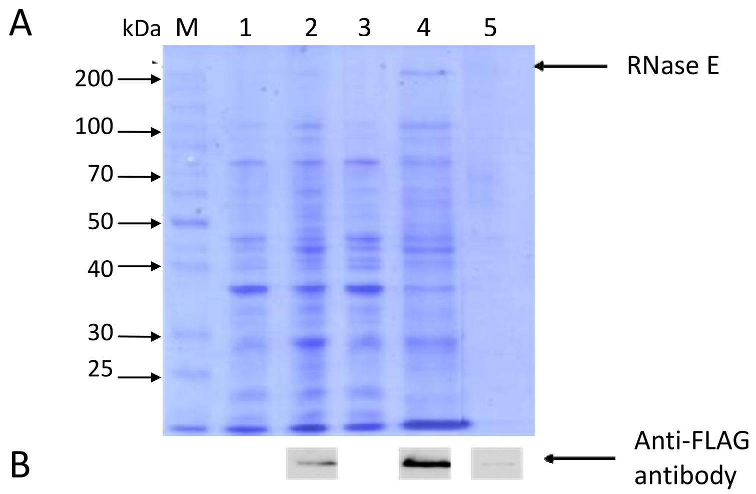

Figure 2.8 Purification of FLAG-tagged RNase E
10% educing SDS-PAGE analysis of FLAG-tagged purification of pRE196 (A). Western blot analysis of FLAG-tagged purification of pRE196 (B). (A) *E. coli* BL21 (DE3) transformed with pRE196, cells harvested 90 minutes after induction and FLAG-purification conducted. Lane M: molecular weight marker, lane 1: negative control of untransformed *E. coli* BL21 cells, lane 2: sample of culture grown to 90 minutes after induction, lane 3: uninduced sample, lane 4: sample of centrifuged supernatant after cells had been treated with 0.1 % PEI and sonicated, lane 5: sample eluted with excess FLAG peptide. (B) RNase E of 180 kDa was identified by means of an anti-FLAG antibody using western blot analysis.

unexpected since previous researchers had managed to do so successfully using the FLAG fusion protein. Previous researchers successfully purified the RNA degradosome using detergent Triton X-100, and an ammonium sulphate precipitation ranging from 40 – 60 % (Miczak *et al.*, 1996; Klein *et al.*, 2005). In this study the initial native purification experiments using Triton X-100 did not solubilize RNase E, which lead to an unsuccessful purification (results not shown). Additional detergents were tested, including PEI and sarcosyl. The FLAG-tagged RNase E purification protocol used in this study was based on the purification protocol used by Miczak *et al.* (2003). However, purification of the RNA degradosome using FLAG-tagged RNase E was not successful.

2.3.6 Purification of His-tagged RNase E

Having found that purification using the FLAG epitope was unsuccessful a different purification method was used. Metal affinity chromatography is based on the principle that the amino acid histidine binds to the metal ion nickel (Petty, 2001). RNase E has 6 His residues at the C-terminus that will bind to nickel ions, which through chelation is bound to agarose beads. The ions are immobilized to the resin through nitrilotriacetic acid (NTA) (Petty, 2001).

The His-tagged purification of RNase E was performed to purify the *E. coli* RNA degradosome (Figure 2.9). As determined by the solubility study in Section 2.3.4 the detergent PEI appeared to best solubilize RNase E and thus used in the RNase E purification (Figure 2.9, panel A). In addition to PEI, sarcosyl was used in a second RNase E purification to determine if RNase E could successfully be purified from a protein extract that had been treated with this detergent (Figure 2.9, panel B). In the His-purification using PEI, RNase E protein was visible in the sonicated cells, the soluble supernatant as well as, surprisingly, the insoluble pellet (Figure 2.9, panel A, lanes 1 – 3). The presence of RNase E protein in both the supernatant and pellet indicates that the detergent PEI had not completely solubilized RNase E. The protein was present in low levels in the RNase E protein extract incubated with beads overnight (Figure 2.9, panel A, lanes 4). A 50 mM imidazole wash removed non-specific proteins from binding to the beads; no RNase E protein is visible, showing that no protein had been washed off the beads (Figure 2.9, panel A, lane 5). RNase E protein was present in the first two elutions of 750 mM and 1 M imidazole but in the third wash of 1 M again (Figure 2.9, panel A, lanes 6 – 8, respectively). Traces of RNase E and the same proteins as in the elution remained bound to the beads (Figure 2.9, panel A, lane 9).

RNase E of 180 kDa was identified by means of an anti-His antibody using western blot analysis (Figure 2.9, panel C). The protein bands in the elution lane at approximately 70 and 80 kDa could be DnaK and PNPase (Figure 2.9, panel B, lane 6). The protein band that is faintly visible between 40 and 50 kDa may be enolase. Western blot analysis using anti-DnaK, -PNPase and –enolase would verify the presence of these proteins. The

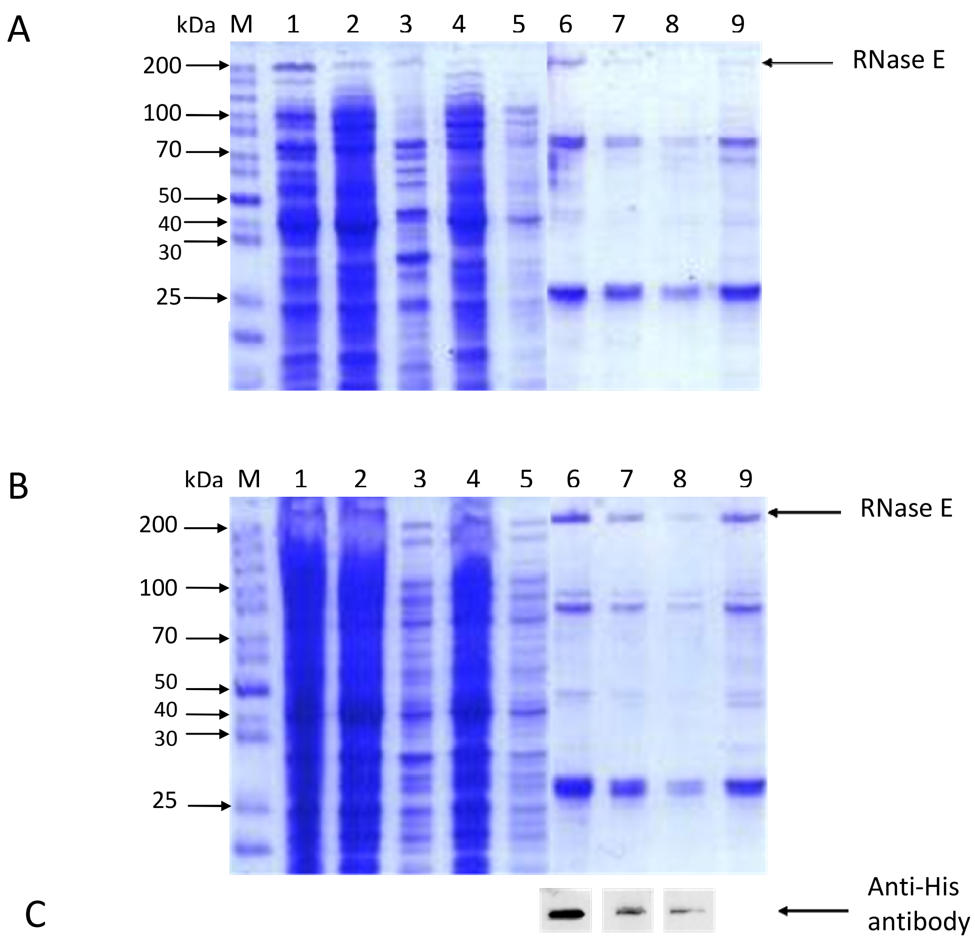

Figure 2.9 Purification of His-tagged RNase E from *E. coli* BL21cells
His-tagged purified PEI-treated RNase E using 10% reducing SDS-PAGE analysis (A), His-tagged purified sarcosyl-treated RNase E using 10% reducing SDS-PAGE analysis (B) and western blot analysis of RNase E (A) Once harvested *E. coli* BL21 (DE3) [pRE296] was treated with 0.1 % PEI prior to sonication and then purified with nickel-affinity chromatography. Lane M: molecular weight marker, lane 1: PEI-treated sonicated cells, lane 2: centrifuged supernatant after sonication, lane 3: pellet resuspended in PBS, lane 4: RNase E incubated with beads overnight, lane 5: first wash with 50 mM imidazole, lane 6: first elution with 750 mM imidazole, lane 7: second elution with 1M imidazole, lane 8: third elution, lane 9: beads after elution sample. (B) Harvested *E. coli* BL21 (DE3) [pRE296] was treated with 7.5 % sarcosyl prior to sonication and then purified with nickel-affinity chromatography. Lane M: molecular weight marker, lane 1: sarcosyl-treated sonicated sample, lane 2: centrifuged supernatant after sonication, lane 3: pellet resuspended in PBS, lane 4: RNase E incubated with beads overnight, lane 5: first wash with 50 mM imidazole, lane 6: first elution with 750 mM imidazole, lane 7: second elution with 1M imidazole, lane 8: third elution, lane 9: beads after elution sample. (C) RNase E of 180 kDa was identified by means of an anti-His antibody using western blot analysis.

RNase E purification, when compared to the reconstitution of the RNA degradosome performed by Worrall *et al.* (2008) shows the presence of what may be all major RNA degradosome components, albeit that RhlB helicase and enolase are expressed at lower protein levels. However, in the purification of RNase E, two additional protein bands are present in the region of 25 – 30 kDa. These two proteins may be constituents of the RNA degradosome, RraA and 30S ribosomal protein, but have as of yet not been identified (Gao *et al.*, 2006). It may be that these two proteins are by-products of the purification either when using His-tagged RNase, or the use of sarcosyl to solubilize RNase E. A control such as a vector plasmid would help determine whether RraA and 30S ribosomal protein are constituents of the RNA degradosome, or simply by-products of the purification protocol.

To rule out that the protein bands are not *E. coli* contaminating proteins, *E. coli* cells must be transformed with an empty vector; the control experiment should have no proteins bound to the beads. One of the proteins retained by His-tags columns is peptidyl prolyl isomerase (SlyD), with a mobility of 25 kDa (Hengen, 1995). To rule out SlyD as the protein band migrating at 25 – 30 kDa in panel A, His-tagged RNase E either has to be purified using a cobalt based resin (which does not bind SlyD) or the protein has to be produced in a SlyD-deficient *E. coli* strain (Hengen, 1995). These experimental controls need to be conducted to confirm the identity of the small protein bands.

The purification of RNase E using the detergent PEI was not consistent. Previous results showed variations in the level of RNase E solubility (results not shown). RNase E was thus purified using the solubilizing agent sarcosyl. (Figure 2.9, panel B). The sonicated cells and supernatant are both poorly resolved, and the pellet appears to contain RNase E protein (Figure 2.9, lane B, lanes 1 – 3). Not being able to determine if RNase E is present in the soluble fraction, it cannot be concluded that RNase E has or has not been solubilized, or if it has only been partially solubilized. RNase E was incubated with beads overnight, again showing poorly resolved protein bands which are inconclusive (Figure 2.9, lane B, lane 4). In contrast to the PEI purification in Figure 2.9, panel A, the 50 mM imidazole wash appears to have washed off a substantial level of RNase E protein from the beads (Figure 2.9, lane B, lane 5). The 750 mM and two elutions of 1 M allowed RNase E to be eluted from the beads, producing high levels of purified RNase E (Figure

2.9, lane B, lanes 6 - 8). The same protein bands appear in the sarcosyl purification as in the PEI purification, but are more concentrated. RNase E and the same proteins as in the elutions remained bound to the beads, indicating that the purification procedure can be optimized further to allow no loss of protein. The sarcosyl purification of His-tagged RNase E produced a higher concentration of purified protein than that of the PEI purified protein. Sarcosyl is thus the preferred detergent and RNase E was successfully purified.

2.4 <u>Determination of co-purified degradosome components</u>

To verify the successful purification of the *E. coli* RNA degradosome each of the constituents of the degradosome (PNPase, RhlB helicase and enolase) needs to be detected with their respective antibodies using western blot analysis. However, not having produced antibodies for each of the proteins, a different approach was used. All of the proteins were available as FLAG-tagged fusion proteins, and upon producing these proteins and comparing the resulting protein bands with those obtained in the RNase E elution, the presence of the degradosome could be confirmed.

Upon the successful purification of His-tagged RNase E, the constituents of the degradosome were expressed and compared to the unidentified protein bands found in the SDS-PAGE analysis of purified RNase E. Western blot analysis using anti-FLAG antibody was used to identify RNase E at 180 kDa. RhlB helicase at an expected mobility of 50 kDa and enolase at 47 kDa corresponded to the protein bands in the RNase E purified lane (Figure 2.10, panel A, lanes H, E and El, respectively). The proteins, PNPase, DnaK, RhlB helicase and enolase, were purified separately and the lanes were cut out of separate SDS-PAGE gels in order to determine if all the constituents of the RNA degradosome were present in the purification. (Figure 2.10, panel A, lanes P, D, H, E and E1, respectively). This was necessary due to a lack of antibodies. PNPase, DnaK, helicase and enolase of 85 kDa, 70 kDa, 50 kDa and 47 kDa, respectively, was identified by means of anti-FLAG antibodies using western blot analysis (Figure 2.10, panel B, lanes P, D, H and E, respectively). In the lane of purified RNase E, protein bands of PNPase, helicase and enolase showed very low protein levels when compared to that of RNase E and DnaK (Figure 2.10, panel A, lane El). There are two protein bands that have not been identified, with a size between 25 and 30 kDa, as was discussed in Section 2.3.6. These unknown proteins may be proteins of interest or components of the RNA degradosome that have not yet been identified. Further research needs to be conducted to determine whether they are related to the RNA degradosome or whether they may be proteins of interest. The two protein bands with mobilities between 25 and 30 kDa were suggested to be 30 S ribosomal protein and RraA protein by Gao *et al.*, (2006).

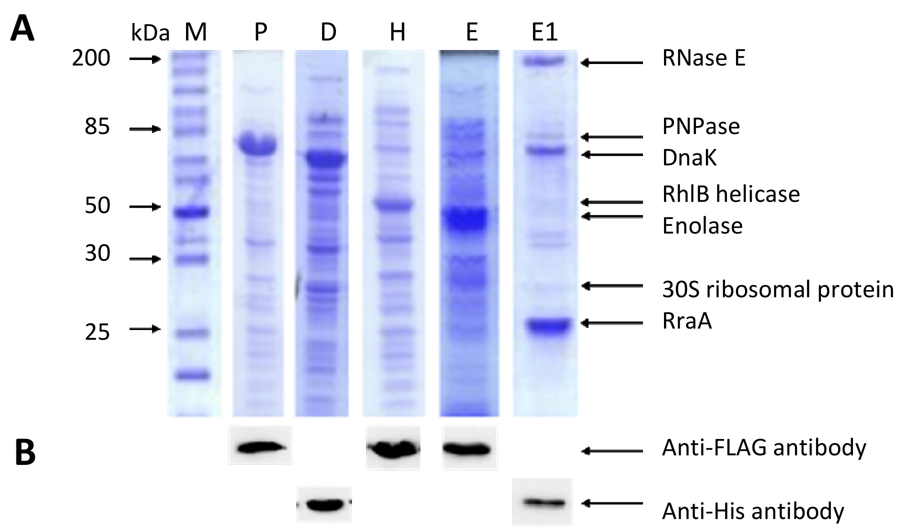

Figure 2.10 Purification of the *E. coli* RNA degradosome
10% reducing SDS-PAGE analysis of the degradosome components (A). Western blot analysis of the degradosome components (B). (A) Lane M: marker, lane P:sample of PNPase 5 hours after induction (79 kDa), lane D: sample of DnaK 5 hours after induction (70 kDa), lane H: sample of RhlB Helicase 5 hours after induction (50 kDa), lane E: sample of enolase 5 hours after induction (47 kDa), and lane E1: RNase E elution (180 kDa).

The RNA degradosome was purified to use it as a tool to investigate what some of the components response would be to certain environmental conditions. For instance, the effect of DnaK on enolase can be determined using the purified RNA degradosome under heat shock by enolase assays. Another point to investigate would be to determine whether GroEL could functionally replace DnaK under heat shock. This would be achieved by *in vivo* heat shocking of the purified RNA degradosome from *E. coli* BL21 cells and from an *E. coli danK* minus strain, using western blot analysis with anti-GroEL and anti-DnaK.

2.5 <u>Conclusion</u>

Through restriction analysis and agarose gel electrophoresis the plasmids were successfully verified. Protein production after induction was monitored to determine the time points of optimal protein production and analyzed by SDS-PAGE and western blot analysis. Protein production was conducted at 30ºC as RNase E protein production levels were found to be less at 37°C (results not shown). It was found that RNase E protein production levels reached an optimal production 40 minutes after induction with IPTG. Induction over a longer period of time showed a decrease in protein production levels which could be due to auto-regulation of RNase E. This finding was supported by Miczak *et al.* (1996) who showed that RNase E enzyme concentration started to decrease after having reached maximum protein production after only 25 minutes. The low protein levels prompted a concentration of the induced samples prior to SDS-PAGE analysis. The other components of the degradosome, however, were over expressed at 37°C. Optimal protein production of enolase, RhlB helicase and PNPase were shown to be 5 hours after induction and their molecular mass was identified by means of anti-FLAG antibodies using western blot analysis.

RNase E was not over expressed during the protein production study. RNase E controls its own production levels by cleaving its mRNA (Mudd and Higgens, 1993; Jain and Belasco, 1995). The discovery that RraA (regulator of ribonuclease activity A) prevents the endonucleolytic cleavage of RNase E and therefore in its presence allows restored growth to *E. coli* cells with an over production of RNase E led to the finding that overproducing the *E. coli* RNase E protein results in a decrease of cellular growth (Claverie-Martin *et al.*, 1991; Lee *et al.*, 2003; Yeom and Lee, 2006). This could possibly explain why the highest protein production occurs within only 30 minutes for His-tagged RNase E after induction with inducer IPTG. FLAG-tagged RNase E showed an optimal protein production 90 minutes after induction.

The fact that the membrane bound scaffold protein was found to be insoluble made it necessary to determine which treatment or detergent to use to remove RNase E from the cytoplasmic membrane. Literature indicated the use of Triton X-100 to solubilize RNase

E (Miczak *et al.,* 1996), but surprisingly we could not achieve a successful purification using this detergent. In the solubility study of RNase E, PEI showed the best result by leaving no apparent traces of RNase E in the pellet containing cellular debris, rendering it soluble. However, the result was not easily reproducible. Experiments showed that PEI could not render RNase E soluble every time, leaving traces of protein in the insoluble pellet fraction. Upon further testing the anionic detergent N-lauroyl sarcosine was found to be the optimal reagent to solubilize RNase E, even though traces of protein were found in the pellet. The sarcosyl-treated cellular extract produced a higher purified protein yield than that treated by PEI.

Protein purification using fusion proteins have advantages such as elongation of the target protein which increases the proteins' solubility and the yield of produced protein, as well as purification of the target protein in a one-step purification procedure through the use of affinity matrices and allowing easy identification (Einhauer and Jungbauer, 2001). In this study the FLAG-tagged purification of RNase E was not successful. The FLAG tag shows the disadvantages of immunoaffinity purification, including that of having a low binding ability (Einhauer and Jungbauer, 2001), which may have contributed to obtaining a very low yield of purified *E. coli* RNase E. This protein, being a protein that can't be over expressed without the presence of the RraA protein, was difficult to purify with a procedure that had been used by previous researchers, surprisingly even after having used the detergents that they achieved successful purification with (Miczak *et al.,* 1996).

The successful purification of RNase E was achieved through the metal affinity chromatography purification procedure. Both PEI and sarcosyl-treated protein extracts allowed the purification of RNase E; however, sarcosyl produced a higher yield of purified protein. Not only was RNase E purified but the *E. coli* RNA degradosome constituents attached to the scaffold protein were co-purified alongside RNase E. Protein production of each of the components were compared with the purified product and found to correspond to the protein bands, thereby verifying the purification of the degradosome. Therefore, in conclusion, RNase E and the components of the *E. coli* RNA degradosome were successfully verified and expressed, and the purification of the scaffold protein was optimized in order to allow further study of the degradosome.

Future work would include producing antibodies against PNPase, RhlB helicase and enolase to facilitate further research concerning the RNA degradosome. Another aspect of future work would include obtaining the RraA protein inhibitor to allow the over production of RNase E protein, which would allow optimization of the RNA degradosome purification. Optimization, in turn, would allow continuing analysis of the components of RNA degradosome, such as the levels of molecular chaperones upon heat stress to elucidate their role; and investigating new proteins in the RNA degradosome which have not yet been annotated.

Chapter 3

Analysis of heat shock proteins in the *E. coli* RNA degradosome

----

3.1 Introduction

Miczak *et al.* (1996) discovered DnaK to be associated with the degradosome in substoichiometric amounts over a decade ago. DnaK and its co-chaperones DnaJ and GrpE are involved in the heat shock response. Steady-state protein levels in *E. coli* cells do not differ noticeably within the normal range of 20°C to 37°C implying that the composition of the cell stays similar over this range (Lemaux *et al.*, 1978). However, upon a temperature shift to the high range (above 40°C) or the low range (below 20°C), physiological changes become evident, implying that the cellular composition differs when moving between the normal temperature range and the extreme ranges. One of these changes at a temperature range above 37°C is the induction of the heat shock response. The production of heat shock proteins, elicited by the increased temperature, is an adaptive physiological change that is vital for cellular growth (Neidhart and VanBogelen, 1987). The heat shock regulon, through the transcription factor σ^{32}, is modulated through DnaK, DnaJ and GrpE (Bukau, 1993). In the presence of ATP, DnaK and DnaJ interact with σ^{32}, thereby preventing the transcription factor from associating to RNA polymerase, turning off gene expression mediated by the heat shock response (Liberek and Georgopoulos, 1993). Failure of DnaK to fold proteins at elevated temperatures may mark the proteins for degradation (Sherman and Goldberg, 1992). Miczak *et al.* (1996) proposed that degradation of the remaining mRNA by the degradosome scaffold protein, RNase E, would render the heat shock response more complete. Another component of the degradosome, GroEL, is a heat shock protein that is essential for *E. coli* cells to grow at all temperatures.

