Science can purify religion from error and superstition. Religion can purify science from idolatry and false absolutes.

- Blessed Pope John Paul II

University of Alberta

Molecular Studies on the RelA-Mediated (p)ppGpp Synthesis Mechanism

by

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Abstract

In *Escherichia coli*, the enzyme RelA catalyses the synthesis of (p)ppGpp in response to amino acid starvation. RelA activation requires the codon specific binding of a deacylated tRNA to the ribosomal A-site. By a poorly understood regulatory mechanism, RelA alternates between an inactive ribosome-bound and an active ribosome-free state. RelA activation on the ribosome terminates with the dissociation of the deacylated tRNA from the A-site. Inactivation of RelA off the ribosome is presumed to be via a conformational change in the C-terminal region of RelA

We use an *in vitro* assay in combination with standard molecular mutagenesis techniques to gain further insight into two aspects of the RelA mediated (p)ppGpp synthesis mechanism: the first is the influence tRNA species has on the duration of (p)ppGpp synthesis. The second is the involvement of the ACT domain, a common regulatory domain of metabolic proteins, in the interaction with the ribosome and its function as a potential allosteric regulatory site in RelA.

A tRNA can function as both a monitor of nutrient status in the cell and a co-activator of the enzyme RelA. *E. coli* contains 47 different tRNAs each with its own unique feature, one of which is the differences in A-site dissociation rates. We are the first to report that this idiosyncratic feature of the tRNA does indeed have an effect on the duration of (p)ppGpp synthesis *in vitro*.

The C-terminal region of RelA is comprised of two domains with undefined function in RelA. In our characterization of these domains, we not only identified a function to the ACT domain in RelA but also a novel mechanism of regulation where amino acid methionine is an allosteric inhibitor of RelA activity.

Our molecular studies into the RelA mediated (p)ppGpp synthesis addresses two aspect of this mechanism that until now has been left unexplored, and thus has significantly contributed to our knowledge of this potent survival response in bacteria.

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70S	Tight-couple 50S subunit and 30S subunit
ACT	Domain whose name is derived from asparata kinase, chorismate mutase and TyrA
aminoacyl-tRNA	aminoacylated tRNA
A-site	Aminoacyl-tRNA acceptor site of 70S ribosomse
ATP	Adenosine triphosphate
βME	β-mercaptoethanol
B. subtilis	*Bacillus subtilis*
charged tRNA	Aminoacylated tRNA
CTD	C-terminal domain
deacyl-tRNA	Deacylated tRNA
DksA	transcription regulator
ΔL11-RAC	RACs assembled with mutant ribosomes that have no L11 protein
depL7-RAC	RACs assembled with mutant ribosomes that have been depleted of ribosomal protein L7/L12
E. coli	*Escherichia coli*
EF-G	Elongation factor G
EF-Tu	Elongation Factor Tu
EDTA	Ethylenediaminetetraacetic acid
GDP	Guanosine diphosphate
GST	Glutatione S-transferase
GTP	Guanosine triphoshphate
HD	Domain rich in histidine and aspartates
HCl	Hydrochloric acid
HEPES	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr(s)	Hour(s)
IF2	Initiation factor 2

IPTG	Isopropyl β-D-thiogalactopyranoside
KOH	Potassium hydroxide
L11	Ribosomal protein L11
L7/L12	Ribosomal protein L7/L12
LB	Luria Bertani or Luria Broth
LdcI	Inducible lysine decarboxylase
LC-MS	Liquid chromatography-mass spectrometry
L. pneumophila	*Legionella pneumophila*
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
min(s)	minute(s)
mRNA	Messenger RNA
NaOH	Sodium hydroxide
N693A	Asparagine residue in position 693 is mutated to alanine
NT	Nucleotidyl-transferase
NTD	N-terminal domain
OD	Optical Density
PA buffer	Polyamine buffer
PAGE	Polyacrylamine gel electrophroesis
PCR	Polymerase chain reaction
PDB	Protein Data Bank
Pi	Inorganic phosphate
ppGpp	Tetrephosphate guanosine or 5'-3' bis- diphosphate guanosine
pppGpp	Pentaphosphate guanosine or 5' triphosphate and 3' diphosphate guanosine
PPX	Exopolyphosphate
PNPase	Polynucleotide Phosphorylase
RAC	RelA Activating Complex

RelA	*E. coli* (p)ppGpp synthetase that catalysis ppGpp during amino acid starvation
SDS	Sodium dodecyl sulphate
secs	Seconds
S. aureus	*Staphylococcus aureus*
Uncharged tRNA	Deacylated tRNA
TGS	Domain whose name is derived from Threonine tRNA synthetase, GTPases, and SpoT
TLC	Thin layer chromotography
TRIS	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA

Chapter 1

Introduction.