PNPase, the exoribonuclease of the degradosome, is, in contrast to DnaK, a cold shock protein (Jones and Inouye, 1994). *E. coli* growth temporarily stops when the cell is exposed to cold shock and production of cold shock proteins is highly induced. PNPase is required to suppress the specific cold shock proteins at the end of the acclimation phase so that cell growth can be restarted and non-cold shock protein production can be continued (Yamanaka and Inouye, 2001). RNase E binds both cold shock and heat shock proteins suggesting that these proteins engage in the folding of the degradosome complex in reaction to varying environmental conditions (Miczak *et al.,* 1996). This present study, however, will only focus on heat shock and the role of heat shock proteins.

Obtaining an *E. coli dnaK* minus strain would allow it to serve as a tool to study the degradosome in the absence of DnaK. The *E. coli* BM271 (DE3) strain was constructed in order to be able to use T7 promoter-based expression system to allow over-production of recombinant proteins (Sugimoto *et al.,* 2008). The *dnaK* minus strain was developed through a series of steps. The Δ*dnaK52* mutant, BM271, could not be infected by λDE3 because functional DnaK was not present. pEcoDnaK, encoding DnaK, was transformed into BM271 to allow lysogenization with λDE3 to occur. Spot tests were used to test the thermo sensitivity of the transformant. BM271 was infected by λDE3 using a λDE3 lysogenization kit (Novagen). The lysogen was called BM271 Δ*dnaK* (DE3) (Sugimoto *et al.,* 2007). Through plasmid curing the pEcoDnaK plasmid was removed from the strain resulting in BM271 (DE3) with resistance to chloramphenicol (Sugimoto *et al.*, 2008).

The aim of this study was to investigate the role of heat shock proteins in the assembly of the degradosome under conditions of stress. Does DnaK become more abundant in the RNA degradosome upon heat shock? Is GroEL present in the RNA degradosome when DnaK is absent? Can GroEL functionally replace DnaK in the RNA degradosome? A protein production study of *E. coli* DnaK will be used to determine optimal protein production using western blot analysis. The second objective was to purify His-tagged DnaK using metal affinity chromatography. The study was also focused on the *in vivo* analysis of DnaK protein levels upon heat shock. Western blot analysis was used to identify the molecular mass of DnaK using anti-His antibody and as a result detect change in levels of DnaK in *E. coli* BL21 cells transformed with RNase E. Another objective was the *in vivo* analysis of GroEL protein levels upon heat shock. Firstly, RNase E and the RNA degradosome would have to be purified from the *dnaK* minus strain. Western blot analysis will be used to detect changes in levels of GroEL in the RNA degradosome purified from the *dnaK* minus strain. Secondly, a subcellular fractionation in *E. coli* BL21 cells at 37°C and under heat shock would be used to determine GroEL levels using western blot analysis.

3.2 Materials and Methods

3.2.1 Materials

Reagents used were purchased from Sigma Chemicals Co. (St. Louis, Mo U.S.A.), Merck Chemicals (Darmstadt, Germany), BioRad (U.S.A.) or Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Hybond^{TM} Cextra chemiluminescence nitrocellulose was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Anti-GroEL anitibody produced in rabbit was purchased from Sigma. Monoclonal Mouse anti-His was purchased from Amersham. Monoclonal anti-DnaK antibody was purchased from Stressgen BIOCOM Biotech. The *E. coli* strain BM271 (DE3) was a kind donation from Prof. K. Sonomoto *(Department of Bioscience and Biotechnology, Kyushu University, Japan).* Plasmid pEKJ30 was constructed by Dr A. Boshoff.

Table 3.1 Description of *E. coli* strains and plasmids

Plasmid	Description	Reference
pRE296	pGM102 encoding *E. coli* His-tagged RNase E, Amp^R	Miczak *et al.* (1996)
pFLAG-ENO	pGP1–2 encoding *E. coli* enolase, Amp^R	Liou *et al.* (2002)
pEKJ30	pQE30 encoding *E. coli dnaK*, Amp^R	Boshoff *et al.* (2008)

Strains	Description	Reference
E. coli BL21 (DE3)	F- ompT gal [dcm] [lon] hsdSB λDEs	Studier *et al* (1990)
E. coli BM271 (DE3)	BM271 (MC4100Δ*dnaK*52::Cm^R) infected by λDE3	Sugimoto *et al*. (2008)
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r)].	Bullock *et al.* (1987)

3.2.2 Methods

3.2.2.1 Transformation of competent cells

Competent *E. coli* BM271 (DE3) cells were transformed with His-tagged RNase E (pRE296) as described in Section 2.2.2.5. Competent *E. coli* XL1 Blue cells were transformed with His-tagged DnaK (pEKJ30).

3.2.2.2 Determination of optimal protein production

The protein production studies were conducted as described in Section 2.2.2.6. For RNase E produced from *E. coli* BM271 (DE3) cells both 100 µg/ml ampicillin as well as 20 µg/ml chloramphenicol was used; ampicillin for the RNase E plasmid antibiotic resistance and chloramphenicol for the *dnaK* mutant strain. For DnaK produced from *E. coli* XL1-Blue cells 100 µg/ml ampicillin was used and the cell culture was grown at 37°C.

3.2.2.3 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed as described in Section 2.2.2.7.

3.2.2.4 Western blot analysis of proteins

Western blot analysis was performed as described in Section 2.2.2.8. Monoclonal mouse anti-His antibody was used to detect His-tagged RNase E with ECL anti-mouse IgG HRP-linked species specific whole antibody. Anti-DnaK and anti-GroEL antibodies produced in rabbit were used to detect DnaK and GroEL proteins using the same secondary antibody.

3.2.2.5 His-tagged protein purification

The purification of RNase E from the *E. coli dnaK* minus strain as well as the *E. coli* DnaK purification from XL1-Blue cells was performed as described in Section 2.2.2.11. Both 100 µg/ml ampicillin as well as 20 µg/ml chloramphenicol was used for the RNase E purification. Ampicillin only was used for the purification of DnaK.

3.2.2.6 Subcellular fractionation after heat stress

The subcellular fractionation was performed as described in Hatzixanthis *et al.* (2003). An overnight culture was prepared by growing *E. coli* BL21 (DE3) cells transformed with pflag-ENO in 25 ml 2 x YT broth with 100 µg/ml ampicillin at 37ºC. The overnight culture was inoculated into 275 ml 2 x YT broth with 100 µg/ml ampicillin and grown to an optical density of A_{600nm} = 0.3. Protein production was induced by the addition of IPTG to a final concentration of 1 mM. The culture was divided and one half was heat shocked for 10 minutes at 42ºC, the other kept at 37ºC. The two cultures were harvested at 4 000 g for 20 minutes at 4ºC. The pellets were separately resuspended in 7.5 ml periplasting buffer (20 % sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 500 µg/ml lysozyme) and incubated on ice for 5 minutes. A volume of 7.5 ml cold water was added to each sample, mixed with slow pipetting and again incubated on ice for 5 minutes. A pellet was produced by centrifuging for 20 minutes at 4 000 g at 4ºC and resuspended in 15 ml detergent lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1 % deoxycholate) and left to incubate at room temperature. The supernatant was kept as the periplasmic fraction. The solutions were sonicated at 60 Hz (20 x 10 seconds) and centrifuged twice for 20 minutes at 4 000 g at 4ºC to produce a supernatant. The supernatants were ultra-centrifuged at 44 800 g for 1 hour at 4ºC. The pellet, in which the membrane fraction is found, was resuspended in 1 ml detergent lysis buffer with no added deoxycholate. The supernatant was kept as the cytoplasmic fraction. The level of protein in the different cell organelles were analyzed by SDS-PAGE and the production of GroEL was confirmed using western blot analysis and anti-GroEL antibody.

3.2.2.7 *In vivo* heat shocking of RNase E and levels of chaperone production

To study protein levels of DnaK an overnight culture of *E. coli* BL21 (DE3) transformed with pRE296 was diluted 1 : 10 into 2x YT broth, incubated at 30ºC and grown to an optical density of A_{600nm} = 0.6. Protein production was induced by adding IPTG to a final concentration of 1 mM. The cells were grown for 40 minutes after induction followed by heat shocking at 42°C for 30 minutes. SDS-PAGE and western blot analysis using anti-His antibody was conducted. To study GroEL levels of protein an overnight culture of *E. coli* BL21 (DE3) transformed with enolase was diluted into 275 ml 2x YT broth, incubated at 37ºC and grown to an optical density of A_{600nm} = 0.3. Protein production was induced by adding IPTG after which the cells were heat shocked at 42°C for 10 minutes before performing a subcellular fractionation. SDS-PAGE and western blot analysis using anti-GroEL antibody was performed.

3.3 Results and discussion

3.3.1 *E. coli* DnaK production and purification

A protein production study of DnaK in *E. coli* XL1 Blue cells was performed to determine at what point cell culture should be harvested to obtain the highest quantity of protein. SDS-PAGE analysis indicated that DnaK (70 kDa) was over expressed and present even in the absence of an inducer (Figure 3.1, panel A). DnaK protein was clearly visible over the duration of 5 hours and after overnight growth (Figure 3.1, panel A, lanes 2 – 6, 8, respectively). Western blot analysis was performed to identify the 70 kDa protein band of DnaK using anti-His antibody on the third and fourth hours after induction and on the overnight sample (Figure 3.1, panel B, lanes 4, 5 and 8).

E. coli DnaK was purified to be used in the enolase activity assays (discussed in Section 4.3.4) to determine if there is an interaction between DnaK and enolase in the degradosome. Upon IPTG induction of *E. coli* XL1 Blue cells with His-tagged DnaK, SDS-PAGE analysis showed a distinct protein band at 70 kDa, DnaK of 70 kDa was identified by means of an anti-His antibody using western blot analysis (Figure 3.2, panels A and B, lane 2). Three elutions were performed, the first two with 750 mM imidazole, and the third with 1 M imidazole.

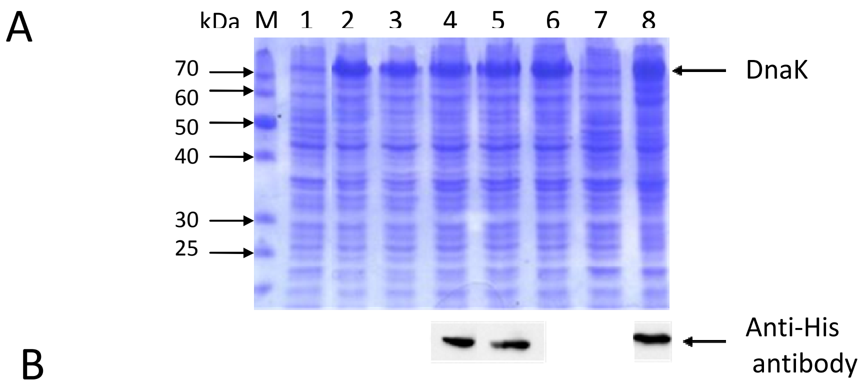

Figure 3.1 Protein production study and western blot analysis of DnaK in *E. coli* XL1 Blue cells
12% reducing SDS-PAGE (A) and western blot analysis (B) of optimal protein production levels of His-tagged DnaK in XL1 Blue cells. (A) Lane M: molecular weight marker; lane 1: untransformed *E. coli* XL1 Blue cells; lane 2: sample taken 1 hour after induction; lane 3: 2 hours after induction; lane 4: 3 hours after induction; lane 5: 4 hours after induction; lane 6: 5 hours after induction; lane 7: transformed cells before induction; lane 8: overnight growth after induction. (B) DnaK of 70 kDa was identified by means of an anti-His antibody using western blot analysis.

A very high DnaK protein concentration was obtained in the first elution, the molecular mass of 70 kDa which was identified using anti-His antibody during western blot analysis (Figure 3.2, panels A and B, lane 3). The second and third elutions produced slightly lower protein concentrations (Figure 3.2, panels A and B, lanes 4 and 5). The *E. coli* DnaK His-tagged purification was thus successful and enough protein was produced to use in enolase assays. The protein concentration was determined prior to conducting the assays using the Bradford assay (discussed in Section 4.2.2.7). Due to a satisfactory DnaK protein concentration, optimization of the purification of DnaK was not required.

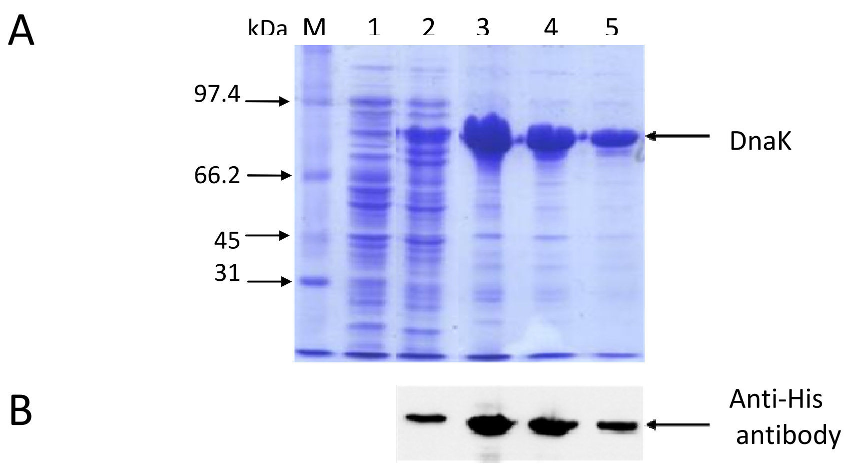

Figure 3.2 His-tagged purification of *E. coli* DnaK
10% reducing SDS-PAGE (A) and western blot analysis (B) of His-tagged purification of DnaK in *E. coli* XL1-Blue. (A) Lane M: molecular weight marker; lane 1: untransformed *E. coli* XL1-Blue cells; lane 2: transformed cells after overnight growth; lane 3: first elution with 750 mM imidazole; lane 4: second elution with 750 mM imidazole; lane 5: third elution with 1 M imidazole. (B) DnaK of 70 kDa was identified by means of an anti-His antibody using western blot analysis.

3.3.2 RNase E production and purification in the *E. coli dnaK* minus strain

To determine whether DnaK is functionally replaced by GroEL in the RNA degradosome, the *E. coli dnaK* minus strain was used to purify RNase E and the RNA degradosome, as the components of the degradosome associate to the scaffold protein RNase E. His-tagged RNase E (180 kDa) was produced in the *E. coli dnaK* minus strain, BM271 (DE3) and was studied using western blot analysis (Figure 3.3, panels A and B). No RNase E protein was visible prior to or after IPTG induction in the SDS-PAGE analysis (Figure 3.3, panel A, lanes 1 – 8). However, RNase E of 180 kDa was identified by means of anti-His antibody using western blot analysis albeit at low protein levels (Figure 3.3, panel B, lanes 2, 3 and 5). RNase E was detected 30, 40 and 60 minutes after IPTG induction, the highest RNase E protein concentration appeared to be 40 minutes after induction (Figure 3.3, panel B, lanes 2, 3 and 5). The absence of RNase E 50 minutes after induction is likely due to a poor western blot analysis in which an air bubble may have hindered the transfer of protein from the SDS-PAGE to the nitrocellulose membrane (Figure 3.3, panel B, lane 5).

Nickel affinity chromatography was used for the purification of His-tagged RNase E from the *dnaK* minus strain and all the other constituents that associate to this scaffold protein to form the degradosome. The detection of GroEL was conducted using western blot analysis. Previous studies (Section 2.3.4) have indicated that sarcosyl solubilises membrane-bound

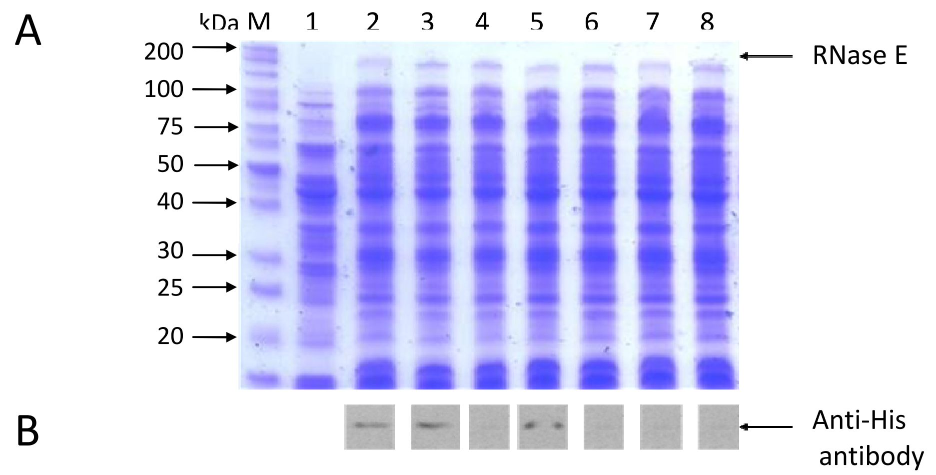

Figure 3.3 Protein production study of RNase E in *E. coli* BM271 (DE3) cells
10% reducing SDS-PAGE (A) and western blot analysis (B) of optimal protein production levels of His-tagged RNase E in the DnaK minus strain upon ITPG induction. (A) Lane M: molecular weight marker; lane 1: untransformed BM271 cells; lane 2: sample taken 30 minutes after IPTG induction; lane 3: 40 minutes after induction; lane 4: 50 minutes after induction; lane 5: 60 minutes after induction; lane 6: 90 minutes after induction; lane 7: 120 minutes after induction; lane 8: 180 minutes after induction. (B) RNase E of 180 kDa was identified by means of an anti-His antibody using western blot analysis.

RNase E. The soluble supernatant after treatment with ionic detergent sarcosyl does not show the RNase E protein band (180 kDa) to be present, yet through previous studies (Figure 3.4, panels A and B) it was determined that even though the protein is not visible on SDS-PAGE analysis, western blot analysis does confirm its presence, albeit at low levels of production. The molecular mass of 180 kDa of RNase E was identified by means of anti-His antibody but at a surprisingly low level of protein (Figure 3.4, panels A and B, lanes 1 and 2).

Having determined that RNase E was successfully purified from the *dnaK* minus strain, albeit at very low protein levels, western blot analysis was used to detect for GroEL. Neither the supernatant nor the RNase E elution showed any GroEL to be present (Figure 3.4, panel C, lanes 1 and 2). Purified GroEL was used as a positive control to verify the western blot analysis procedure, which showed a clear band at 60 kDa in the SDS-PAGE and was identified using western blot analysis (Figure 3.4, panels A and C, lane 3).

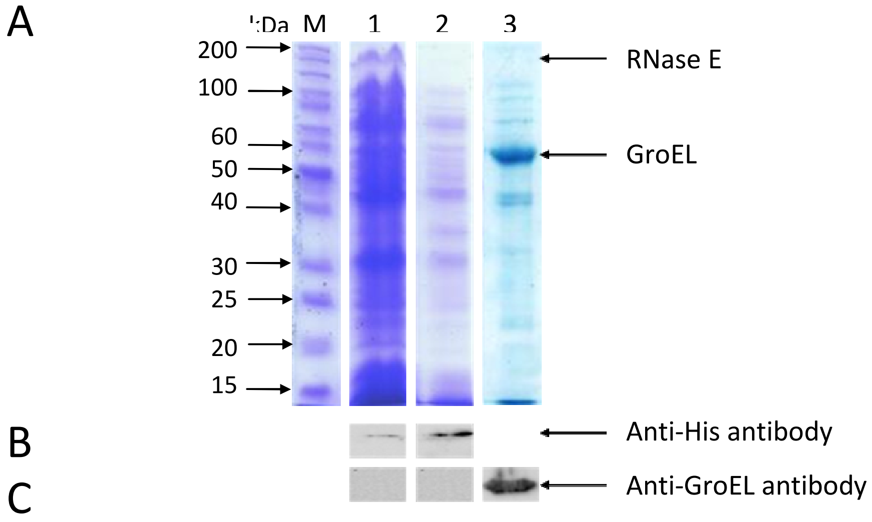

Figure 3.4 Purification of His-tagged RNase E in *E. coli* BM271 (DE3) cells
10% reducing SDS-PAGE of His-tagged purification of RNase E in the DnaK minus strain (A) and western blot analysis of RNase E (B) and GroEL as a degradosome constituent associated to RNase E (C). (A) Lane M: molecular weight marker; lane 1: soluble supernatant after sonication; lane 2: elution of RNase E (180 kDa) with 1 M imidazole; lane 3: positive control of GroEL, 5 hours after induction (60 kDa). (B) RNase E of 180 kDa was identified by means of an anti-His antibody using western blot analysis. (C) Using anti-GroEL antibody, western blot analysis did not confirm the presence of GroEL.

Upon a second attempt at the RNase E purification in the *dnaK* minus strain, neither RNase E nor GroEL were detected using western blot analysis. *E. coli* BM271 (D3) was a very difficult strain to work with due to its inconsistent growth patterns. Growth period of colonies varied from over night to over 2 days. The time it took for the culture to grow to mid log phase varied from a day to a few days, and occasionally there would be no growth beyond a certain absorbance reading. The absence of GroEL in the *dnaK* minus strain was unexpected. GroEL along with its co-chaperone GroES are the only chaperones that are required by *E. coli* for cellular growth at all temperatures, and thus should have been present (Fayet *et al.*, 1989). The purified RNase E protein levels may have been too low to allow the formation of the degradosome, and GroEL could thus be present at undetectable levels of protein. A way to combat this problem would be to induce the over production of RNase E by introducing its protein inhibitor RraA (previously discussed in Section 2.5). Another point to consider is that endogenous GroEL levels may be low as a norm. A control to determine if this is the case would be to use anti-GroEL antibody in western blot analysis to detect for GroEL in untransformed *E. coli dnaK* minus cells.

3.3.3 Subcellular fractionation after heat shock

The subcellular fractionation allowed the determination of the location of GroEL upon inducing heat shock. Having performed the fractionation in *E. coli* BL21 (DE3) cells, the presence of GroEL at 37°C and under heat stress (42°C) was investigated using western blot analysis and anti-GroEL antibody. The SDS-PAGE analysis shows no distinct protein band at 60 kDa under heat shock or at 37°C. However, western blot analysis indicates that GroEL is present in the fractionation, and the positive control of purified GroEL confirmed detection of GroEL (Figure 3.5, panel B, lane 9). In order to gauge the success of the fractionation it is necessary to have antibodies to known protein markers in each fraction. β-lactamase, chloramphenicol transacetylase and mannitol permease-alkaline phosphatase hybrid protein are used as markers for periplasmic, cytoplasmic and membrane fractions respectively (Sugiyama *et al.*, 1991). Based on the lack of these antibodies it was impossible to determine the success of the fractionation. The western blot analysis showed no GroEL was present in the non-heat shocked whole cell fraction, or in the non-heat shocked periplasm (Figure 3.5, panel B, lanes 1 and 2).

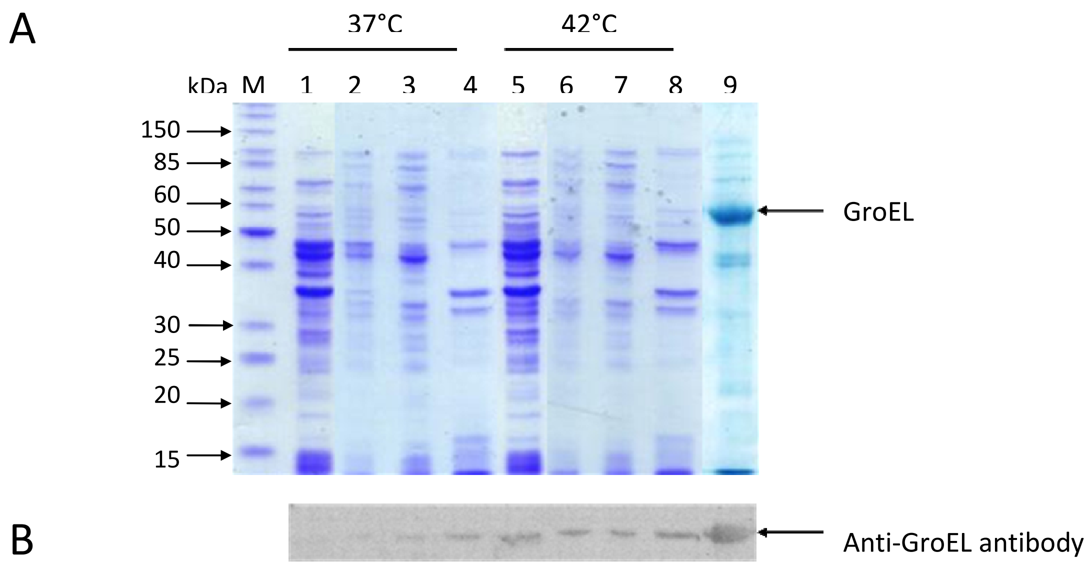

Figure 3.5 GroEL protein levels upon heat shock in a subcellular fractionation
10% reducing SDS-PAGE (A) and western blot analysis (B) of the subcellular localization of GroEL. (B) Lane 1: whole cells; lane 2: periplasmic fraction; lane 3: cytoplasmic fraction; lane 4: membrane fraction. Lanes 1 – 4 contain the fractions at 37°C. Lane 5: whole cells; lane 6: periplasmic fraction; lane 7: cytoplasmic fraction; lane 8: membrane fraction; lane 9: positive control of GroEL, 5 hours after induction (60 kDa). Lanes 5 – 8 contain fractions after heat shock. The subcellular fractions from the culture grown at 37°C were compared to those placed under heat stress.

The protein levels of the non-heat shocked cytoplasm and membrane appeared to be lower than that of the heat shocked whole cells, periplasm and cytoplasm protein levels (Figure 3.5, panel B, lanes 3 – 7). The heat shocked membrane fraction has a higher GroEL protein level than the other fractions. Importantly, the heat shocked membrane fraction has a higher GroEL protein concentration than the membrane from the culture grown at 37°C. This fascinating result implicates that GroEL is recruited to the membrane upon heat shock and by extension that GroEL is recruited to the RNA degradosome during times of stress. It is very possible that GroEL replaces DnaK functionally in the RNA degradosome in the *dnaK* minus strain. However, these are only preliminary results, and further experiments need to be performed to prove that GroEL replaces DnaK. For instance, the GroEL production levels in this study need to be compared to endogenous GroEL production levels to determine whether its absence in the non-heat shocked whole cells is characteristic. None the less, this find could lead to further knowledge of the functioning of molecular chaperones in the RNA degradosome.

3.3.4 <u>The effect of heat shock as DnaK associated with the RNA degradosome</u>

The aim of the experiment was to determine the protein levels of DnaK in the assembly of the degradosome under conditions of stress. Elevated temperatures elicit the heat shock response upon which the heat shock proteins are over produced (Bukau, 1993). Detecting DnaK in an *in vivo* heat shocked RNase E culture would allow the investigation of a change in DnaK protein levels from a non-heat shocked culture to a heat shocked culture. A control of untransformed *E. coli* BL21 (DE3) cells at 30°C and heat shocked at 42°C was performed to monitor the endogenous protein levels of DnaK upon inducing heat stress. SDS-PAGE analysis showed no evident change in protein levels before or after 30 minutes of heat shock. Anti-His identified the 70 kDa protein band of DnaK and showed increased DnaK protein levels after 30 minutes of heat shock when compared to a non-heat shocked sample (Figure 3.6, panel B, lanes 2 and 1, respectively). These changed protein levels of DnaK showed that under heat stress endogenous levels of DnaK increase due to the heat shock response. *E. coli* BL21 (DE3) cells transformed with His-tagged RNase E were grown 40 minutes after IPTG induction before heat stress was induced for half an hour (Figure 3.6, panels A and B, lane 3). Western blot analysis, by means of anti-His antibody identifying the 70 kDa protein band of DnaK, indicated that the protein levels of DnaK distinctly increased after heat shock; the DnaK concentration was highest 30 minutes after heat shock (Figure 3.6, panel B, lanes 4 – 6). The positive control of purified DnaK with molecular mass of 70 kDa was identified by means of an anti-His antibody using western blot analysis (Figure 3.6, panel B, lane 7). This exciting result indicates that DnaK associated with the RNA degradosome does indeed increase after heat shock and is most likely facilitating the heat shock response.

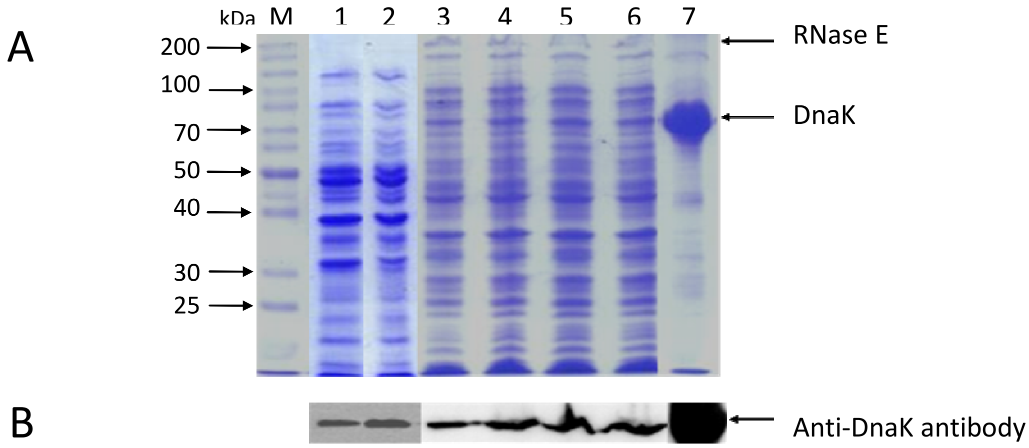

Figure 3.6 The effect of heat shock as DnaK associated with the RNA degradosome
10% reducing SDS-PAGE (A) of RNase E under heat stress and western blot analysis (B) of DnaK associated to RNase E. (A) Lane M: molecular weight marker; lane 1: non-heat shocked untransformed *E. coli* BL21 cells; lane 2: heat shocked untransformed *E. coli* BL21 cells; lane 3: RNase E cells 40 minutes after IPTG induction at 30°C; lane 4: RNase E cells 10 minutes after heat shock at 42°C; lane 5: RNase E cells 20 minutes after heat shock; lane 6: RNase E cells 30 minutes after heat shock; lane 6: cells transformed with DnaK. (B) DnaK of 70 kDa was identified by means of anti-His antibody using western blot analysis.

To further investigate the protein levels of DnaK upon heat stress, the culture was grown at a lower temperature prior to heat shock. Would a larger difference between the heat shock and cell growth temperatures induce a higher level of protein over-production? An *in vivo* heat shock experiment was performed in which *E. coli* BL21 (DE3) cells transformed with RNase E was cultured at 25°C and heat shocked at 42°C to investigate the effect of a larger temperature difference on the DnaK protein levels (Figure 3.7). The purpose of this particular study was to determine if a greater difference between DnaK concentrations could be obtained at a greater temperature difference. The culturing temperature of 25°C resulted in a lower DnaK concentration when compared with that of 30 °C (Figure 3.7, panel B, lane 1; Figure 3.6, panel B, lane 3, respectively). When comparing the DnaK concentrations after heat shock, the 30°C culture appeared to produce a higher DnaK concentration than DnaK cultured at 25°C (Figure 3.6, panel B, lanes3 – 6; Figure 3.7, panel B, lanes 1 – 4, respectively). A control that could be considered would be to compare the purified degradosome that was heat shocked to the purified degradosome cultured at 30°C and to use western blot analysis using anti-DnaK.

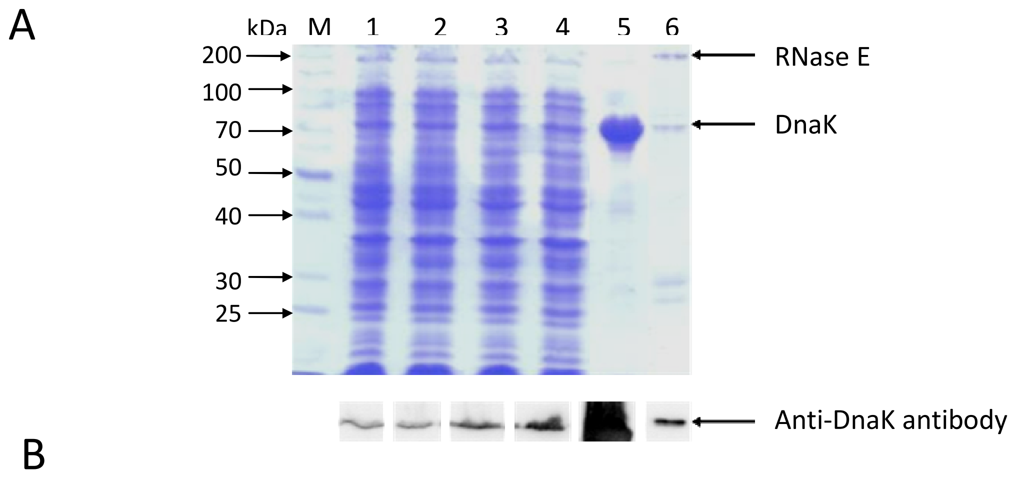

Figure 3.7 Heat shock of RNase E in *E. coli* BL21cells and western blot analysis of DnaK production
10% reducing SDS-PAGE (A) of RNase E induced at 25° C and placed under heat stress (42°C) and western blot analysis (B) of DnaK associated to RNase E. Panel A and B lane 1: uninduced *E. coli* BL21 transformed with RNase E; lane 2: cells 40 minutes after IPTG induction; lane 3:10 minutes after heat shock; lane 4: 20 minutes after heat shock; lane 5: positive control of purified DnaK; lane 6: RNase E purified in *E. coli* BL21 cells. Western blot analysis confirmed the presence of DnaK using anti-DnaK antibody. Western blot analysis confirmed the presence of DnaK.

3.4 Conclusion

It was determined that His-tagged RNase E protein could successfully be produced in the *E. coli dnaK* minus strain albeit with low yields of RNase E. However, the purification of RNase E from the mutant strain was successful only once thus not allowing investigation of whether or not GroEL functionally replaces DnaK in the degradosome. RNase E protein was difficult to grow and purify from the *dnaK* minus strain. Cell colonies inoculated into broth did not grow to mid-log phase on every occasion, and when growth was successful the time span ranged from a day to two days to reach mid-log phase. Further research would need to be conducted to optimise production and purification of RNase E from the *dnaK* mutant strain. The availability of ribosomal protein L4, which inhibits the ability of RNase E to auto regulate its mRNA levels, could allow over-production of RNase E and perhaps allow purification of RNase E from the *E. coli dnaK* minus strain to be reproducible.

L4 binds to the scaffold domain of RNase E and inhibits RNase E cleavage of selected mRNA’s, including those encoding stress-related proteins. This RNase E inhibition results in an accumulation of the stress-related proteins (Singh *et al.*, 2009). L4 binding RNase E appears to be critical for the bacteria to adapt to stresses such as elevated temperature. This study set out to determine the protein levels of molecular chaperones in the degradosome upon conditions of heat stress.

Very interestingly DnaK showed an increase in protein production during the assembly of the RNA degradosome when heat shock was induced. There is a distinct increase in DnaK protein levels after heat stress. RNase E cleaves its cognate mRNA through auto-regulation (Mudd and Higgens, 1993). Literature suggests that RNase E activity is not fully repressed during the heat shock response, therefore DnaK is regulated and cannot be over produced. Ribosomal protein L4 in *E. coli* is essential for regulation of transcription and translation of its own operon, S10 (Lindahl and Zengel, 1979). Singh *et al.*, (2009) showed that *E. coli* L4 regulates RNase E activity through its interaction with RNase E, by changing the steady-state level and degradation of regulatory and mRNA's. L4 is suggested to bind the C-terminus of RNase E, thereby inhibiting RNase E activity, causing not only half-lives of RNAI to be extended but also resulting in full-length endogenous RNase E being more abundant. However, 5S rRNA, tRNA, 6S RNA and M1 RNA processing by RNase E appeared to remain unaffected by the presence of L4. Singh *et al.* (2009) also found that L4 can stabilize mRNA's encoding stress-induced proteins; inhibition of RNase by L4 occurs in association with an increased level of stress-responsive transcripts. L4 was found to have a similar action on RNase E as that of RraA and RraB, which are also inhibitors of RNase E (Gao *et al.*, 2006). However, L4 does not change the degradosome composition as RraA was found to do upon RNase inhibition. Basal levels of endogenous L4 are present in *E. coli* and by implication full inhibition of RNase E activity upon cell stress is not carried out. Therefore the reason why DnaK is not over-produced after heat shock is explained.

An interesting find by Regonesi *et al.* (2006) determined that DnaK abundance in the degradosome varies under different conditions. By studying the degradosome composition in different preparations it was discovered that DnaK was more abundant in IPTG-induced degradosome over-expressing FLAG-tagged RNase E when compared to low basal levels of RNase E. Regonesi *et al.* (2006) also determined DnaK to be more abundant in a non-complemented PNPase null mutant. This is due to the fact that PNPase is a cold shock protein (Jones and Inouye, 1994). The *E. coli* cold shock response to a lowered temperature results in the induction of cold shock proteins, transcription and translational protein synthesis and, importantly, the suppression of heat shock proteins (Jones and Inouye, 1994). Regonesi *et al.*

(2006) further established DnaK to associate to immature degradosome intermediates during times of stress. DnaK was shown to prefer intermediates in which either RhlB or PNPase was absent, or present in reduced amounts.

Upon optimizing the over-production of RNase E by the addition of RNase E activity inhibitors such as ribosomal protein L4 or protein inhibitors RraA and RraB; and by extension the purification of the degradosome, over-production of DnaK may be achieved, thereby allowing further investigation of the role of DnaK and GroEL in the *E. coli* RNA degradosome. If over-production of DnaK is induced in the degradosome in response to the heat shock response, is DnaK present in the RNA degradosome under normal conditions? Is DnaK always present in the RNA degradosome? How does DnaK interact with the RNA degradosome? Its hasn't been determined where DnaK binds to RNase E. Vanzo *et al,* (1998) implicated DnaK binding to the central region of RNase, or suggested binding to RhlB helicase or enolase. Does DnaK associate to the degradosome though interaction with enolase? The next study will be focused on determining if there is a relationship or an interaction between DnaK and enolase.

Chapter 4

The role of enolase in the *E. coli* RNA degradosome

4.1 Introduction

The first discovery of enolase in 1934 occurred whilst the conversion of 3-phosphoglycerate to pyruvate was studied in muscle cells (Lohman and Meyerhof, 1934). Since then enolase has been found to be plentiful in the biological world where it plays key roles in the processes of fermentation and glycolysis. The metalloenzyme α-enolase, also known as 2-phospho-D-glycerate hydrolyase, catalyzes the dehydration of 2-phospho-D-glycerate (2-PGA) to phosphoenolpyruvate (PEP) in the catabolic pathway of the glycolytic cycle (Wold, 1971). Gluconeogenesis occurs through the reverse anabolic pathway in which PEP is hydrated to 2-PGA by α-enolase. Enolase is identified as a metalloenzyme because of its need for metal ions for its activity. However, divalent magnesium ions bind enolase weakly and so Valle (1955) claimed enolase should be identified as a metal-ion-activated enzyme complex instead. Magnesium ions bind at two binding sites that influence enolase activity (Faller *et al.*, 1977). The conformational site allows binding of a substrate by incurring changes in the conformation of the active site of enolase. The second is called the catalytic site and is a requirement of the catalytic machinery. The second magnesium ion binds to it once the first ion is firmly bound to the first site. Conformational site binding is always stronger than catalytic site binding (Brewer, 1985).

A continuous spectrophotometric rate determination assay can be used to determine activity of enolase. Coenzyme NADH will absorb UV light it its reduced form, but not in its oxidized form. The assay containing an oxidoreductase thus uses NADH as substrate in order to monitor the decrease in UV absorbance as the coenzyme is consumed (Bergmeyer, 1974). The transformation of $NADH{\bullet}H^+$ to NAD^+ was used to determine the enolase activity through three reactions (Figure 4.1). Enolase catalyzes 2-PGA to PEP. A base in the active site of enolase removes a proton from C2 of 2-PGA creating a carbanion enolate intermediate. Enolase catalyzes the removal of a hydroxyl group from C3 of the intermediate producing PEP. The conversion of PEP to pyruvate, with the release of adenosine triphosphate (ATP), is catalysed by pyruvate kinase. Oxidoreductase lactate dehydrogenase finally converts pyruvate to lactate, and oxidises NADH.

$$\text{2-Phosphoglycerate} + H_2O \xrightarrow{\textbf{Enolase}} \text{Phosphoenolpyruvate}$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\textbf{Pyruvate kinase}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH}\bullet\text{H}^+ \xrightarrow{\textbf{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

Figure 4.1 Sequential catalysis of 2-phosphoglycerate to lactate
Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP). PEP is converted to pyruvate with the addition of ADP and pyruvate kinase and ATP is released. Pyruvate and NADH•H$^+$ are catalyzed by lactate dehydrogenase to form lactate and NAD$^+$.

Multifunctional enolase has been described to have "moonlighting" functions, referring to the non-glycolytic roles that enolase assumes. Enolase production changes due to pathophysiological, developemental or metabolic states of the cell and so is expressed by a non-housekeeping gene (McAlister and Holland, 1982). Major components of eye lens are crystallins, which have been shown to contribute 20 – 60 % of the wet weight of eye lens. The amino acid sequence of τ-crystallin, a principle lens protein in reptiles, fish, lamprey and birds, showed a high level of similarity with human and yeast enolase. Alpha-enolase was shown as a τ-crystallin protein with a structural role in lens and cataracts (Wistow *et al.*, 1988). τ-crystallin has low enolase activity which could be due to its monomeric form. The second Mg^{2+}, required for dimerization (which is essential for enzyme activity), is absent (Wistow *et al.*, 1988). Alpha-enolase has been shown as a Myc-binding protein (MBP1), relating its function to regulation of cellular growth and differentiation (Subramanian and Miller, 2000). What is interesting is that, even though strongly similar in structure, MBP1 does not have glycolytic activity due to the fact that it does not have the first 96 amino acids of α-enolase. The N-terminus of α-enolase is thus vital for glycolytic activity, and not essential for c-myc binding (Subramanian and Miller, 2000).

In *Saccharomyces cerevisiae* alpha-enolase has been identified as a heat shock protein, HSP48, which is produced by the *eno1* gene (Iida and Yahara, 1985). The *eno1* gene product HSP48 is not only involved with thermal tolerance but also in control of cell growth. *HSR1* *(heat shock resistant)* negatively regulates *eno1* gene expression; the HSR1 mutant provides extreme heat shock resistance to the organism (Iida and Yahara, 1984). HAPs (hypoxia associated proteins) are a set of five cell-associated stress proteins that are up-regulated during a stress such as chronic hypoxia (Sunstrom and Aliaga, 1992). Enolase has also been identified as an endothelial hypoxic stress protein, functioning to increase anaerobic metabolism thereby offering cellular protection (Aaronson *et al.*, 1995).

Enolase, commonly known as a glycolytic enzyme, is one of the major components of the *E. coli* RNA degradosome, a multi-enzyme complex involved with the degradation of mRNA. The primary constituents PNPase, RhlB helicase and enolase associate to the C-terminus of the scaffold protein RNase E to form the "decay machine" (Carpousis *et al.*, 1994). All of the major components were shown to function together in the general decay of RNA except for enolase. Enolase, as part of the degradosome, had not had a role annotated to it until Morita *et al.* (2004) showed its presence is critical for the regulation of the degradation of *ptsG* mRNA under conditions of phosphosugar stress.

The uptake of glucose involves the transfer of phosphor groups through the phosphotransferase system (PTS). The *ptsG* mRNA encodes the major glucose transporter ($IICB^{Glc}$) which is a membrane-bound enzyme part of the PTS. The three components of PTS involved in the transport of glucose are E1, HPr and $EIIA^{Glc}$. Phosphoryl transfer: phosphoenolpyruvate – E1 – HPr – $EIIA^{Glc}$ – $IICB^{Glc}$ – glucose (Nam *et al.*, 2001). Upon blocked glycolysis ($IICB^{Glc}$) is destabilized; RNase E mediates the immediate degradation of *ptsG* mRNA (Kimata *et al.*, 2001). Increased glucose 6-phosphate (G6P) or fructose 6-phosphate (F6P) results in RNase E degrading *ptsG* mRNA (Morita *et al.*, 2003). The rapid degradation of *ptsG* mRNA was not eliminated through the assembly of the degradosome, as thought previously, but interestingly rather through reduced enolase levels (Morita *et al.*, 2004). Enolase is speculated to co-ordinate the action of RNase E *ptsG* mRNA in response to phosphosugar stress (Morita *et al.*, 2004).