1.1 Stress Responses in Bacteria

In the natural environment, depletion of nutrients or changes in abiotic conditions could be detrimental if the bacterial cell is unable to efficiently adapt. Depending on the type of stress encountered, the cell responds by altering its metabolic processes. All known stress responses of bacteria can be divided into two broad categories: the specific stress response and the general stress response **(Figure 1.1)** (Storz & Hengge-Aronis 2000). The specific stress response is associated with one particular type of insult to cell homeostasis, such as heat-shock, envelope response, or oxidative stress response. The general stress response is triggered under conditions of nutritional depletion and leads to global changes to metabolism and gene expression. Activation of the general stress response is controlled by either one or a few key regulators such as cyclic-AMP and polyphosphate guanosine (Ullman & Danchin 1980; Botsford & Harman 1992; Buettner et al. 1973; Hengge-Aronis 2002; Storz & Hengge-Aronis 2000; Cashel 1975; Gallant 1979). Polyphosphate guanosine is also known as the activator of the stringent response which in turn can lead to the activation of the general stress response (Loewen et al. 1998).

The stringent response is thought to be initiated as an early warning system, just before or at the start of starvation (Szalewska-Pałasz & Potrykus 2011; Nyström 2004). As a result, the cell either accommodates the shortage of nutrients (via the expression biosynthetic pathways) or prepares for long term survival (Dalebroux & Swanson 2012; Potrykus & Cashel 2008; Gallant 1979; Wu & Xie 2009; Szalewska-Pałasz & Potrykus 2011). Although initially identified in *Escherichia coli (E. coli)* (Stent & Brenner 1961; Cashel 1969) the physiological significance of the stringent response has been explored in many other types of bacteria. Particular interest has been given to the role in the virulence and long term persistence of pathogenic bacteria (Taylor et al. 2002; Godfrey et al. 2002; Nascimento et al. 2008; Dalebroux et al. 2010; Haralalka et al. 2003; Gaynor et

al. 2005; Vogt et al. 2011; Kazmierczak et al. 2009; Klinkenberg et al. 2010). The activation of the stringent response is known to be necessary for persistence in *Mycobacterium tuberculosis* (Primm et al. 2000; Klinkenberg et al. 2010), for *Legionella pneumophila* transmission in macrophages (Dalebroux et al. 2009), for *Yersinia pestis* lung dissemination (W. Sun et al. 2009), for adhesion in *Listeria monocytogene,* (Taylor et al. 2002), and for intracellular invasion in *Salmonella typhimurium* (Ramachandran et al. 2012).

1.2 Why call it "The Stringent Response"?

The term "stringent" was used to describe the correlation between the strict regulation of stable RNA species, which are the transfer RNAs (tRNA) and ribosomal RNAs (rRNA) (Neidhardt 1964; Deutscher 2006; Deutscher 2003), during amino acid starvation (Stent & Brenner 1961; Cashel 1969; Kaczanowska & Rydén-Aulin 2007). Wildtype *E. coli* cells exhibit a rapid decrease in rRNA synthesis as soon as they were deprived of amino acids (Metzger, Schreiber, et al. 1989; Cashel 1969; Stent & Brenner 1961; Borek & Ryan 1958; Borek et al. 1956). If the cell continued to synthesise RNA, even after amino acid deprivation, the phenotype would be defined as being relaxed (Borek et al. 1956; Borek & Ryan 1958; Cashel et al. 1996; Stent & Brenner 1961). From then on, whenever mutant genes that lead to the relaxed phenotype were identified, they were termed *rel* gene, for example*: relA* gene – the gene for the protein RelA (Cashel et al. 1996; Stent & Brenner 1961; Metzger, Schreiber, et al. 1989; Metzger et al. 1988; Fiil & Friesen 1968; Parker et al. 1976). RelA is the enzymes involved in the catalyses of the polyphosphate guanosine signalling molecule to during amino acid starvation.

Classically, the stringent response was thought to be a specific stress response to amino acid starvation. However, subsequent studies have shown that the stringent response is activated during other nutritional stresses,