The 47-kDa protein enolase is expressed abundantly in the cytoplasm where it functions to catalyze the processes of fermentation and glycolysis, but has also been shown to be present on the cell surface (Miles *et al.*, 1991). This glycolytic enzyme has been shown to be expressed on the surface of hematopoetic cells, epithelial cells and neuronal cells as a strong plasminogen-binding receptor (Dudani *et al.*, 1993; Nakajima *et al.*, 1994; Redlitz *et al.*, 1995). Enolase has not only been reported as a surface protein on various eukaryotic cells, but on bacterial cells as well. It was identified as a surface protein strongly binding plasminogen in group *A. streptococci* (Pancholi and Fischetti, 1998). Hydrophobic domains and post translational modifications such as acylation and phosphorylation are thought to assist cytoplasmic enolase in membrane association and surface production (Pancholi, 2001). Interestingly Myc-binding protein (MBP-1), structurally very alike to α-enolase, has a nuclear localization (Subramaniam and Miller, 2000). Multifunctional enolase has thus shown to have multiple locations by various researchers.

To determine the localization of enolase upon heat shock a subcellular fractionation study will be used. The protocol of preparing membrane vesicles from Gram-negative bacteria was adapted from Pool, (1993). This method allows the isolation of a high yield of the subcellular membrane, with its structural and functional integrity. The procedure relies on the disruption of the cell so that the subcellular fraction of interest contains no contaminating structures. This is achieved through sonication, breaking open the cells releasing the spheroplasts. Osmotic lysis allows the production of membrane vesicles from the spheroplasts. Swelling and bursting of the spheroplasts is caused by exposure to a hypo-osmotic solution. Detergent lysis buffer and ultra-centrifugation subsequently allow the release of the cytoplasm and the membrane fractions.

Heat shock proteins DnaK and GroEL associate to the degradosome in substoichiometric amounts. They are known to facilitate protein folding and may have a role in the assembly of the degradosome. Vanzo *et al.* (1998) indicated that DnaK binds to the central region of RNase E, but it has not been determined whether DnaK binds directly to RNase E or whether its presence in the degradosome is through enolase or RhlB helicase. The interaction of DnaK and enolase has not been investigated prior to this study. The binding site of heat shock protein DnaK to RNase E has not been determined. Could DnaK and enolase be interacting? Do they have similar roles in the degradosome? These are questions that are still unanswered. This study was used to determine if DnaK influences the activity of enolase through the use of enolase activity assays. As of yet the role of enolase in the *E. coli* RNA degradosome has not been elucidated. Does enolase function as a heat shock protein in the degradosome, does it direct compartmentalization, and does it interact with any of the other degradosome constituents?

The aim of this study was to investigate the role of enolase in the *E. coli* RNA degradosome. The first objective would be to use bioinformatics tools to perform BLAST searches and sequence alignments to investigate the degree of similarity between homologues. The second objective was to analyze the sequence similarity between *E. coli* enolase and *Homo sapiens* enolase. The study was then focused on the localization of FLAG-tagged enolase produced in *E. coli* under normal conditions as well as under heat stress by an *in vivo* subcellular fractionation. The fourth objective of the study was to use an *in vitro* enolase assay to monitor the enolase activity in the presence and absence of purified *E. coli* DnaK.

4.2 <u>Materials and methods</u>

4.2.1 <u>Materials</u>

Reagents used were purchased from Sigma Chemicals Co. (St. Louis, Mo U.S.A.), Merck Chemicals (Darmstadt, Germany), BioRad (U.S.A.) or Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Hybond^{TM} Cextra chemiluminescence nitrocellulose was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A). Commercial enolase from rabbit muscle was purchased from Sigma. Monoclonal anti-FLAG antibody was purchased from Sigma. Plasmid pFLAG-Eno was a generous donation from Prof. S. Lin-Chao *(Institute of Molecular Biology, Academia Sinica, Taiwan)* (Table 4.1). Plasmid pEKJ30 was constructed by Dr A. Boshoff.

Table 4.1 Description of *E. coli* strains and plasmids

Plasmid	**Description**	**Reference**
pFLAG-ENO	pGP1–2 encoding *E. coli* enolase, Amp^R	Liou *et al.* (2002)
pEKJ30	pQE30 encoding *E. coli dnaK*, Amp^R	Boshoff *et al.* (2008)
Strains	**Description**	**Reference**
E. coli BL21 (DE3)	F- ompT gal [dcm] [lon] hsdSB λDEs	Studier *et al* (1990)
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)].	Bullock *et al.*(1987)

4.2.2 Methods

4.2.2.1 Bioinformatic analysis of *E. coli* and *Homo sapiens* enolase

A comparative investigation of enolase from two different organisms was conducted using sequence analysis tools. The comparison was made using amino acid sequences of the protein from each organism. The *E. coli* and *H. sapiens* sequences were downloaded from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The sequences were aligned using the CLUSTALW multiple sequence alignment tool from GenomeNet (http://align.genome.jp/). The alignment was shaded using the Mobyl@pasteur Boxshade v.3.0 (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=boxshade).

4.2.2.2 Transformation of competent cells

Competent *E. coli* BL (DE3) cells were transformed with FLAG-tagged enolase (pFlag-ENO) and competent *E. coli* XL1 Blue cells were transformed with His-tagged DnaK (pEKJ30) as described in Section 2.2.2.5.

4.2.2.3 Determination of optimal protein production

An overnight culture was prepared by growing *E. coli* BL21 (DE3) cells transformed with plasmid pFlag-ENO in 25 ml 2 x YT broth with 100 µg/ml ampicillin at 37ºC. The overnight culture was inoculated into 225 ml 2 x YT with 100 µg/ml ampicillin and grown to an optical density of A_{600nm} = 0.4. Protein production was then induced by the addition of IPTG to a final concentration of 1 mM. Protein samples were taken every hour for 5 hours after induction. The samples were centrifuged for 1 minute at 12 000 g and the pellet resuspended in a volume of PBS as determined by the absorbance reading where 150 µl of PBS was added for each 0.5 OD_{600nm} absorbance unit. The samples were analyzed on a 10 % SDS-PAGE gel. The protein production study of PNPase, enolase and RhlB helicase differed in that protein samples were taken over hourly periods for duration of 5 hours. Western blot analysis identified 79 kDa, 47 kDa and 50 kDa protein bands, respectively, using anti-FLAG antibodies. M2 monoclonal anti-FLAG antibody allowed for the detection of the FLAG-tagged proteins with ECL anti-mouse IgG HRP-linked species-specific whole antibody.

4.2.2.4 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed as described in Section 2.2.2.7.

4.2.2.5 Western blot analysis of proteins

Western blot analysis was performed as described in Section 2.2.2.8. Monoclonal anti-FLAG antibody was used to detect FLAG-tagged enolase with ECL anti-mouse IgG HRP-linked species specific whole antibody. Monoclonal mouse anti-His antibodies were used to detect His-tagged DnaK with ECL anti-mouse IgG HRP-linked species-specific whole antibody.

4.2.2.6 Subcellular fractionation of enolase under heat shock

An overnight culture was prepared by growing *E. coli* BL21 (DE3) cells transformed with pflag-ENO in 25 ml 2 x YT broth with 100 µg/ml ampicillin at 37ºC. The overnight culture was inoculated into 275 ml 2 x YT broth with 100 µg/ml ampicillin and grown to an optical density of A_{600nm} = 0.4. Protein production was induced by the addition of IPTG to a final concentration of 1 mM. The culture was divided and one half was heat shocked for 10 minutes at 42ºC, the other kept at 37ºC. The two cultures were harvested at 4 000 g for 20 minutes at 4ºC. The pellets were separately resuspended in 7.5 ml periplasting buffer (20 % sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 500 µg/ml lysozyme) and incubated on ice for 5 minutes. A volume of 7.5 ml cold water was added to each sample, mixed with slow pipetting and again incubated on ice for 5 minutes. A pellet was produced by centrifuging for 20 minutes at 4 000 g at 4ºC and resuspended in 15 ml detergent lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1 % deoxycholate) and left to incubate at room temperature. The solutions were sonicated at 60 Hz (20 x 10 seconds) and centrifuged twice for 20 minutes at 4 000 g at 4ºC to produce a supernatant. The supernatants were ultra-centrifuged at 44 800 g for 1 hour at 4ºC. The pellet was resuspended in 1 ml detergent lysis buffer with no added deoxycholate. The total protein in the different cell components were analyzed by SDS-PAGE (Section 2.2.2.7) and FLAG-tagged enolase of 47 kDa was identified by means of anti-FLAG antibody using western blot analysis (Section 2.2.2.8).

4.2.2.7 Determination of protein concentration using the Bradford assay

The Bradford assay was performed in a microtitre plate at an absorbance of 595 nm using a PowerWave microtitre plate reader (Biotek) (Bradford, 1976). Bradford reagent to a volume of 1 ml was added to 20 µl *E. coli* DnaK protein sample and incubated at room temperature for 30 minutes before taking the absorbance reading. A standard curve was prepared using a concentration linear range of 0.125 to 1.0 mg/ml bovine serum albumin (Figure 4.2) (BSA). Bradford reagent (1 ml) was added to the BSA standards 20 µl, incubated at room temperature for 30 minutes and the absorbance reading taken. The experiment was conducted in triplicate. The DnaK protein concentration was determined by comparing the unknown to the prepared standard curve.

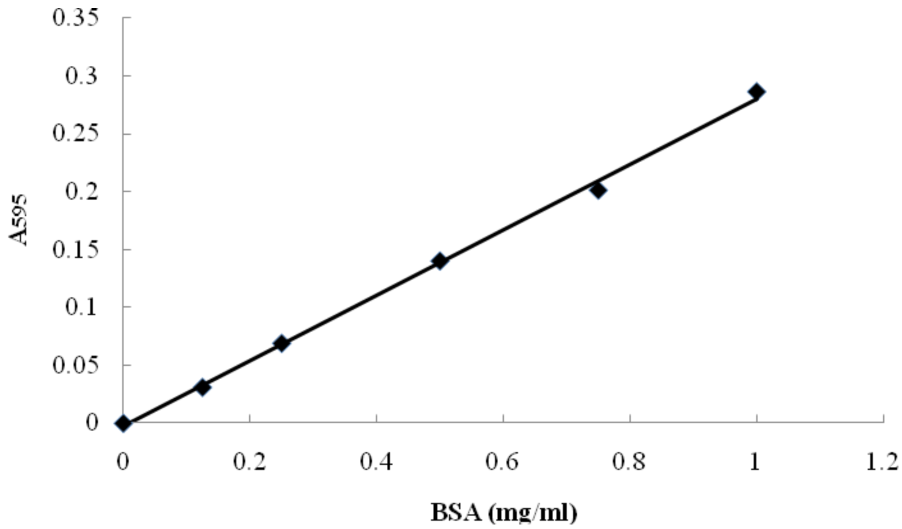

Figure 4.2 Standard curve for protein determination using the Bradford assay
Bradford assay using BSA to produce a standard curve where y = 0.2827x – 0.0025 and R^2 = 0.998.

4.2.2.8 Enolase activity assays

The assay mix (3 ml) was prepared prior to each assay: 2.29 ml of 100 mM HEPES, pH 7.4; 0.1 ml of 56 mM 2-phosphoglycerate (DPG); 50 µl of 7 mM NADH; 0.15 ml of 500 mM $MgSO_4$; 0.2 ml of 2 M KCl; 0.2 ml of 20 mM ADP; 2.75 µl of 2000 units/ml polyphosphate kinase (PK); 1.5 µl of 5500 units/ml lactate dehydrogenase (LDH); and varied DnaK volumes: 1 µl, 5.3 µl, 10 µl, 20 µl and 50 µl of 1.4 mg/ml DnaK. With each different assay, the HEPES buffer volume was adjusted to a total of 3 ml for the reaction. To start the reaction, 15 µl of 27 units/mg enolase, was added. The reaction was mixed by inversion and incubated at 25ºC for 5 minutes whilst the A_{340nm} was monitored. The reaction was followed on a ThermoSpectronic spectrophotometer (Merck). A reaction was set up in the same manner with 1.5 µl of 15 mM Tris-HCl, pH 7.4, 0.02% BSA replacing enolase to serve as a negative control for the activity of enolase. A positive control experiment was set up in the same manner except that DnaK was not added to the reaction mix. Five experiments were carried out before using newly thawed purified protein. For every experiment each assay was carried out in duplicate.

The enolase activity for each assay was calculated as µmol/min/ml according to the following relationship:

$$(\Delta A.Vt)/(\varepsilon.Ve.t)$$

where ΔA is the total change in absorbance at 340 nm; Vt is the total volume of the reaction mixture; Ve is the volume of enzyme extract; t = time (5 min); ε = extinction coefficient = 6.22 ml^{-1}. min^{-1}.

4.3 Results and discussion

4.3.1 Multiple sequence alignment of eukaryotic and prokaryotic enolase

A multiple sequence alignment of the amino acid residues of bacterial and human enolase was performed to determine conserved regions amongst the different sequences. BLAST analysis allowed the identification of identical and similar sequences. The alignment between *E. coli* enolase and human alpha enolase showed a 52 % identity and that of *E. coli* enolase and human beta-enolase a 55 % identity. Human alpha-enolase shares an 83 % identity with human beta-enolase. Human alpha- and beta-enolase are considered close homologues of *E. coli* enolase due to a percentage identity higher than 50 % (Russel *et al.*, 1997). The level of conservation across the N-terminus and the enolase principle domain, the TIM-barrel, appears to remain consistent (Figure 4.3). The longest helix, H5, is conserved across *E. coli* and human enolase. The mainly hydrophobic helix, H11, is very well conserved, with a sequence identity of 100 % across human and *E. coli* enolase. The alignment between *E. coli* and Human enolase was made due to the fact that the eukaryotic exosome (a complex very similar to the RNA degradosome) is present in Human. Enolase is also highly conserved and the high percentage identity between *E. coli* and Human enolase was an interesting find.

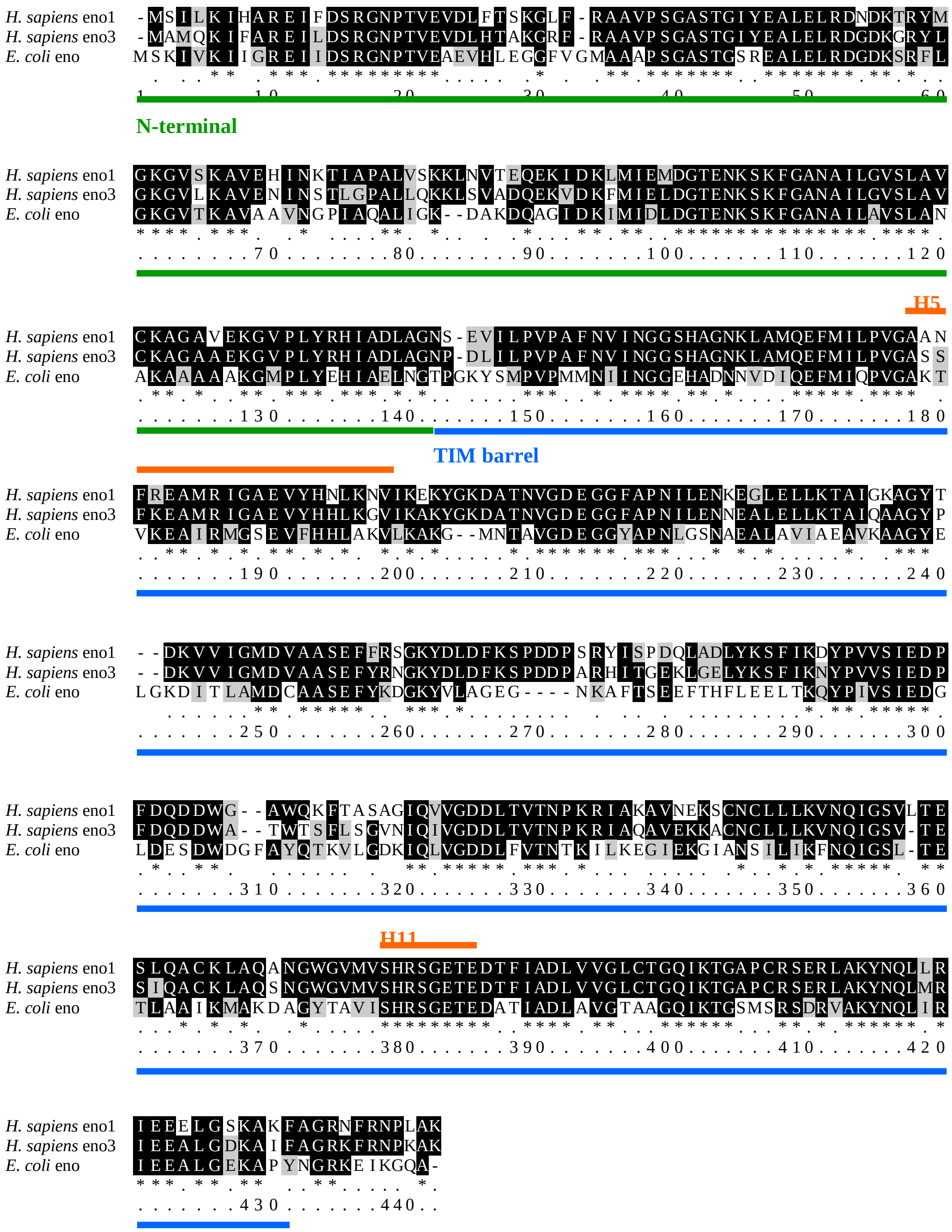

Figure 4.3 Multiple sequence alignment of *E. coli* enolase and *Homo sapiens* alpha-enolase and beta-enolase

E.COLI: *E. coli* enolase protein (GenBank EU896509), H.SAPIENS 1: *H. sapiens* alpha-enolase protein (GenBank NP001419) and H.SAPIENS 3: *H. sapiens* beta-enolase protein (GenBank NP001967). Green: *E. coli* N-terminus; blue: *E .coli* TIM barrel; orange: *E. coli* helix 5(H5) and helix 11 (H11).

4.3.2 Protein production study of FLAG-tagged enolase

The protein production study of FLAG-tagged enolase, of size 47 kDa, is shown in Figure 4.4. Enolase was successfully produced in *E. coli* BL21 cells and appeared to produce protein at increasing levels over the duration of 5 hours after induction with IPTG (Figure 4.4, panel A, lanes 2 to 7).

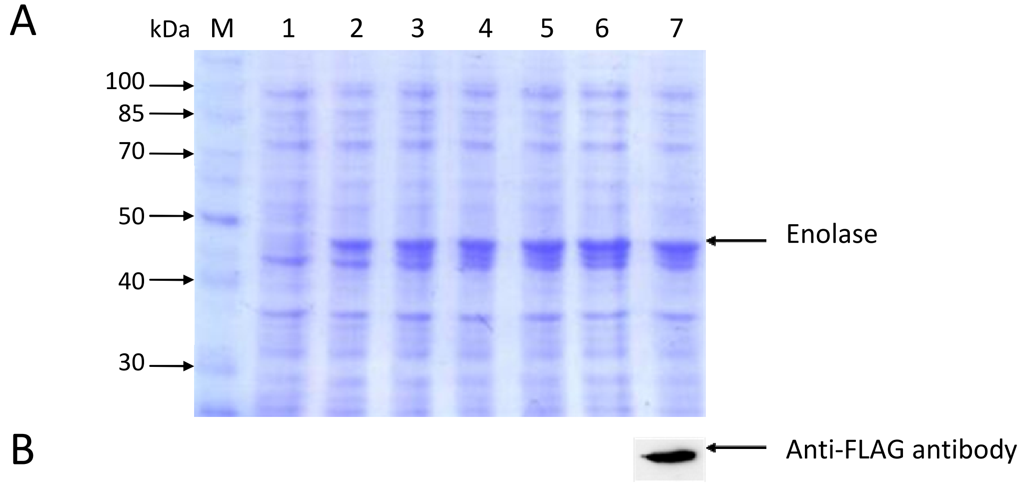

Figure 4.4 Protein production study and western blot analysis of FLAG-tagged enolase. Study of optimal protein production of *E. coli* BL21 (DE3) [pflag-ENO] through 10% reducing SDS-PAGE analysis (A) and western blot analysis (B). (A) lane 1: negative control of untransformed *E. coli* BL21 cells; lane 2: uninduced cells transformed with FLAG-tagged enolase; lane 3: transformed cells 1 hour after IPTG induction; lane 4: 2 hours after induction; lane 5: 3 hours after induction; lane 6: 4 hours after induction; lane 7: 5 hours after induction. The enolase protein band has a mobility of 47 kDa. (B) Anti-FLAG antibody was used to identify the 47 kDa protein band of enolase using western blot analysis.

No enolase protein is visible in the untransformed *E. coli* BL21 cells. Western blot analysis was used to identify the molecular mass of 47 kDa of enolase using an anti-FLAG antibody 5 hours after IPTG induction (Figure 4.4, panel B, lane 7).

4.3.3 Subcellular fractionation of enolase under heat shock

The aim of this study was to investigate if enolase was recruited to the membrane under conditions of stress. This finding would determine if enolase recruits to the *E. coli* RNA degradosome that is tethered to the membrane through its scaffold protein RNase E upon heat stress. It was thus necessary to determine the localization of enolase in the presence and absence of heat stress using a subcellular fractionation. This experiment would allow the divisions of the cell into its organelles, and through western blot analysis identify where the 47 kDa protein band of enolase is most abundant. *E. coli* cells were divided into periplasmic, cytoplasmic and membrane fractions through the use of lysozyme digestion, osmotic shock and ultracentrifugation. Each lane of the SDS-PAGE shows the level of enolase protein production present in whole cells, periplasmic, cytoplasmic and membrane fractions from a heat shocked culture and from a culture grown at 37° (Figure 4.5, panel A). Western blot analysis confirmed the presence of enolase and showed the differences in relative concentrations of enolase protein in the different fractions (Figure 4.5, panel B). At 37ºC, enolase was most abundant in the cytoplasmic fraction, and least abundant in the membrane fraction (Figure 4.5, panel B, lanes 7 and 8). Upon the environmental stress of heat shock both the cytoplasmic and membrane fractions showed an increase of protein content (Figure 4.5, panel B, lanes 3 and 4). The periplasm showed no distinct difference between the heat shocked and non-heat shocked fractions (Figure 4.5, panel B, lanes 2 and 6).

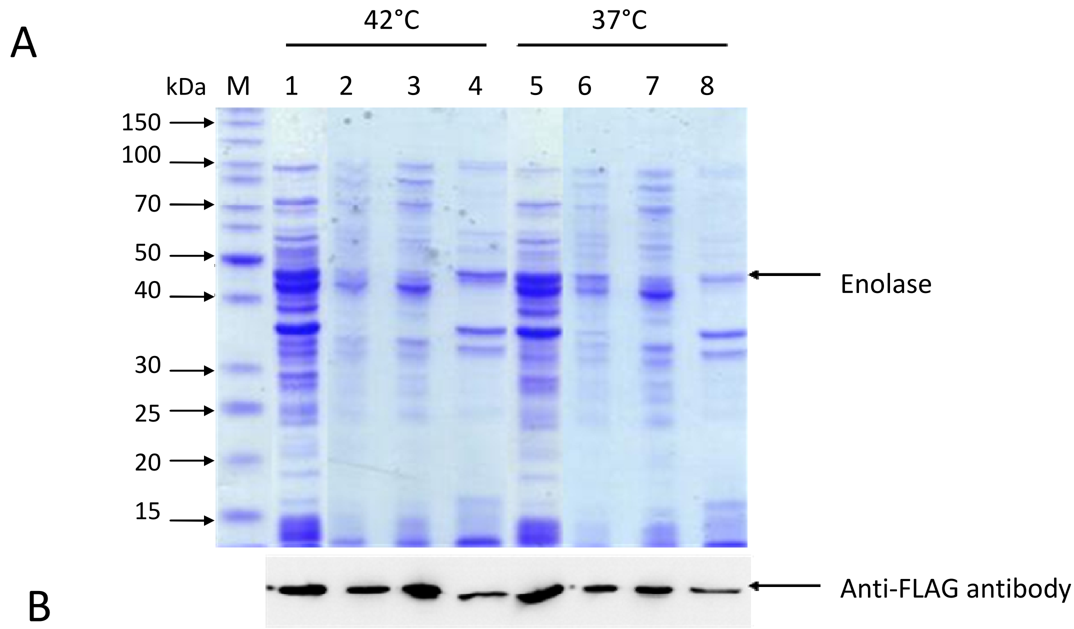

Figure 4.5 Subcellular fractionation of enolase under heat stress
10% reducing SDS-PAGE (A) and western blot analysis (B) of the subcellular localization of enolase. (B) Lane 1: whole cells containing enolase; lane 2: periplasmic fraction containing enolase; lane 3: cytoplasmic fraction containing enolase; lane 4: membrane fraction with enolase present. Lanes 1 – 4 contain the fractions after heat shock. Lane 5: whole cells; lane 6: periplasmic fraction; lane 7: cytoplasmic fraction; lane 8: membrane fraction. Lanes 5 – 8 contain the fractions at 37°C. The subcellular fractions from the culture placed under heat stress were compared to that from a culture grown at 37ºC.

This result, interestingly, showed increased levels of enolase in the cytoplasm as well as in the membrane after heat shock. Enolase may very well be membrane-associated; increased protein levels in the membrane could be due to enolase recruiting to the degradosome when the cell is placed under heat stress. Enolase was not over produced in the whole cells. If a higher level of enolase protein production could be achieved prior to the fractionation, there may be a larger difference between the membrane fractions before and after heat shock.

Enolase over production may also then show a movement of enolase from the cytoplasm to the membrane upon heat shock, as opposed to the current result where both the membrane and cytoplasmic fractions show increased yields of enolase. Another point to consider would be to heat shock the *E. coli* cell culture over a longer duration. This could also result in a larger difference in the membrane enolase protein levels before and after heat shock, and may reflect a translocation of enolase from the cytoplasm to the membrane. Western blot analysis is used to quantify the differences in proteins by the intensity of fluorescence; therefore each sample must contain the same amount of protein.

4.3.4 Enolase activity assays

The activity assays were optimised to that of an enolase concentration of 0.0175 mg/ml. Enolase was found to have enzyme activity at both the concentrations 0.035 mg/ml and 0.0175 mg/ml. The lower enzyme concentration resulted in the reaction occurring over a longer period of time and in a higher enolase activity (6.9969 μmol/min/ml) than that of 0.035 mg/ml which produced an enolase activity of 5.509 μmol/min/ml (results not shown). The blank, with no added enolase, showed no signs of activity (results not shown). An enolase concentration of 0.0175 mg/ml was thus used for all further activity assays. Bovine serum albumin (BSA) was used as a negative protein control because it does not affect enzymes that do not need it for stabilization, and BSA produced no change in enolase activity.

E. coli DnaK was purified (discussed in Section 3.3.1) for the purpose of adding it to the enolase assays to determine if DnaK influences enolase activity. A wide range of molarities was investigated to determine at what concentration, if any, DnaK would influence enolase. Using a molar ratio of DnaK to enolase of 1:37, an increased enolase activity of 8.3 μmol/min/ml (standard error of 15.8 %) was observed (Figure 4.6). However, an error of 18.5 % amounts to approximately 1 unit of activity. The enolase activity with and without DnaK differed by 1.4 units. Thus, the result obtained at a molar ratio of 1:37 is not statistically relevant. Using a DnaK concentration of 0.002 mg/ml in the enolase activity assay (DnaK to enolase molar ratio of 1:13) resulted in a statistically significant increase in enolase activity of 9.5 μmol/min/ml. This result indicated that DnaK does in fact influence enolase activity.

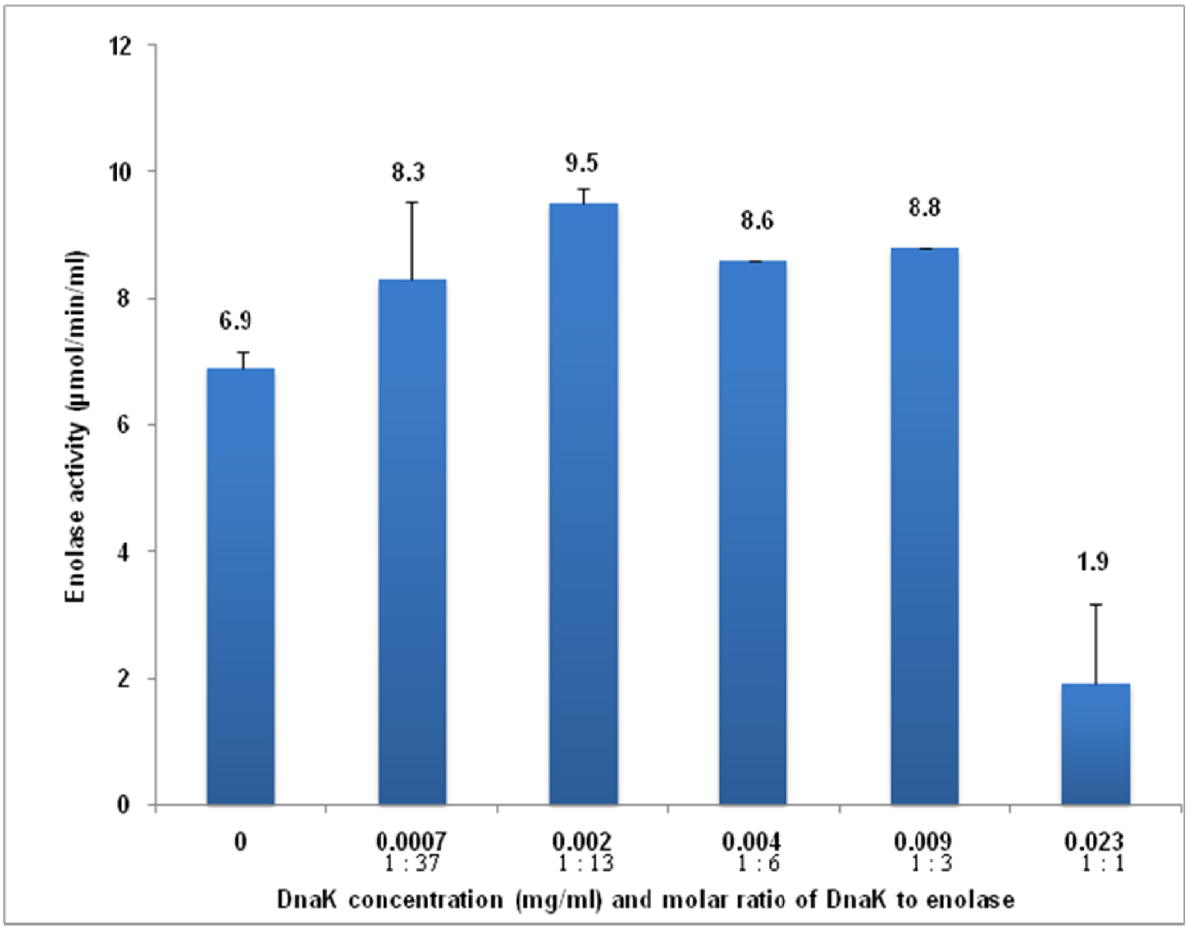

Figure 4.6 Analysis of enolase activity in the presence and absence of DnaK
Enolase activity was measured at a constant enolase concentration (0.0175 mg/ml) and varied DnaK concentrations: 0.0007 mg/ml, 0.002 mg/ml, 0.004 mg/ml, 0.009 mg/ml and 0.023 mg/ml. Each assay was conducted in triplicate. Molar ratios of DnaK to enolase at increasing DnaK concentrations were calculated: 1:37 (0.0007 mg/ml); 1:13 (0.002 mg/ml); 1:6 (0.004 mg/ml); 1:3 (0.009 mg/ml) and 1:1 (0.023 mg/ml).

Upon increasing the DnaK concentration further, at molar ratios of 1:6 and 1:3, enolase activity decreased slightly in comparison to the activity at the molar ratio of 1:37 (DnaK : enolase). However, changing the molar ratio of DnaK to enolase to 1:1 resulted in approximately one third of the activity of enolase in the absence of DnaK (Figure 4.6). Thus, these results show, for the first time, that substoichiometric amounts of DnaK increase enolase activity. Substoichiometric concentrations of DnaK appeared to enhance the folding of enolase resulting in an increase of activity, while equimolar concentrations of DnaK resulted in a decrease of activity. It is thus possible that DnaK is acting as a holdase at equimolar concentrations, and inhibiting the activity of enolase.

Commercial eukaryotic enolase was used in the enolase assays. The multiple sequence alignment of prokaryotic and eukaryotic enolase in Figure 4.3 indicated a 55 % identity between the two organisms. Despite the fact that it would have been preferable to use *E. coli* enolase, the percentage identity indicates that they are close homologues.

4.4 Conclusion

Two of the three structurally similar isoforms of enolase are alpha- and beta-enolase. Ubiquitous alpha-enolase (non-neuronal enolase) is expressed in the early stages of embryonic development, whereas beta-enolase genes are expressed in the adult skeletal and cardiac muscle cells (Terrier *et al.,* 2007). Alpha-enolase is highly conserved, ranging from 40 – 90 % identity between different organisms (Pancholi, 2001). *E. coli* enolase and human alpha-enolase share a 52 % identity. *E. coli* enolase and human beta-enolase share a 55 % identity and human beta-enolase and human alpha-enolase share 83 % homology. The evolutionary relationship between two sequences is inferred based on the high sequence identity between them. Two proteins are considered medium homologues if the percentage identity between them is greater than 25 % and less than 50 % (Russel *et al.,* 1997). Close homologues share a percentage identity greater than 50 % (Russel *et al.,* 1997). *E. coli* enolase can thus be termed a close homologue of both human alpha- and beta-enolase.

In the *in vivo* fractionation study, the *E. coli* cells were fractionated to produce periplasmic, cytoplasmic and membrane fractions, with the aim of determining the localization of enolase upon inducing heat stress. Enolase appeared to localize towards both the membrane and cytoplasm. This finding indicates that enolase is recruited to the membrane, and by implication to the *E. coli* RNA degradosome, under heat stress. It is possible that increasing the period of heat shock may result in greater localization of enolase to the membrane. Enolase has previously been shown to be expressed on the cell surface as a strong plasminogen binding protein in both eukaryotes and prokaryotes (Dudani *et al.,* 1993; Pancholi and Fischetti, 1998). Enolase is very diverse in its functioning across species and has a wide variety of moonlighting functions. Further research could answer the question as to why enolase localizes to the membrane where the scaffold protein is located. Could enolase be functioning as a heat shock protein? The role of enolase can further be elucidated by determining if post-translational modifications such as phosphorylation recruit this protein to the degradosome through membrane translocation.

During the *in vitro* analysis of enolase activity in the presence and absence of DnaK, it was found that enolase activity was either stimulated or inhibited, depending on the DnaK concentration. Substoichiometric amounts of DnaK enhanced the activity of enolase while equimolar DnaK concentrations inhibited enolase activity. Future work will include the use of enolase purified from *E. coli* cells and the addition of DnaJ and GrpE, which are co-chaperones of DnaK. The addition of co-chaperones may further enhance the activity of enolase. The chaperone binding site on RNase E has not been determined. It was, however, speculated that DnaK could associate with the central part of RNase E, or with either enolase or RhlB helicase as part of the degradosome (Vanzo *et al.*, 1998). From this study it is possible that the chaperones associate with RNase E via enolase. Could the binding of DnaK facilitate the binding of enolase to the RNA degradosome? This research poses many fascinating and new exciting areas of study.

Chapter 5
Conclusions and future work

The major components of the *E. coli* RNA degradosome have been studied and characterized (Miczak *et al.,* 1996). The role of RNase E, PNPase and RhlB helicase in mRNA processing and degradation in the RNA degradosome are known (Miczak *et al.,* 1996; Vanzo *et al.,* 1998). However, the specific functions of all the components of the RNA degradosome have not been elucidated. Enolase, a major component, has not been shown to have a specific function in the process of mRNA degradation, even though it is a “moonlighting” protein with a wide variety of functions across different organelles and different organisms. Molecular chaperones, minor components of the RNA degradosome, were not considered significant, until recently, so that no specific function in the RNA degradosome has been found for them.

The RNA degradosome could not be purified using the FLAG-tagged purification. The level of RNase E protein obtained was low and not consistent. The FLAG-tagged purification would have allowed purification of all the major as well as the minor components of the RNA degradosome. The RNA degradosome was thus purified using the His-tagged purification. However, communications with our collaborators lead us to the understanding that the His-purification is not ideal for purification of the RNA degradosome as not all the components are present. RNase E catalytic activity is auto-regulated. Inhibitors of RNase E activity include the proteins RraA and L4, both of which prevent endonucleolytic cleavage of RNase E. RraA inhibits RNase E activity by altering the composition of the RNA degradosome, whereas L4 inhibition of RNase E does not result in a modification of the RNA degradosome (Gao *et al.* 2006). L4 can thus be used to over-produce RNase E and facilitate the purification of the RNA degradosome.

Proteins associating with the RNA degradosome in substoichiometric amounts were previously considered to be insignificant, but recently a different view point has been adopted. Some of the minor constituents of the RNA degradosome were suggested to participate in the response to changing environmental conditions. The question of whether DnaK and GroEL are essential in the assembly of the RNA degradosome under elevated temperatures still remains unanswered. The purified RNA degradosome was heat shocked to investigate levels of DnaK and it was found that DnaK protein production increased in the RNA degradosome after heat stress. The role of DnaK was not fully elucidated. The molecular chaperones GroEL/GroES are the only chaperones required for *E. coli* viability at all temperatures. Very interestingly, GroEL was shown, in preliminary results, to localize in the membrane fraction after heat shock. An *E. coli dnaK* minus strain was specifically obtained to allow the purification of the RNA degradosome and determine whether GroEL could functionally replace DnaK in this multi-protein complex. However, the RNA degradosome purified from the *dnaK* minus strain produced a very low yield of RNase E protein and an incomplete degradosome; GroEL was not present. Further research using the L4 protein would allow the specific function of DnaK as well as GroEL in the RNA degradosome to be determined. Does the chaperone, GroEL, that binds non-native polypeptides, replace DnaK functionally? How do the molecular chaperones integrate to function within the RNA degradosome?

Enolase has been shown to have a wide variety of functions. This glycolytic protein was shown to have a specific function of directing compartmentalization of the RNA degradosome to the plasma membrane for the *ptsG* mRNA to then be degraded (Morita *et al.,* 2004). As of yet (2010) the there is no evidence to suggest that enolase has a role in mRNA degradation. Its specific function in the assembly of the RNA degradosome under conditions of heat stress has not been determined. Investigating enolase provided exciting results. Enolase protein production was found to increase in abundance in the membrane fraction after heat shock. By extension it is suggested that enolase may be directed towards the membrane-tethered scaffold protein RNase E, to which all the components of the RNA degradosome associate. Further research will determine whether enolase localizes in the cellular membrane or whether enolase is specifically recruited towards the RNA degradosome upon heat shock. Does enolase act as a heat shock protein in the RNA degradosome? A study was performed to determine whether DnaK influenced enolase activity. Commercial eukaryotic enolase was used for enolase assays to which purified DnaK was added. Eukaryotic enolase was used because enolase could not be purified using the FLAG-tagged purification. A set of preliminary results was produced showing very interestingly that substoichiometric amounts of DnaK increased the activity of enolase. A molar ratio of DnaK to enolase of 1 : 1, however, showed that enolase activity was inhibited by DnaK. It may be possible that change in enolase activity at varied DnaK concentrations is due to the chaperone functioning of DnaK, acting as a hold/folding protein. Does DnaK refold enolase into a better conformation that enhances enolase activity? Vanzo *et al.* (1998) ruled out the possibility of DnaK binding to PNPase in associating with the RNA degradosome, and suggested DnaK associated with the RNA degradosome by binding to either the central region of RNase E, or to RhlB or enolase. Further research could determine why DnaK binds to enolase, and if the binding of DnaK facilitates the binding of enolase to the degradosome. An interesting study would be to determine what the mechanism of action is when DnaK interacts with enolase and whether DnaJ and GrpE influence DnaK's activity on enolase.

Future work should be started by successfully over-producing RNase E in the presence of protein inhibitor RraA. Over-production of RNase E would allow FLAG-tagged purification of the RNA degradosome. The next important point to consider would be the positive identification of the recombinantly expressed proteins through obtaining antibodies specific to each protein associated to the scaffold protein. The full RNA degradosome could then be compared to the degradosome purified from the *E. coli dnaK* minus strain under conditions of heat stress. *E. coli e*nolase must be purified to further research the effect of DnaK and also DnaJ on enolase activity. An enolase plasmid that encodes for a His-tag therefore needs to be constructed to allow the purification of *E. coli* enolase to be used in the activity assays. Antibodies against enolase, as well as PNPase and RhlB helicase need to be prepared so that the major components of the RNA degradosome can be identified under varied conditions. This study was at a disadvantage because these antibodies were not available to investigate if the full RNA degradosome had been purified. The association of RhlB helicase to the degradosome increases its ATPase activity fifteen fold, but it has not been determined how the interaction of RNase E activates RhlB (Vanzo, 1998). Future work could include determining the mechanism of action of RNase E on RhlB.

Future work could also include elucidating the role of enolase by determining if a post-transcriptional regulation, such as phosphorylation, recruits this protein to the RNA degradosome. The majority of enzymes involved in degradative pathways have been shown to be active in the phosphorylated state and inactive in the dephosphorylated state (Ranjeva and Boudet, 1987; Randall and Blevins, 1990). Using enolase activity assays, the phosphorylated state of enolase can be determined by measuring the activity of purified enolase upon treatment with alkaline phosphatase. It can then be compared to the enolase activity from the purified degradosome treated with alkaline phosphatase.

Enolase was shown to be a validated target for drug design in *Trypanosoma brucei*. Disruption of the degradosome could be incurred using platinum nanoparticles. Could research on enolase from the disrupted RNA degradosome possibly lead to drug targeting?

Anti-alpha-enolase antibodies have been found in many autoimmune and antiflammatory diseases such as multiple sclerosis, rheumatoid arthritis, paraneoplastic retinopathy etc. These antibodies may come about due to microbial infection or uncontrolled cellular growth in pathophysiological conditions. In infections, these antibodies could function in decreasing microbial tissue invasion. Autoimmune and inflammatory diseases results in endothelial injury induced by the anti-alpha-enolase antibodies and in apoptotic cell death (Terrier *et al.,* 2006). Thus a thorough study of *E. coli* enolase could enhance our understanding of its role in human diseases.

References

---

1. Aaronson, R.M., Graven, K.K., Tucci, M., McDonald, R.J. & Farber, H.W. (1995). Non-neuronal enolase is an endothelial hypoxic stress protein. *J. Biol. Chem.* **270:** 27752-27757
2. Alberts, B. (1998). The cell as a collection of protein machines: preparing the next generation of molecular biologists. *Cell* **92:** 291–294
3. Allmang, C., Petfalski, E., Podtelejnikov, A., Mann, M., Tollervey, D. & Mitchell, P. (1999). The yeast exosome and human PM/Scl are related complexes of 3'-5' exonucleases. *Genes dev.* **13:** 2148-2158
4. Angiolella, L., Facchin, M., Stringaro, B., Maras, N., Simonetti, N. & Cassone, A. (1996). Identification of a glucan-associated enolase as a mian cell wall protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. *J. Infect. Dis.* **173:** 684-690
5. Apirion D (1978). Isolation, genetic mapping and some characterization of a mutation in Escherichia coli that affects the processing of ribonuleic acid. *Genetics* **90:** 659-71
6. Arifuzzaman, M., Oshima, T., Nakade, S., Mori, H. (2002) Characterization of HscC (Hsc62), homologue of Hsp70 in *Escherichia coli*: over-expression of HscC modulates the activity of housekeeping sigma factor sigma70. *Genes Cells*. **7:** 553-566
7. Arsene, F., Tomoyasu, T. & Bukau, B. (2000). The heat shock response of *Escherichia coli. Int. J. Food Micro.* **55:** 3-9
8. Bakau, B. (1993). Regulation of the *Escherichia coli* heat- shock response. *Mol. Microbiol.* **9:** 671-680
9. Baker, T. A. & Bell, S. P. (1998). Polymerases and the repliosome: machines within machines. *Cell* **92:** 295–305
10. Banecki, B. & Zylicz, M. (1996). Real time kinetics of the DnaK/DnaJ/GrpE molecular chaperone machine action. *J. Biol. Chem.* **271:** 6137–6143
11. Bardwell, J.C.A. & Craig, E.A. (1984). Major heat shock protein of *Drosophila* and the *Escherichia coli* heat inducible *dna*K gene are homologous. *Proc. Natl. Acad. Sci. USA* **81:** 848-852
12. Barraclough, R. & Ellis, R.J. (1980). Protein synthesis in chloroplasts. IX. Assembly of newly synthesized large subunits into ribuloase bisphosphate carboxylase in isolated intact chloroplasts. *Biochim. Biophys. Acta* **608:** 19-31

13. Baumeister, W., Walz, J., Zuhl, F. & Seemuller, E. (1998). The proteosome: paradigm of a self-compartmentalizing protease. *Cell* **92:** 367–380
14. Bechhofer, D.H., Oussenko, I.A., Deikus, G., Yao, S., Mathy, N. & Condon, C. (2008). Analysis of mRNA decay in *Bacillus subtilis. Methods in Enzymology* **447:** 259-276
15. Beckmann, R.P., Mizzen, L.E. & Welch, W.J. (1990). Interaction of Hsp70 with newly synthesized proteins: implications for protein folding and assembly. *Science*, **248:** 850-854
16. Belasco, J.G. (1993). Control of Messenger RNA Stability. *Academic Press, San diego, California:* 3-12
17. Bennet, J. (1991). Protein phosphorylation in green plant chloroplasts. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 281-311
18. Beran RK, Simons RW (2001). Cold-temperature induction of Escherichia coli polynucleotide phosphorylase occurs by reversal of its autoregulation. *Mol Microbiol* **39:** 112-25
19. Bercovich, B., Stancovski, I., Mayer, A., Blumenfeld, N., Laszlo, A., Schwartz, A.L. & Ciechanover, A. (1997). Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70. *J. Biol. Chem.* **272:** 9002
20. Bergmeyer, H. U. "Methods of Enzymatic Analysis", Vol. 4, Academic Press (New York, NY:1974), pp.2066-2072
21. Bernstein, J.A., Lin, P., Cohen, S.N. & Lin-Chao, S. (2003). Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays, *PNAS* **101:** 2758-2763
22. Bessarab DA, Kaberdin VR, Wei CL, Liou GG, Lin-Chao S (1998). RNA components of Escherichia coli degradosome: evidence for rRNA decay. *Proc. Natl. Acad. Sci. USA* **95:** 3157-3161
23. Blevins, T., Rajeswaran, R., Shivaprasad, P.V., Beknazariants, D., Si-Ammour, A., Park, H., Vazquez, F., Robertson, D., Meins, F., Hohn, T. & Pooggin, M.M. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res.* **34:** 6233–46
24. Blum, E., Py, B., Carpousis, A.J., & Higgens, C.F. (1997). Polyphosphate kinase is a component of the Escherichia coli RNA degradosome. *Mol. Microbiol.* **26:** 387-398
25. Bochtler, M., Hartmann, C., Song, H.K., Bourenkov, G.P., Batunik, H.D. & Huber, R. (2000). The structures of HslU and the ATP-dependent protease HslU-HslV. *Nature* **403:** 800-805

26. Boshoff, A., Hennessy, F. & Blatch, G.L. (2004). The in vivo and in vitro characterization of DnaK from *Agrobacterium tumefaciens* RUOR. *Prot. Expres. Purif.* **38:** 161-169
27. Boshoff, A., Nicoll, W.S., Hennessy, F., Ludeiwg, M.H., Daniel, S., Modisakeng, K.W., Shonhai, A., McNamara, C., Bradley, G. & Blatch, G.L. (2004). Molecular chaperones in biology, medicine and protein biotechnology. *S.A. J. Sci.* **100:** 1-13
28. Bouvet, P. & Belasco, J.G. (1992). Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in *Escherichia coli*. *Nature* **360:** 488-491
29. Braun F, Le Derout J, Regnier P (1998). Ribosomes inhibit an RNase E cleavage which induces the decay of the rpsO mRNA of Escherichia coli. *EMBO J.* **17:** 4790-4797
30. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254
31. Brehmer, D., Rudiger, S., Gassler, C.S., Klostermeier, D., Packschies, L., Reinstein, J., Mayer, M.P. & Bukau, B. (2001). Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. *Nat. Struct. Biol.* **8:** 427–432
32. Brewer, J.M. (1985). Specificity and mechanism of action of metal ions in yeast enolase. *FEBS Letters* **182:** 8-14
33. Bricker, A., Scheufler, C., von der Mülbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F. (2002). Ligand Discrimination By TPR Domains .*J. Biol Chem.* **277:** 19265-19275
34. Briegel, K., Baker, A. & Jain, C. (2005). Identification and analysis of *Escherichia coli* ribonuclease E dominant-negative mutants. *Genetics* **172:** 7-15
35. Brizzard, B.L., Chubet, R.G. & Vizard, D.L. (1994). Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* **16:** 730-735
36. Brodsky, J.L. & McCracken, A.A. (1999). ER protein quality control and proteasome-mediated protein degradation. *Sem. Cel. Dev. Biol.* **10:** 507-513
37. Brouwer, R., Allmang, C., Raijmakers, R., van Aarssen, Y., Vree Egberts, W., Petflaski, E., van Venrooij, W.J., Tollervey, D. & Pruijn, G.J. (2001). Three novel components of the human exosome. *J. Biol. Chem.* **276:** 6177-6180
38. Brown, J.T., Yang, X. & Johnson, A.W. (2000). Inhibition of mRNA Turnover in Yeast by an *xrn1* Mutation Enhances the Requirement for eIF4E Binding to eIF4G and for Proper Capping of Transcripts by Ceg1p. *Genetics* **155:** 31-42

39. Buchberger, A., Theyssen, H., Schroder, H., McCarty, J.S., Virgallita, G., Milkereit, P., Reinstein, J. & Bukau, B. (1995). Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidencefor interdomain communication. *J. Biol. Chem.* **270:** 16903-16910
40. Bukau,B. (1993). Regulation of the *Escherichia coli* heat-shock response. *Mol. Microbiol.* **9:** 671–680
41. Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5:** 376–378
42. Burkholder, W.F., Panagiotidis, C.A., Silverstein, S.J., Cegielska, A., Gottesman, M.E. & Gaitanaris, G.A. (1994). Isolation and characterization of an *Escherichia coli* DnaK mutant with impaired ATPase activity. *J. Mol. Biology* **242:** 364-377
43. Bycroft, M., Hubbard, T.J., Proctor, M., Freund, S.M. & Murzin, A.G. (1997). "The solution structure of the S1 RNA binding domain: a member of an ancient nucleic acid-binding fold." *Cell* **88:** 235-242
44. Callaghan, A.J., Grossmann, J.G., Redko, Y.U., Ilag, L.L., Moncrieffe, M.C., Symmons, M.F., Robinson, C.V., McDowall, K.J. & Luisi, B.F. (2003). Quaternary structure and catalytic activity of the Escherichia coli ribonuclease E amino-terminal catalytic domain. *Biochemistry* **42:** 13848-13855
45. Callaghan, A.J., Marcaida, M.J., Stead, J.A., McDowall, K.J., Scott, W.G. & Luisi, B.F. (2005). Structure of Escherichia coli RNase E catalytic domain and implications for RNA turnover. *Nature* **437:** 1187-1191
46. Callaghan, .AJ, Redko, Y., Murphy, L.M., Grossmann, J.G., Yates, D., Garman, E., Ilag, L.L., Robinson, C.V., Symmons, M.F., McDowall, K.J. & Luisi, B.F. (2005). “Zn-Link": a metal-sharing interface that organizes the quaternary structure and catalytic site of the endoribonuclease, RNase E. *Biochemistry* **44:** 4667-4675
47. Caplen, N. J., Parrish, S., Imani, F., Fire, A. & Morgan, R. (2001). Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* **98:** 9742–9747
48. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S. & Cerami, A. (1986). Sequences controlling histone H4 mRNA abundance. *EMBO J.* **6:** 1825-1831
49. Carpousis, A.J. (2002). The *Escherichia coli* RNA degradosome: structure, function and relationship to other ribonucleolytic multienzyme complexes. *Biochemical Society Transactions* **30:** 150-154

50. Carpousis, A.J. (2007). The RNA degradosome of *Escherichia coli:* an mRNA-degrading machine assembled on RNase E. *Annu. Rev. Microbiol.* **61:** 71-87
51. Carpousis, A.J., Khemici, V., Ait-Bara, S. & Poljak, L. (2008). Co-immunopurification of multiprotein complexes containing RNA-degrading enzymes. *Methods in Enzymology* **447:** 65-82
52. Carpousis, A.J., Leroy, A., Vanzo, N. & Khemici, V. (2001). *Escherichia coli* RNA Degradosome. *Methods in Enzymology* **342:** 333-345
53. Carpousis, A.J., Van Houwe, G., Ehretsmann, C. & Krisch, H.M. (1994). Copurification of E. coli RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell* **76:** 889-900
54. Carpousis, A. J., Vanzo, N. F. & Raynal, L. C. (1999). mRAN degradation. A tale of poly(A) and multiprotein machines. *Trends Genet.* **15:** 24–28
55. Carvalho, A.L., Diaz, F.M.V., Prates, J.A.M., Nagy, T., Gilbert, H.G., Davies, G.J., Ferreira, L.M.A., Romao, M.J. & Fontes, C.M.G.A. (2003). Cellulosome assembly revealed by the crystal structure of the cohesion-dockerin complex. *PNAS* **100:** 13809-13814
56. Carzaniga, T., Briani, F., Zangrossi, S., Merlino, G., Marchi, P. & Deho, G. (2009). Autogenous regulation of Escherichia coli polynucleotide phosphorylase expression revisited. *J. Bacteriol.* **191:** 1738-1748
57. Casaregola, S., Jacq, A., Laoudj, D., McGurk, G., Margar-son, S., Tempete, M., Norris, V. & Holland, I.B. (1992). Cloning and analysis of the entire *Escherichia coli ams* gene, *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. *J. Mol. Biol.* **228:** 30-40
58. Cashel, M., and K. E. Rudd. 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
59. Chamberlin, M. & Ring, J. (1973). Characterization of T7-specific ribonucleic acid polymerase. 1. General properties of the enzymatic reaction and the template specificity of the enzyme. *J. Biol. Chem.* **248:** 2235-2244
60. Chandran, V. & Luisi, B.F. (2006). Recognition of Enolase in the *Escherichia coli* RNA Degradosome. *J. Mol. Biol.* **358:** 8-15

61. Chaney, S.G. & Boyer, P.D. (1972). Incorporation of water oxygens into intracellular nucleotides and RNA. II. Predominantly hydrolytic RNA turnover in *Escherichia coli*. *J. Mol. Biol.* **64:** 581-591
62. Chekanova, J.A., Shaw, R.J., Wills, M.A., & Belostotsky, D.A. (2000). Poly(A) tail-dependent exonuclease AtRrp41p from Arabidopsis thaliana rescues 5.8S rRNA processing and mRNA decay defects of the yeast ski6 mutant and is found in an exosome-sized complex in plant and yeast cells. *J. Biol. Chem.* **275:** 33158-33166
63. Chevalier, C., Huntzinger, E., Fechter, P., Boisset, S., Vandenesch, F., Romby, P. & Geissmann, T. (2008). *Staphylococcus aureus* endoribonuclease III: purification and properties. *Methods in Enzymology* **447:** 309-327
64. Chiang, C.M. & Roeder, R.G. (1993). Expressiona dn purification of general transcription factors by Flag epitope tagging and peptide elution. *Pept. Res.* **6:** 62-64
65. Chirico, W.J., Waters, M.G. & Blobel, G. (1988). 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* **332:** 805-810
66. Claverie-martin, F., Diaz-torres, M.R., Yancey, S.D. & Kushner, S.R. (1991). Analysis of the altered mRNA stability (*ams*) gene from *Escherichia coli. J. Biol. Chem.* **266:** 2843-2851
67. Coburn, G.A. & Mackie, G.A. (1999). Degradation of mRNA in Escherichia coli: an old problem with some new twists. *Prog. Nucleic Acids Res. Mol. Biol.* **62:** 55-108
68. Cohen, S.N. & McDowall, K.J. (1997). RNase E: still a wonderfully mysterious enzyme. *Mol. Microbiol.* **23:** 1099-1106
69. Condon, C. & Putzer, H. (2002). The phylogenetic distribution of bacterial ribonucleases. *Nucleic Acids Res.* **30:** 5339-5346
70. Cordin, O., Banroques, J., Tanner, N. K. & Linder, P. (2006). The DEAD-box protein family of RNA helicases. *Gene* **367:** 17–37
71. Coyle, J.E., Texter, F.L., Ashcroft, A.E., Masselos, D., Robinson, C.V. & Radford, S.E. (1999). GroEL accelerates the refolding of hen lysozyme without changing its folding mechanism. *Nature Struct. Biol.* **6:** 683-690
72. Creighton, 1993. T.E. Creighton, Proteins: Structures & Molecular Properties. In: (2nd edn ed.), W. H. Freeman & Co., New York, USA (1993), pp. 293–296
73. Cyr, D.M., Langer, T. & Douglas, M.G. (1994). DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends in Biochem. Sci.* **19:** 176-181
74. Dawson, R. M. C., et al., Data for Biochemical Research, 3rd ed., Oxford University Press (New York, NY: 1986), pp. 284-285.

75. De Maio, A. (1999). Heat shock proteins: facts, thoughts, and dreams. *Shock* **11** (1): 1-12
76. Deutscher, M.P. & Li, Z. (2001). Exoribonucleases and their multiple roles in RNA metabolism. *Prog. Nucleic Acids Res. Mol. Biol.* **66:** 67-105
77. Deutscher, M.P. & Reuven, N.B. (1991). Enzymatic basis for hydrolytic versus phosphorylytic mRNA degradation in *Esscherichia coli* and *Bacillus subtilis. Proc. Natl. Acad. Sci. USA* **88:** 3277-3280
78. Diamant, S. & Goloubinoff, P. (1998). Temperature-controlled activity of DnaK-DnaJ-GrpE chaperones: protein-folding arrest and recovery during and after heat shock depends on the substrate protein and the GrpE concentration. *Biochem.* **37:** 9688-9694
79. Dirheimer, G. & Ebel, J.P. (1965). Characterization of polyphosphate-AMP phosphotransferase in *Corynebacterium serosis. C. R. Acad. Sci. (Paris)* **260:** 3787-3790
80. Dirheimer, G. & Ebel, J.P. (1968). Purification and properties of a polyphosphate: glucose and glucosamine 6-phosphotransferase from *Corynebacterium xerosis. Bull. Soc. Chim. Biol.* **50:** 1933-1947
81. DiTullio, R. A., Mochan, T. A., Venere, M., Bartkova, J., Sehested, M., Bartek, J. & Halazonetis, T. D. (2002) 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat. Cell Biol.* **4:** 998–1002
82. Donovan, W.P. & Kushner, S.R. (1986). Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. U.S.A.* **83:** 124-124
83. Du, T. & Zamore, P.D. (2005). microPrimer: the biogenesis and function of microRNA. *Development* **132:** 4645-4652
84. Dudani, A.K., Cummings, C., Hashemi, S. & Ganz, P.R. (1993). Isolation of a novel 45 kDa plasminogen receptor from human endothelial cells. *Thromb. Res.* **69:** 185-196
85. Duffy, J.J., Chaney, S.G. & Boyer, P.D. (1972). Incorporation of water oxygens into intracellular nucleotides and RNA. I. Predominantly non-hydrolytic RNA turnover in *Bacillus subtilis. J. Mol. Biol.* **64:** 565-579
86. Dumont, S., Cheng, W., Serebrov, V., Beran, R. K., Tinoco, I., Pyle, A. M. & Bustamante, C. (2006). RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* **439:** 105–108
87. Dziembowski, A. & Stepien, P.P. (2001). Genetic and biochemical approaches for analysis of mitochondrial degradosome from *Saccharomyces cerevisiae*. *Methods Enzymol.* **342:** 367-378

88. Eggers, D.K., Welch, W.J. & Hansen, W.J. (1997). Complexes between nascent polypeptides and their molecular chaperones in the cytosol of mammalian cells. *Mol. Biol. Cell* **8:** 1559-1573
89. Ehretsmann, C.P., Carpousis, A.J. & Krisch, H.M. (1992). Specificity of Escherichia coli endoribonuclease RNase E: in vivo and in vitro analysis of mutants in a bacteriophage T4 mRNA processing site. *Genes Dev.* **6:** 149-159
90. Einhauer, A. & Jungbauer, A. (2001). The FLAG^{TM} peptide, a versatile fusion tag for the purification of recombinant proteins. *J. Biochem. Biophys. Methods* **49:** 455–465
91. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494–498
92. Ellis, R.J. (1987). Proteins as molecular chaperones. *Nature* **328:** 378-379
93. Ellis, R.J. (2001). Molecular chaperones: inside and outside the Anfinsen cage. *Curr. Biol*. **11:** 1038–1040
94. Ellis, R.J. (2006). Molecular chaperones: assisting assembly in addition to folding. *TRENDS in Biochem. Sci.* **31:** 395-401
95. Engels, B.M. & Hutvagner, G. (2006). Principles and effects of microRNA-mediated post0transcriptional gene regulation. *Oncogene* **25:** 6163-6169
96. Estévez, A. M., Kempf, T. & Clayton, C. (2001). The exosome of *Trypanosoma brucei. EMBO J.* **20:** 3831–3839
97. Evhuenieva-Hackenberg, E., Wagner, S. & Klug, G. (2008). *In vivo* and *in vitro* studies of RNA degrading activities in Archaea. *Methods in Enzymology* **447:** 381-416
98. Fairman, M. E., Maroney, P. A., Wang, W., Bowers, H.A., Gollnick, P., Nilsen, T. W. & Jankowsky, E. (2004).Protein displacement by DexH/D “RNA helicases” without duplex unwinding. Science **304:** 730–734
99. Faller, L.D., Baroudy, B.M., Johnson, A.M. & Ewall, R.X. (1977). Magnesium ion requirements for yeast enolase reactivity. *Biochemistry* **16:** 3864-3869
100. Fayet, O., Ziegelhoffer, T. & Georgopoulos, C. (1989). The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171:** 1379-1385
101. Feldheim, D., Rothblatt, J. and Schekman, R. (1992). Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Molecular and Cellular Biology* **12:** 3288-3296

102. Feng, Y., Huang, H., Liao, J. & Cohen, S.N. (2001). *Escherichia coli* Poly(A)-binding Proteins That Interact with Components of Degradosomes or Impede RNA Decay Mediated by Polynucleotide Phosphorylase and RNase E. *J. Biol. Chem.* **276:** 31651-31656
103. Fenton, W.A. & Horwich, A.L. (1997). GroEL-mediated protein folding. *Protein Sci*. **6:** 743–760
104. Fink, A.L. (1999). Chaperone-mediated protein folding. *Physiol. Rev.* **79:** 425-449
105. Finkelstein, D.B., Strausberg, S. & McAlister, L.(1982). Alterations of transcription during heat shock of *Saccharomyces cerevisiae. J. Biol. Chem.* **257:** 8405-8411
106. Fitt, P. S. & See, Y. P. (1970). Guinea-pig liver polynucleotide phosphorylase. *Biochem. J.* **116:** 309–311
107. Flaherty, K.M., Wilbanks, S.M., De Luca-Flaherty, C. and McKay, D.B. (1990). Three-dimensional structure of the ATPase fragment of a 70 kDa heat shock cognate protein. *Nature* **346:** 623-628
108. Fontan, P.A., Pancholi, V., Nociari, M.M. & Fischetti, V.A. (2000). Antibodies to streptococcal surface enolase react with human alphaenolase: implications in poststreptococcal sequelae. *J Infect Dis* **182:** 1712–21
109. Freeman, B.C., Myers, M.P., Schumacher, R. & Morimoto, R.I. (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO J.* **14:** 2281
110. Friedman, D.I., Olson, E.R., Georgopoulos, C., Tilly, K., Herkowitz, I. & anuett, F. (1984). Interactions of bacteriophage and host macromolecules in the growth of bacteriophage I. *Micro. Reviews* **48:** 299-325
111. Fuller, C. W., Beauchamp, B. B., Engler, M. J., Lechner, R. L., Matson, S. W., Tabor, S., White, J. H. & Richardson, C. C. (1983) Cold Spring Harbor Symp. Quant. Biol. **48:** 669-679
112. Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J.S., Rüdiger, S., Schönfeld, J., Schirra, C., Bujard, H. & Bukau, B. (1996). A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *Escherichia coli* heat shock transcription factor 32. *EMBO J.* **15:** 607–617
113. Gao, J., Lee, K., Zhao, M., Qiu, J., Saxena, A., Moore, C.J., Cohen, S.N. & Georgiou, G. (2006). Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that later the composition of the degradosome. *Mol. Micro.* **61:** 394-406

114. Gässler, C.S., Buchberger, A., Laufer, T., Mayer, M.P., Schröder, H., Valencia, A. & Bukau, B. (1998). Mutations in the DnaK chaperone aVecting interaction with the DnaJ cochaperone. *Proc. Natl. Acad. Sci. USA* **95:** 15229–15234
115. Gengenheimer, P., Watson, N. & Apirion, D. (1977). Multiple pathways for primary processing of ribosomal RNA in *Escherichia coli*. *J. Biol. Chem.* **252:** 3064-3073
116. Ghora, B.K. & Apirion, D. (1978). Structural analysis and in vitro processing to p5 rRNA of a 9S RNA molecule isolated from an rne mutant of *E. coli*. *Cell* **15**: 1055-1066
117. Giallongo, A., Feo, S., Moore, R., Croce, C.M. & Showe, L.C. (1986). Molecular cloning and nucleotide sequence of a full-length cDNA for human α enolase. *Proc. Natl. Acad. Sci. USA* **83:** 6741-6745
118. Gillet, R. & Felden, B. (2001). Emerging views on tmRNA-mediated protein tagging and ribosome rescue. *Mol. Micro.* **42** (4): 879–885
119. Gottesman, S., Maurizi, M.R. & Wickner, S. (1997). Regulatory subunits of energy-dependent proteases. *Cell* **91:** 435-439
120. Green, R. & Noller, H. F. (1997) *Annu. Rev. Biochem.* **66:** 679–716.
121. Gregarov, A., Zeng, L., Zhao, X. Burkholder, W. And Gottesmann, M.E. (1994). Specificity of DnaK-peptide binding. *J Mol Biol.* **235:** 848–854
122. Gross, C.A. (1996). Function and regulation of the heat shock proteins in *Escherichia coli* and *Salmonella.* ed. F.C. Neidhardt, pp. 1382-1399. ASM Press, Washington, D.C.
123. Guimarães, M.J., Peterson, D., Vicari, A., Cocks, B.G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Ferrick, D.A., Kastelein, R.A., Bazan, J.F. & Zlotnik, A. (1996). Identification of a novel selD homolog from eukaryotes, bacteria, and archaea: is there an autoregulatory mechanism in selenocysteine metabolism? *Proc. Natl. Acad. Sci. U S A* **93:** 15086-15091
124. Guissani, A. & Portier, C. (1976). Study on the structure-function relationship of polynucleotide phosphorylase: model of a proteolytic degraded polynucleotide phosphorylase. *Nucleic Acids Res.* **3:** 3015-3024
125. Gulick, A.M., Hubbard, B.K., Gerlt, J.A. & Rayment,I. (2000). Evolution of enzymatic activities in the enolase superfamily: crystallographic and mutagenesis studies of the reaction catalyzed by D-glycerate dehydratase from *Escherichia coli. Biochemistry,* **39:** 4590-4602
126. Hajnsdorf, E., Steier, O., Coscoy, L., Teysset, L. & Regnier, P. (1994). Roles of RNase E, RNase II and PNPase in the degradation of the rpsO transcripts of Escherichia coli:

stabilizing function of RNase II and evidence for efficient degradation in an ams pnp rnb mutant. *EMBO J.* **13:** 3368-3377

127. Hammon, B.D., Hendershot, L.M. & Johnson, A.E. (1998). BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in the translocation. *Cell* **92:** 747-758
128. Han, W. & Christen, P. (2003). Mechanism of the targeting action of DnaJ in the DnaK molecular chaperone system. *J. Biol. Chem*. **278:** 19038–19043
129. Har-El, R., Silberstein, A., Kuhn, J. & Tal, M. (1979). Synthesis and degradation of *lac* mRNA in *E. coli* depleted of 30S ribosomal subunits. *Mol. Gen. Genet.* **167:** 135-144
130. Harrison, C.J., Hayer-Hartl, M., Di Liberto, M., Hartl, F. & Kuriyan, J. (1997). Crystal structure of the nucleotide exchange factor GrpE bound to the *ATPase* domain of the molecular chaperone DnaK. *Science* **276:** 431–435
131. Hartl, F. U. (1996) Molecular chaperones in cellular protein folding. *Nature*. **381:** 571-580
132. Hartl, F.U. & Hayer-Hartl, M. (2009). Converging concepts of protein folding *in vitro* and *in vivo*. *Nature Structural & Molecular Biology* **16:** 574-581
133. Hatzixanthis, K., Palmer, T. & Sargent, F. (2003). A subset of bacterial inner membrane proteins integrated by the twin-arginine translocase. *Molecular Microbiology* **49:** 1377-1390.
134. Hendershot, L., Wei, J., Gaut, J., Melnick, J.S Aviel, S. & Argon, Y. (1996). Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. *Proc. Natl. Acad. Sci. USA* **93:** 5269–5274
135. Hendrick, J.P. & Hartl, F.U. (1993). Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem.* **62:** 349-384
136. Hengen, P.N. (1995). Purification of His-Tag fusion proteins from *Escherichia coli. Trends Biochem Sci.* **20:** 285-6
137. Hesterkamp, T. & Bukau, B. (1998). Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E. coli. EMBO J.* **17:** 4818-4828
138. Hochuli, E., Bannwarth, W., D6beli, H., Gentz, R. & Stt~ber, D. (1988). Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology* **6:** 1321-1325
139. Hoff, K. G., Silberg, J. J., and Vickery, L. E. (2000) Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97:** 7790–7795

140. Hoffmann-Ostenhof, O., Kenedy, J., Keck, K., Gabriel, O. & Schonfellinger, H.W. (1954). A new transphosphorylase from yeast. *Biochem. Biophys. Acta.* **14:** 285

141. Horwich, A. L., Brooks Low, K., Fenton, W.A., Hirshfield, I.N. & Furtak, K. (1993). Folding *in vivo* of bacterial cytoplasmic proteins: role of GroEL. *Cell* **74:** 909-917

142. Horwich, M.D., Li C., Matranga, C., Vagin, V., Farley, G., Wang, P. & Zamore, P.D. (2007). The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Current Biology* **17:** 1265–72

143. Horwick, A.L., Weber-Ban, E.U. & Finley, D. (1999). Chaperone rings in protein folding and degradation. *Proc. Natl. Acad. Sci. USA* **96:** 11033

144. Houry, W.A., Frishman, D., Eckerskorn, C., Lottspeich, F. & Hartl, F.U. (1999). Identification of *in vivo* substrates of the chaperonin GroEL. *Nature* **402:** 147-154

145. lida, H. & Yahara, 1. (1984). A heat-shock resistant mutant of *Saccharomyces cerevisiaei* shows constitutive synthesis of two heat shock proteins and altered growth. *J. Cell. Biol.* **99:** 1441-1450

146. lida, H. & Yahara, 1. (1985). Yeast heat-shock protein of MR 48,000 is an isoprotein of enolase. Nature **315:** 688-690

147. Ingolia, T. & Craig, E.A. (1982). *Drosophila* gene related to the major heat shock-induced gene is transcribed at normal temperatures and not induced by heat shock. *Natl. Acad. Sci. USA* **79:** 525-529

148. Jäger, S., Fuhrmann, O., Heck, C., Hebermehl, M., Schiltz, E., Rauhut, R. & Klug, G. (2001). An mRNA degrading complex in *Rhodobacter capsulatus. Nucleic Acids Res.* **29:** 4581-4588

149. Jagessar, K.L. & Jain, C. (2010). Functional and molecular analysis of *Escherichia coli* strains lacking multiple DEAD-box helicases. *RNA* **16:** 1386-1392

150. Jain, C., Deana, A. & Belasco, J.G. (2002). Consequences of RNase E scarcity in *Escherichia coli. Mol. Microbiol.* **43:** 1053-1064

151. Jakob, U., Lilie, H., Meyer, I. & Buchner, J. (1995). Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock *in vivo. J. Biol. Chem.* **270:** 7288-7294

152. Jarrige A.C., Brechemier-Baey D, Mathy N, Duche O, Portier C (2002). Mutational analysis of polynucleotide phosphorylase from Escherichia coli. *J. Mol. Biol.* **321:** 397-409

153. Jarrige, A.C., Mathy, N. & Portier, C. (2001). PNPase autocontrols its expression by degrading a double-stranded structure in the pnp mRNA leader. *EMBO J.* **20:** 6845-6855

154. Jeffery, C.J. (1999). Moonlighting proteins. *Trends Biochem. Sci*. **24:** 8–11

155. Jones, P.G. & Inouye, M. (1994). The cold-shock response – a hot topic. *Molecul. Microbiol.* **11:** 811-818

156. Kaberdin, V.R., Miczak, A., Jakobsen, J.S., Lin-Chao, S., McDowall, K.J. & von Gabain, A. (1998). *Proc. Natl. Acad. Sci. USA* **95:** 11637-11642

157. Kaldis, A., Atkinson, B.G. & Heikkila, J.J. (2004). Molecular chaperone function of the *Rana catesbeiana* small heat shock protein, hsp30. *Comp. Biochem. Physiol.* **139:** 175-182

158. Kandror, O., Busconi, L., Sherman, M. & Goldberg, A.L. (1994). Rapid degradation of an abnormal protein in *Escherichia coli* involves the chaperones GroEL and GroES. *J. Biol. Chem.* **269:** 23575-23582

159. Kaplan, R. & Apirion, D. (1974). The involvement of ribonuclease I, ribonuclease II, and polynucleotide phosphorylase in the degradation of stable ribonucleic acid during carbon starvation in *Escherichia coli*. *J. Biol. Chem.* **249:** 149-151

160. Kaplan, R. & Apirion, D. (1975). Decay of ribosomal ribonucleic acid in *Escherichia coli* cells starved for various nutrients. *J. Biol. Chem.* **250:** 3174-3178

161. Karunakaran, K. P., Noguchi, Y., Read, T. D., Cherkasov, A., Kwee, J., Shen, C., Nelson, C. C. & Brunham, R. C. (2003). Molecular analysis of the multiple GroEL proteins of Chlamydiae. *J. Bacterio.* **185:** 1958 - 1966

162. Kerner, M.J., Naylor, D.J., Ishihama, Y., Maier, T., Chang, H., Stines, A.P., Georgopoulos, C., Frishman, D., Hayer-Hartl, M., Mann, M. & Hartl, F. (2005). Proteome-Wide Analysis of Chaperonin-Dependent Protein Folding in *Escherichia coli*. *Cell* **122:** 209–220

163. Kessler, B. & Chen, D. (1964). Distribution, properties and specificity of polynucleotide phosphorylase in wheat roots. *Biochim. Biophys. Acta* **80:** 533–541

164. Khemici, V. & Carpousis, A.J. (2004). The RNA degradosome and poly(A) polymerase of *Escherichia coli* are required in vivo for the degradation of small mRNA decay intermediates containing REP-stabilizers. *Mol. Microbiol.* **51:** 777-790

165. Khemici, V., Poljak, L., Toesca, I. & Carpousis, A.J. (2005). Evidence in vivo that the DEAD-box RNA helicase RhlB facilitates the degradation of ribosome-free mRNA by Rnase E. *Proc. Natl. Acad. Sci.* **102:** 6913-6918

166. Kido, M., Yamanaka, K., Mitani, T., Niki, H, Ogura, T. & Hiraga, S. (1996). RNase E polypeptides lacking a carboxyl-terminal half suppress a mukB mutation in *Escherochia coli*. *J. Bacteriol.* **178:** 3917-3925

167. Kim, K.S. & Lee, Y. (2004). Regulation of 6S RNA biogenesis by switching utilization of both sigma factors and endoribonucleases. *Nucleic Acids Res.* **32:** 6057-6068

168. Kimata, K., Tanaka, Y., Inada, T. & Aiba, H. (2001). Expression of the glucose transporter gene, ptsG, is regulated at the mRNA degradation step in response to glycolytic flux in *Eshcerichia coli*. *EMBO J.* **20:** 3587-3595

169. Kinscherf, T.G. & Apirion, D. (1975). Polynucleotide phosphorylase can participate in decay of mRNA in *Escherichia coli* in the absence of ribonuclease II. *Mol. Gen. Genet.* **139:** 357-362

170. Klein, S., Geiger, T., Linchevski, I., Lebendiker, M., Itkin, A., Assayag, K. & Levitzki, A. (2005). Expression and puirification of active PBK kinase from *Escherichia coli*. *Prot. Expr. Purif.* **41:** 162-169

171. Klug, G., Adams, C.W., Belasco, J.G., Doerge, B. & Cohen, S.N. (1987). Biological consequences of segmental alterations in mRNA stability: Effect of deletion of the intercistronic hairpin-loop region of the *R. capsulatus puf* operon. *EMBO].* **6:** 3515-3520

172. Kornberg, S.R. (1957). Adenosine triphoshpate synthesis from polyphosphate by an enzyme from *Escherichia coli*. *Biochem. Biophys. Acta* **26:** 294-300

173. Kuhnel, K. & Luisi, B.F. (2001). Crystal Structure of the *Escherichia coli* RNA Degradosome Component Enolase. *J. Mol. Biol.* **313:** 583-592

174. Kulaev, I.S. & Bobyk, M.A. (1971). Detection in *Neurospora crassa* of a new enzyme - 1,3-diphosphoglycerate: polyphosphate phosphotransferase. *Biokhimiya* **36:** 426-429

175. Kushner, S.R. (2002). mRNA decay in *Escherochia coli* comes of age. *J. Bacteriol.* **184:** 4658-4665

176. Kusukawa, N., Yura, T., Ueguchi, C., Akiyama, Y. & Ito, K. (1989). Effects of mutations in heat-shock genes *groES* and *groEL* on protein export in *Escherichia coli*. *EMBO J.* **8:** 3517-3521

177. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685

178. Laminet, A.A., Ziegelhoffer, T., Georgopoulos, C. & Pluckthun, A. (1990). The *Escherichia coli* heat shock proteins GroEL and GroES modulate the folding of the beta-lactamase precursor. *EMBO J.* **9:** 2315-2319

179. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. & Hartl, F.U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356:** 683–689

180. Laskey, R.A., Honda, B.M., Mills, A.D. & Finch, J.T. (1978). Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature* **275:** 416-420

181. LeClair, K.P., Blanar, M.A. & Sharp, P.A. (1993). The p50 subunit of NF-kB associates with the NF-IL6 transcription factor. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 8145-8149

182. Lee, K., Bernstein, J.A. & Cohen, S.N. (2002). RNase G complementation of *rne* null mutation identifies functional inter-relationships with RNase E in *Escherichia coli. Mol. Microbiol.* **43:** 1445-1456

183. Lee, K., Zhan, X., Gao, J., Qiu, J., Feng, Y., Meganathan, R., Cohen, S.N. & Georgiou, G. (2003). RraA. A protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell* **114:** 623–634

184. Lelivelt, M.J. & Kawula, T.H. (1995). Hsc66, an Hsp70 homolog in *Escherichia coli*, is induced by cold shock but not by heat shock. *J. Bacteriol.* **177:** 4900-4907

185. Lemaux, P.G., Herendeens. l., Bloch, P.L. & Neidhardft, C. (1978). Transient rates of synthesis of individual polypeptides in *Escherichia coli* following temperature shifts. *Cell* **13:** 427-434

186. Leroy, A., Vanzo, N.F., Sousa, S., Dreyfus, M. & Carpousis, A.J. (2002). Function in *Escherichia coli* of the non-catalytic part of RNase E: role in the degradation of ribosome-free mRNA. *Mol. Microbiol.* **45:** 1231-1243

187. Levy, E.J., McCarty, J., Bukau, B. & Chirico, W.J. (1995). Conserved ATPase and luciferase refolding activities between bacteria and yeast Hsp70 chaperones and modulators. *FEBS Letters* **368:** 435-440

188. Li, Z., Pandit, S. & Deutscher, M.P. (1999). RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. *EMBO J* **18** (10): 2878-2885

189. Li, Z., Pandit, S. & Deutscher, M.P. (2002). RNase E plays an essential role in the maturation of *Escherichia coli* tRNA precursors. *RNA* **8:** 97-109

190. Liberek, K. & Georgopoulos, C. (1993). Autoregulation of the *Escherichia coli* heat shock response by the DnaK and DnaJ heat shock proteins. *Proc. Natl. Acad. Sci. USA* **90:** 11019-11023

191. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. & Zylicz, M. (1991). *Escherichia coli* DnaJ and GrpE heat proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl. Acad. Sci.* **88:** 2874-2878

192. Lin, Z., Schwarz, F.P. & Eisenstein, E. (1994). The hydrophobic nature of GroEL-substrate binding. *J. Biol. Chem.* **270:** 1011-1014

193. Liou, G.G., Chang, H.Y., Lin, C.S. & Lin-Chao, S. (2002). DEAD box RhlB RNA helicase physically associates with exoribonuclease PNPase to degrade double-stranded

RNA independent of the Degradosome-assembling region of RNase E. *J. Biol. Chem.* **277:** 41157-41162

194. Liou, G.G., Jane, W.N., Cohen, S.N., Lin, N.S. & Lin-Chao, S. (2001). RNA Degradosome exist *in vivo* in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. *Proc. Natl. Acad. Sci. USA* **98:** 63-68
195. Lipinska, B., King, J., Ang, D. & Georgopolous, C. (1988). Sequence analysis and transcriptional regulation of the *Escherichia coli grpE* gene, encoding a heat shock protein. *Nucleic Acids Res* **16:** 7545–7562
196. Lohman, K. & Meyerhof, O. (1934). Enzymatic transformation of phosphoglyceric acid into pyruvic and phosphoric acid. *Biochem. Z.* **273:** 60-72
197. Lund, P. (2001). *Molecular chaperones in the cell.* New York: Oxford University Press
198. Lund, E. & Kjeldgaard, N.O. **(**1972). Metabolism of guanosine tetraphosphate in *Escherichia coli*. Eur. J. Biochem. **28:** 316–326
199. Lundberg, U. & Altman, S. (1995). Processing of the precursor to the catalytic RNA subunit of RNase P from *Escherichia coli*. *RNA* **1:** 327-334
200. Mackie, G.A. (1989). Stabilization of the 3' one-third of Escherichia coli ribosomal protein S20 mRNA in mutants lacking polynucleotide phosphorylase. *J. Bacteriol.* **171:** 4112-4120
201. Mackow, E. R. & Chang, F.N. (1983). Correlation between RNA synthesis and ppGpp content in *Escherichia coli* during temperature shifts. *Mol. Gen. Genet.* **192:** 5–9
202. Maier, T. (2006). Proteome-Wide Analysis of Chaperonin-dependent Protein Folding in *Escherichia coli.* PhD Dissertation, Ludwig-Maximilians-Universität München
203. Marcaida, M.J., DePristo, M.A., Chandran, V., Carpousis, A.J. & Luisi, B.F. (2006). The RNA degradosome: life in the fast lane of adaptive molecular evolution. *TRENDS Biochem. Sci.* **31:** 359-365
204. Mathy, N., Jarrige, A.C., Robert-Le Meur, M. & Portier, C. (2001). Increased expression of Escherichia coli polynucleotide phosphorylase at low temperatures is linked to a decrease in the efficiency of autocontrol. *J. Bacteriol.* **183:** 3848-3854
205. Matus-Ortega, M.E., Regonesi, M.E., Pina-Escobedo, A., Tortora, P., Deho, G. & Garcia-Mena, J. (2007). The KH and S1 domains of Escherichia coli polynucleotide phosphorylase are necessary for autoregulation and growth at low temperature. *Biochim. Biophys. Acta.* **1769:** 194-203

206. Mayer, M.P., Rudiger, S. & Bukau, B. (2000). Molecular basis for interactions of the DnaK chaperone with substrates. *Biol. Chem.* **381:** 877–885
207. Mazumdar, M., Mikami, A., Gee, M.A. & Vallee, R.B. (1996). *In vitro* motility from recombinant dynein heavy chain. *Proc. Natl. Acad. Sci. USA* **93:** 6552-6556
208. McAlister, L. & Holland, M.J. (1982). Targeted deletion of a yeast enolase structural gene. Identification and isolation of yeast enolase isozymes. *J. Biol. Chem.* **257:** 7181-7188
209. McCarty, J.S., Buchberger, A., Reinstein, J. & Bukau, B. (1995). The role of ATP in the functional cycle of the DnaK chaperone system. *J. Mol. Biol.* **249:** 126-137
210. McDowall, K.J. & Cohen, S.N. (1996). The N-terminal domain of the rne gene product has RNase E activity and is non-overlapping with the arginine-rich RNA-binding site. *J. Mol. Biol.* **255:** 349-355
211. McManus, M., Haines, B., Dillon, C. P., Whitehurst, C. E., van Parijs, L., Chen, J. & Sharp, P. A. (2002). Small interfering RNA-mediated gene silencing in T lymphocytes. *J. Immunol.* **169:** 5754–5760
212. McManus, M.T. & Sharp, P.A. (2002). Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Gene.* **10:** 737-747
213. Melefors, O., Lundberg, U. & von Gabain, A. (1993) Control of Messenger RNA Stability. *Academic Press, San diego, California*. 53-70
214. Mhammedi-Alaoui, A., Pato, M., Gama, M.J. & Toussaint, A. (1994). A new component of bacteriophage Mu replicative transposition machinery: the *Escherichia coli* ClpX protein. *Mol. Microbiol.* **11:** 1109-1116
215. Mian, I.S. (1997). Comparative sequences of ribonucleases HII, III, II PH and D. *Nucleic Acids Res.* **25:** 3187-3195
216. Miczak, A., Kaberdin, V.R., Wei, C.L. & Lin-Chao, S. (1996). Proteins associated with RNase E in a multicomponent ribonucleolytic complex. *Proc. Natl. Acad. Sci. USA* **93:** 3865-3869
217. Mikkelsen, D.N. & Gerdes, K. (1997). Sok antisense RNA from plasmid R1 is functionally inactivated by RNase E and polyadenylated by poly(A) polymerase I. *Mol Microbiol* **26:** 311-320
218. Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K. & Plow, E.F. (1991). Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolases as a candidate plasminogen receptor. *Biochemistry* **30:** 1682–1691

219. Minami, Y., Hohfeld, J., Ohtsuka, K. & Hartl, F.U. (1996). Regulation of the heat shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. *J. Biol. Chem.* **271:** 19617-19624

220. Misra, T.K. & Apirion, D. (1979). RNase E, an RNA processing enzyme from *Escherichia coli*. *J. Biol. Chem.* **254:** 11154-11159

221. Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M. & Tollervey, D. (1997). The exosome: a conserved eukaryotic RNA processing complex containing multiple 3’ ->5’ exoribonucleases. *Cell* **91:** 457-466

222. Mohanty, B.K. & Kushner, S.R. (2002). Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol. Microbiol.* **45:** 1315-1324

223. Morita, T., Kawamoto, H., Mizota, T., Inada, T. & Aiba, H. (2004). Enolase in the RNA degradosome plays a crucial role in the rapid decay of glucose transporter mRNA in the response to phosphosugar stress in *Escherichia coli, Mol. Microbiol.* **54:** 1063-1075

224. Motohashi, K., Yohda, M., Endo, I. & Yoshida, M. (1996). A novel factor required for the assembly of the DnaK and DnaJ chaperones of *Thermus thermophilus*. *J. Biol. Chem.* **271:** 17343–17348

225. Mudd, E.A. & Higgins, C.F. (1993). Escherichia coli endoribonuclease RNase E: autoregulation of expression and site-specific cleavage of mRNA. *Mol. Microbiol.* **9:** 557–568

226. Mujer, V.C., Fox, T.C., Williams, A.S., Andrews, D.L., Kennedy, R.A. & Rumpho, M.E. (1995). Purification, Properties and Phosphorylation of Anaerobically Induced Enolase in *Echinochloa phyllopogan* and *E. crus-pavonis. Plant Cell Physiol.* **36:** 1459-1470

227. Munro, S. & Pelham, H.R. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46:** 291-300

228. Murakami, H., Pain, D. & Blobel, G. (1988). 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.* **107:** 2051-2057

229. Musgrove, J.E. & Ellis, R.J. (1986). The rubisco large subunit binding protein. *Philos. Trans. R. Soc. London Ser. B.* **313:** 419-428

230. Nakajima, K., Hamanoue, M., Takemoto, N., Hattori, T., Kato, K. & Kohsaka, S. (1994). Plasminogen binds specifically to a alpha-enolase on rat neuronal plasma membrane. *J. Neuro-Chem.* **63:** 2048-2057

231. Nam, T.W., Cho, S.H., Shin, D., Kim, J.H., Jeong, J.Y., Lee, J.H., Roe, J.H., Peterkofsky, A., Kang, S.O., Ryu, S. & Seok, Y.J. (2001). The *Escherichia coli* glucose transporter enzyme $IICB^{Glc}$ recruits the global repressor Mlc. *EMBO J.* **20:** 194-198

232. Neidhart, F.C, & VanBogelen, R.A. (1987). In *Escherichia coli* and *Salmonella typhimurium*: *Cellular and Molecular Biology*. Neidhardt, F.C. *et al.*, (eds). Washington, D.C: American Society for Microbiology, pp, 1334-1345

233. Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. & Craig, E.A. (1992). The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. *Cell*, **71:** 97-105

234. Neuwald, A.F., Aravind, L., Spouge, J.L. & Koonin, E.V. (1999). AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9:** 27-43

235. Nogueira T, de Smit M, Graffe M, Springer M (2001). The relationship between translational control and mRNA degradation for the Escherichia coli threonyl-tRNA synthetase gene. *J. Mol. Biol.* **310:** 709-722

236. Nomura, M., Gourse, R. & Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. *Annu. Rev. Biochem.* **53:** 75-117

237. Oddis, C.V., Okano, Y., Rudert, W.A., Trucco, M., Duquesnoy, R.J. & Medsger, T.A.J. (1992). Serum autoantibody to the nucleolar antigen PM-Scl. Clinical and immunogenetic associations. *Arthriti.s Rheum.* **35:** 1211–1217

238. Osipiuk, J., Walsh, M.A., Freeman, B.C., Morimoto, R.I. and Joachimiak, A. (1999). Structure of a new crystal form of human Hsp70 ATPase domain. Acta Crystallographica *D55*, 1105-1107

239. Oussenko, I.A. & Bechhofer, D.H. (2000). The *yvaJ* gene of *Bacillus subtilis* encodes a 3' to 5'-exoribonuclease and is not essential in a strain lacking polynucleotide phosphorylase. *J. Bacteriol.* **182:** 2639-2642

240. Packschies L, Theyssen H, Buchberger A, Bukau B, Goody RS, Reinstein J. (1997). GrpE accelerates nucleotide exchange of the molecular chaperone DnaK with an associative displacement mechanism. *Biochemistry* **36:** 3417–3422

241. Pancholi, V. & Fischetti, V.A. (1998). Alpha-enolase, a novel strong plasmin(ogen) binding protein of the surface of pathogenic streptococci. *J. Biol. Chem.* **273:** 14503-14515

242. Pal-Bwomick, I., Vora, H.V. & Jarori, G.K. (2007). Sub-cellular localization of post-translational modifications of the *Plasmodium yoelii* enolase suggest moonlighting functions. *Malaria J.* **6:** 45-51

243. Palleros, D.R., Reid, K., Shi, L. & Fink, A.L. (1993). DnaK ATPase activity revisited. *FEBS Letters* **336:** 124-128

244. Pao, C.C. & Dyess, B.T. (1981). Stringent control of RNA synthesis in the absence of guanosine 59-diphosphate-39-diphosphate. J. Biol. Chem. **256:** 2252–2257

245. Pellecchia, M., Szyperski, T., Wall., D., Georgopoulos, C. & Wuthrich, K. (1996). NMR structure of the J-domain and the Gly / Phe-rich region of the *Escherichia coli* DnaJ chaperone. *J. Mol. Biol.* **260:** 236-250

246. Pepe, C.M., Maslesa-Galic, S. & Simons, R.W. (1994). Decay of the IS10 antisense RNA by 3' exoribonucleases: evidence that RNase II stabilizes RNA-OUT against PNPase attack. *Mol. Microbiol.* **13:** 1133-42

247. Pepin, C.A. & Wood, H.G. (1986). Polyphosphate glucokinase from *Propionibacterium shermanii* Kinetics and demonstration that the mechanism involves both processive and nonprocessive type reactions. *J. Biol. Chem.* **261:** 4476-4480

248. Parsell, D.A., Kowal, A.S., Singer, M.A. & Lindquist, S. (1994). Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* **372:** 475-478

249. Peterkin, P. I. & Fitt, P. S. (1971). Nucleic acid enzymology of extremely haophilic bacteria *Halobacterium cutirubrum* polynucleotide phosphorylase. *Biochem. J.* **121:** 613–620

250. Petty, K.J. (2001). Metal-chelate affinity chromatography. *Curr. Protoc. Mol. Biol.* Chapter 10, Unit 10.11B

251. Plückthun, A. (1992). Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immunol. Rev.* **130:** 151-188

252. Pool, R.K. (1993). In *Methods in molecular biology* (ed. J.M. Graham and J.A. Higgens), Vol. 19, p. 109. Humana Press, Totowa, NJ, USA.

253. Portier, C. (1975). Quaternary structure of Escherichia coli polynucleotide phosphorylase: new evidence for a trimeric structure. *FEBS Lett.* **50:** 79-81

254. Portier, C., Dondon, L., Grunberg-Manago, M. & Regnier, P. (1987). The first step in the functional inactivation of the Escherichia coli polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. *EMBO J* **6:** 2165-2170

255. Prasanth, S. G., Prasanth, K. V. & Stillman, B. (2002). Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science* **297:** 1026–1031

256. Prud'homme-Genereux, a., Beran, R.K., Iost, I., Ramey, C.S., Mackie, G.A. & Simons, R.W. (2004). Physical and functional interactions among RNase E, polynucleotide

phosphorylase and the cold-shock protein, CsdA: evidence for a "cold-shock degradosome". *Mol. Microbiol.* **54:** 1409-1021

257. Py, B., Higgens, C.F., Krisch, H.M. & Carpousis, A.J. (1996). A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* **381:** 169-172
258. Raijmakers, R., Schilders, G. & Pruijn, G.J.M. (2004). The exosome, a molecular machine for controlled RNA degradation in both nucleus and cytoplasm. *Eur. J. Cell Biol.* **83:** 175-183
259. Randall, D.D. & Blevins, D.G. (1990). *Current Topics in Palnt Biochemistry nad Physiology, Palnt Protein Phosphorylation, Protein Kinases, Calcium and Calmodulin,* Vol 9. The interdisciplinary Plant Biochemistry and Physiology Program, university of Missouri, Columbia
260. Ranjeva, R. & Boudet, A.M. (1987) Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. *Annu. Ref. Plant Physiol.* **38:** 73-93
261. Raynal, L.C. & Carpousis, A.J. (1999). Poly(A) polymerase of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. *Mol. Microbiol.* **32:** 765-775
262. Redlitz, A., Fowler, B.J., Plow., E.F. & Miles., L.A. (1995). The role of an enolase-related molecule in plasminogen binding to cells. *Eur. J. Biochem.* **227:** 407-415
263. Regonesi, M.E., Del Favero, M., Basilico, F., Briani, F., Benazzi, L., Tortora, P., Mauri, P. & Gianni, D. (2006). Analysis of the *Escherichia coli* RNA degradosome composition by a proteomic approach. *Biochimie* **88:** 151-161
264. Richarme, G. & Kohiyama, M. (1994). Amino acid specificity of the *Escherichia coli* chaperone GroEL (heat shock protein 60). *J. Biol. Chem.* **269:** 7095-7098
265. Robert-Le Meur, M. & Portier, C. (1992). *E.coli* polynucleotide phosphorylase expression is autoregulated through an RNase III-dependent mechanism. *EMBO J* **11:** 2633-2641
266. Robeva, A.S., Woodard, R., Luthin, D.R., Taylor, H.E. & Linden, (1996). Double tagging recombinant A1- and A2A-adenosine receptors with hexahistidine and the FLAG epitope. Development of an efficient generic protein purification procedure. *J. Biochem. Pharmacol.* **51:** 545-55
267. Ross, J. (1995). mRNA Stability in Mammalian Cells. *Microbiological Reviews* **59:** 423-450

268. Roy, M.K., Singh, B., Ray, B.K. & Apirion, D. (1983). Maturation of 5-S rRNA: ribonuclease E cleavages and their dependence on precursor sequences. *Eur. J. Biochem.* **131:** 119-27

269. Rüdiger, S., Schneider-Mergener, J. & Bukau, B. (2001). Its substrate speci- ficity characterises the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *EMBO J.* **20:** 1042–1050

270. Russel, R.B., Saqi, M.A.S., Sayle. R.A., Bates. P.A. & Sternberg, M.J.E. (1997). Recognition of analogous and homologous protein folds: analysis of sequence and structure conservation. *J. Mol. Biol.* **269:** 423-439

271. Sabbatini A, Dolcher MP, Marchini B, Chimenti D, Moscato S, Pratesi F, *et al.* (1997). Alpha-enolase is a renal-specific antigen associated with kidney involvement in mixed cryoglobulinemia. *Clin. Exp. Rheumatol.* **15:** 655–658

272. Scaria, V. & Jadhav, V. (2007). microRNAs in viral oncogenesis. *Retrovirilogy* **4:** 1-5

273. Schubert, M., Edge, R.E., Lario, P., Cook, M.A., Strynadka, N.C., Mackie, G.A. & McIntosh, L.P. (2004). Structural characterization of the RNase E S1 domain and identification of its oligonucleotide-binding and dimerization interfaces. *J Mol Biol* **341:** 37-54

274. Schuster, M., Wasserbauer, E., Einhauer, A., Ordner, C., Jungbauer, A., Hammerschmidt, F. & Werner, G. (2000). Protein expression strategies for identification of novel target proteins. *J. Biomol. Screen* **5:** 89–97

275. Schwartz, B.D. & Nathenson, S.G. (1971). Isolation of H-2 Alloantigens Solubilized by the Detergent NP-40. *J. Immunol*. **107:** 1363 -1367

276. Semizarov, S., Frost, L., Sarthy, A., Kroeger, P., Halbert, D.N. & Fesik, S.W. (2003). Specificity of short interfering RNA determined through gene expression signatures. *PNAS* **100:** 6347–6352

277. Shermann, M.Y. & Goldberg, A.L. (1992). Involvement of the chaperonin *dnaK* in the rapid degradation of a mutant protein in *Escherichia coli. EMBO J.* **11:** 71-77

278. Shi, Y. & Thomas, J.O. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell Biol.* **12:** 2186

279. Shoham, Y., Lamed, R. & Bayer, E.A. (1999). The cellulosome concept as an effivicient microbial strategy for the degradation of insoluble polysaccharides. *Trends in Microbiol.* **7:** 275-281

280. Schroder, H., Langer, T., Hartl, F.U. & Bukau, B. (1993). DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *EMBO J.* **12:** 4137-4144
281. Shtilerman, M., Lorimer, G.H. & Englander, S.W. (1999). Chaperonin function: folding by forced unfolding. *Science,* **284:** 822-825
282. Sigler, P.B., Zhaohui, X., Rye, H.S., Burston, S.G., Fenton, W.A. & Horwich, A.L. (1998). Structure and function in GroEL-mediated protein folding. *Annu. Rev. Biochem*. **67:** 581–608
283. Silberg, J. J., Hoff, K. G., and Vickery, L. E. (1998) The Hsc66/Hsc20 chaperone system in *Escherichia coli*. chaperone activity and interactions with the DnaK/DnaJ/GrpE system. *J. Bacteriol*. **180**: 6617-6624
284. Simpson, R.J. (2006). Precipitation of proteins by polyethylenimine. *Cold Spring Harb. Protoc.*, doi:10.1101/pdb.prot4312
285. Skowyra, D., Georgopoulos, C. & Zylicz, M. (1990). The *E. coli dnaK* gene product, the hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell* **62:** 939
286. Soreq, H. & Littauer, U.Z. (1977). Purification and characterization of polynucleotide phosphorylase from *Escherichia coli*. Probe for the analysis of 3' sequences of RNA. *J. Biol. Chem.* **252:** 6885-6888
287. Spring, T.G. & Wold, F. (1971). The purification and characterization of *Escherichia coli* enolase. *J. Biol. Chem.* **246:** 6797-6802
288. Squires, C.L., Pederson, S., Ross, B.M. & Squires, C. (1991). ClpB is the *Escherichi coli* heat shock protein F84.1. *J. Bacteriol.* **173:** 4254-4262
289. Squires, C. & Squires, C. (1992). The Clp proteins: proteolysis regulators or molecular chaperones? *J. Bacteriol.* **174:** 1081-1085
290. Subramanian, A. & Miller, D.M. (2000). Structural analysis of alpha-enolase. Mapping the functionally domains involved in down-regulation of the c-myc protooncogene. *J. Biol. Chem.* **275:** 5958-5965
291. Sugimoto, S., Higashi, C., Saruwatari, K. Nakayama, J. & Sonomoto, K. (2007). A gram-negative characteristic segment in *Escherichia coli* DnaK is essential for the ATP-dependent cooperative function with the co-chaperones DnaK and GrpE. *FEBS Letters* **581:** 2993-2999
292. Sugimoto, S., Higashi, C., Yoshida, H. & Sonomoto, K. (2008). Construction of *Escherichia. Coli dnaK*-deletion mutant infected by λDE3 for overexpression and

purification of recombinant GrpE proteins. *Protein Expression and Purification* **60:** 31-36 9603

293. Sugiyama, J., Mahmoodian, S. & Jacobson, G. (1991). Membrane topology analysis of *Escherchia coli* mannitol permease by using a nested-deletion method to create *mtlA-phoA* fusions. *PNAS* **88:**
294. Sundstrom, P. & Aliaga, G.R. (1992). Molecular cloning of cDNA and analysis of protein secondary structure of *Candida albicans* enolase, an abundant, immunodominant glycolytic enzyme. *J. Bacteriol.* **174:** 6789-6799
295. Supplier data (Triton X-100 is a product of Union Carbide).
296. Szabo, A., Korszun, R., Hartl, F.U. & Flanagan, J. (1996). A zinc finger-like domain of the chaperone DnaJ is involved in binding to denatured protein substrates. *EMBO J.* **15:** 408-417
297. Szabo, A., Langer, T., Schroder, H., Flanagan, J., Bukau, B. & Hartl, F.U. (1994). The atp hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system DnaK, DnaJ, and GrpE. *Proc. Natl. Acad. Sci.* **91:** 10345-10349
298. Szymona, M., Kowalska, H. & Pastuszak, I. (1977). Polyphosphate-glucose phosphotransferase. Purification of *Mycobacterium tuberculosis* H37Ra enzyme to apparent homogeneity. *Acta Biochem. Pol.* **24:** 133-142
299. Szymona, M. & Widomski, J. (1974). A kinetic study on inorgance polyphospahet glucokinase from *Mycobacterium tuberculosis* H37RA. *Physiol. Chem. Phys.* **6:** 393-403
300. Takata R, Izuhara M, Hori K (1989). Differential degradation of the Escherichia coli polynucleotide phosphorylase mRNA. *Nucleic Acids Res* **17:** 7441-7451
301. Takata, R., Mukai, T. & Hori, K. (1987). RNA processing by RNase III is involved in the synthesis of Escherichia coli polynucleotide phosphorylase. *Mol. Gen. Genet.* **209:** 28-32
302. Terrier, B., Degand, N., Guilpain, P. Servettaz, A., Guillevin, L. & Mouthon, L. (2007). Alpha-enolase: a target of antibodies in enfectious and autoimmune diseases. *Autoimmunity Reviews* **6:** 176-182
303. Theyssen, H., Schuster, H.P., Packschies, L., Bukau, B. & Reinstein, J. (1996). The second step of ATP binding to DnaK induces peptide release. *Journal of Molecular Biology* **263:** 657-670
304. Tilly, K., Hauser, R., Campbell, J. & Ostheimer, G.J. (1993). Isolation of *dnaJ*, *dnaK* and *grpE* homologues from *Borrelia burgdorferi* and complementation of *Escherichia coli* mutants. *Mol. Microbiol*. **7:** 359–369

305. Tzeng, C.M. & Kornberg, A. (2000). The multiple activities of polyphosphate kinase of *Escherichia coli* and their subunit structure determined by radiation target analysis. *J. Biol. Chem.* **275:** 3977-3988
306. Ungewickell, E. (1985). The 70-kd mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. *EMBO J.* **4:** 3385-3391
307. Valle, B.L. (1955). Zinc and metalloenzymes. *Adv. Prot. Chem.* **10:** 317-384
308. Van Hoof, A. & Parker, R. (1999). The exosome: a proteasome for RNA? *Cell* **99:** 347-350
309. Vanzo, N.F., Li, Y.S., Py, B., Blum, E., Higgens, C.F., Raynal, L.C., Kirsch, H.M. & Carpousis, A.J. (1998). Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. *Genes Dev.* **12:** 2770-2781
310. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S. & Wigley, D. B. (1999). Crystal structures of omplexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism. *Cell* **97:** 75–84
311. Vorderwulbeckea, S., Kramerb, G., Merzc, F., Kurzc, T.A., Rauchc, T., Zachmann-Brandc, B., Bukauc, B. & Deuerlingc, E. (2004). Low temperature or GroEL/ES overproduction permits growth of *Escherichia coli* cells lacking trigger factor and DnaK. *FEBS Letters* **559:** 181-187
312. Wagner E.G., Altuvia S. & Romby P. (2002). Antisense RNAs in bacteria and their genetic elements. *Adv Genet.* **46:** 361–98
313. Wall, D., Zylicz, M. & Georgopoulos, C. (1995). The conserved G/F/ motif of the DnaJ chaperone is necessary for the activation of the substrate binding properties of the DnaK chaperone. *J. Biol. Chem.* **270:** 2139-2144
314. Wang, J. & Chen, L. (2003). Domain motions in GroEL upon binding of an oligopeptide. *J. Mol. Biol.* **334:** 489-499
315. Wang, J., Hartling, J.A. & Flanagan, J.M. (1997). The structure of ClpP at 2.3 Ȧ resolution suggests a model for ATP-dependent proteolysis. *Cell* **91:** 447-456
316. Waugh, D.S. (2005). Making the most of affinity tags. *TRENDS in Biotechnol.* **23:** 316-320
317. Wen, T., Oussenko, I.A., Pellegrini, O., Bechhofer, D.H. & Condon, C. (2005). Ribonuclease PH plays a major role in the exonucleolytic maturation of CCA-containing tRNA precursors in *Bacillus subtilis*. *Nucleic Acids Res.* **33:** 3636-3643

318. Wistow, G.J., Lietman, T., Williams, L.A., Stapel, S.O., de Jong, W.W., Horwitz, J., *et al.*, (1988). Taucrystallin/alpha-enolase: one gene encodes both an enzyme and a lens structural protein. *J. Cell Biol.* **107:** 2729-2736
319. Wold, F. (1971). Enolase. In: The enzymes, pp. 499-538, Boyer, P.D. (ed.), *Academic Press, New York*
320. Wood, J. N. & Hutchinson, D. W. (1976). Thermostable polynucleotide phosphorylases from *Bacillus stearothermophilus* and *Thermus aquaticus. Nucleic Acids Res.* **3:** 219–229
321. Xu, F., Lin-Chao, S. & Cohen, S.N. (1993). The *Escherichia coli* pcnB gene promotes adenylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *Proc. Natl. Acad. Sci. USA* **90**: 6756-6760
322. Xu, Z. & Sigler, P.B. (1998). GroEL/GroES: structure and function of a two-stroke folding machine. *J. Struct. Biol.* **124:** 129-141
323. Yamanaka, K. & Inouye, M. (2001). Selective mRNA degradation by polynucleotide phosphorylase in cold shock adaptation in *Escherichia coli. J. Bacteriol.* **183:** 2808-2816
324. Yehudai-Resheff, S., Hirsh, M. & Schuster, G. (2001). Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol. Cell. Biol.* **24:** 5408-5416
325. Yeom, J.H. & Kangseok, L. (2006). RraA rescues *Escherichia coli* cells over-producing RNase E from growth arrest by modulating the ribonucleolytic activity. *Biochem. Biophys. Res. Comm.* **345:** 1372-1376
326. Yoshimune, K., Yoshimura, T., Nakayama, T., Nishino, T. & Esaki, N. (2002). Hsc62, Hsc56, and GrpE, the third Hsp70 chaperone system of *Escherichia coli. Biochem. Biophys. Res. Commun.* **293:** 1389-1395
327. Young, J.C., Barral, J.M. & Hartl, F.U. (2003). More than folding: localized functions of cytosolic chaperones. *Trends Biochem. Sci.* **28:** 541–547
328. Zangrossi S, Briani F, Ghisotti D, Regonesi ME, Tortora P, Deho G (2000). Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in Escherichia coli. Mol Microbiol **36:** 1470-1480
329. Zeng, L., Cash, V.L., Flint, D.H. & Dean, D.R. (1998). Assembly of iron sulphur clusters. *J. Biol. Chem.* **273:** 13264-13272
330. Zhang, S., Jin, C.-D. & Roux, S.J. (1993). Casein Kinase II-Type Protein Kinase from Pea Cytoplasmand Its lnactivation by Alkaline Phosphatase in Vitro. *Plant Physiol*. **103:** 955-962

331. Zhu, X., Zhao, X., Burkholder, W., Gragerov, A., Ogata, C.M., Gottesman, M.E., *et al.* (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272:** 1606-1614

332. Zilhao, R., Cairrao, F., Regnier, P. & Arraiano, C.M. (1996). PNPase modulates RNase II expression in Escherichia coli: implications for mRNA decay and cell metabolism. *Mol Microbiol* **20:** 1033-42

333. Zou, X., Ray, D., Aziyu, A., Christov, K., Boiko, A. D., Gudkov, A. V. & Kiyokawa, H. (2002). Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence, *Genes Dev.* **15:** 2923–2934

334. Zuber, M., Hoover, T.A., Dertzbaugh, M.T. & Court, D.L. (1995). Analysis of the DnaK molecular chaperone system of *Francisella tularensis*. *Gene* **164:** 149–152