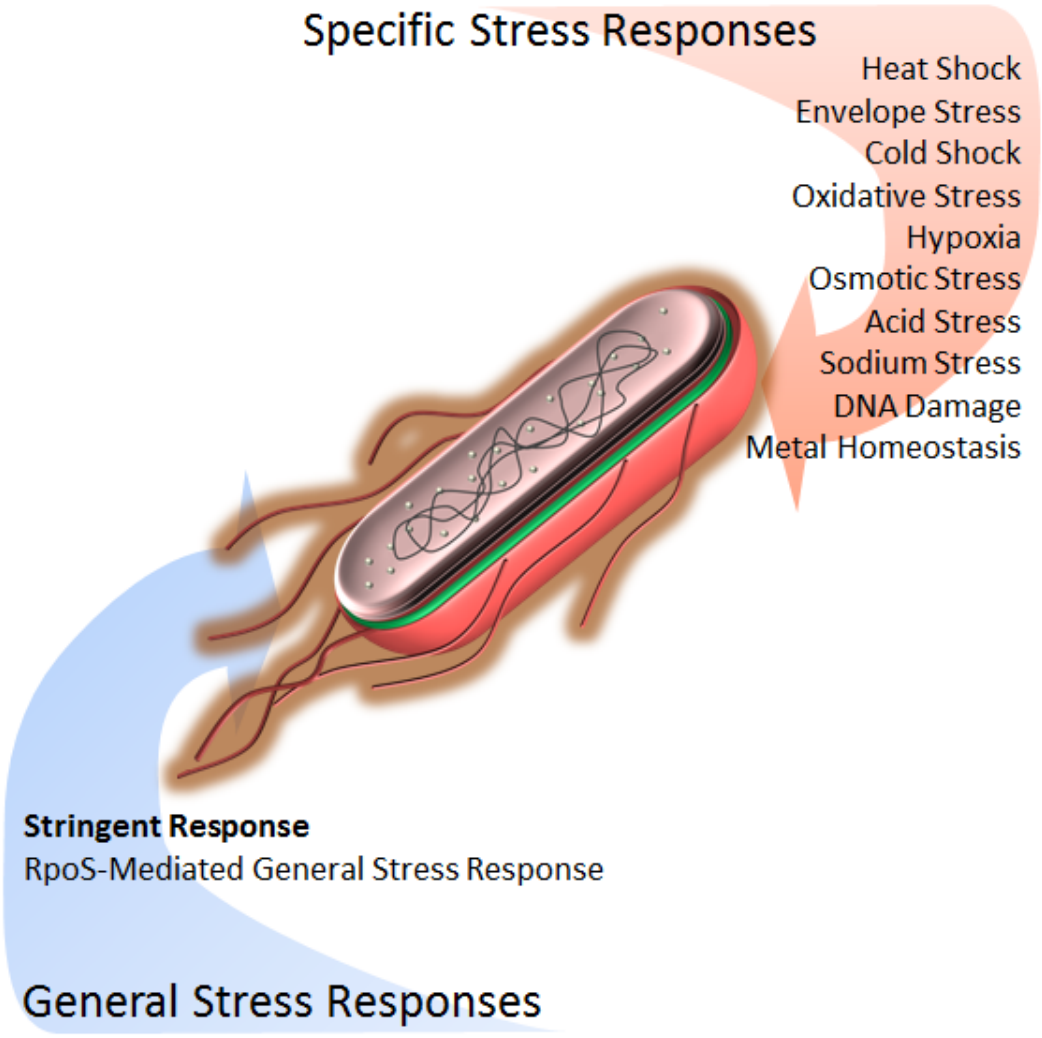


Figure 1.1: Overview of two categories of stress responses in bacteria. Specific stress responses describes those responses that are activated during a one type of stress. General stress response are global responses to nutrient depletion.

such as iron limitation (Vinella et al. 2005), nitrogen (Irr 1972), phosphate (Bougdour & Gottesman 2007) and fatty acid starvation (Battesti & Bouveret 2009).

1.3 Key Regulators of the Stringent Response

The main feature of the stringent response is the accumulation of two alarmones which are unusual derivatives of the high energy molecules GTP and GDP (Cashel 1975; Cashel & Gallant 1969). When acid-soluble extracts of amino acid starved wildtype and *relA* mutant cells were resolved by thin layer chromatography, these unusual nucleotides appeared as spots (Cashel & Gallant 1969). These spots did not appear for the mutants so they were wittily termed the magic spots (Haseltine et al. 1972). Through structural studies, these signalling nucleotides were identified as analogues of GTP and GDP: 5'-triphosphate-3'-diphosphate guanosine (pppGpp) and 5’-3'-dibisphosphate guanosine (ppGpp) (Cashel & Kalbacher 1970), collectively called (p)ppGpp. Although classically defined as a regulator of ribosome biogenesis (Cashel 1969; Cashel & Gallant 1969; Bremer & Ehrenberg 1995), extensive studies done on different bacterial species have shown that (p)ppGpp affects transcription of many other genes (Magnusson et al. 2005; Traxler et al. 2008; Durfee et al. 2008; Kanjee et al. 2012) as well as a number of different cellular processes (V. Hernandez & Bremer 1993; Schreiber et al. 1995; Wang et al. 2007; Ferullo & Lovett 2008; Srivatsan & Wang 2008; Pingoud & W. Block 1981; Mitkevich et al. 2010; Dalebroux et al. 2010; Jain et al. 2006; Chatterji 2001; Kanjee et al. 2012). **Figure 1.2** summarises the cellular processes that are affected by (p)ppGpp.

Downregulated	Upregulated
Cell division	Amino acid biosynthesis
Cell motility	σ^S synthesis
DNA Replication	Universal stress protein synthesis
rRNA and tRNA synthesis	Carbohydrate metabolism
Ribosome biogenesis	Virulence gene expression
Protein Synthesis	Toxin/antitoxin systems
Translation initiation and elongation	Antibiotic resistance
Metabolite transport	Cyclopropane fatty acid synthesis
Phospholipid synthesis	Chaperones and proteolysis systems
Nucleotide biosynthesis	Long-term persistence and virulence
Oxidative metabolism	

Figure 1.2: Cellular processes that are affected by (p)ppGpp.

1.4 *In vivo* (p)ppGpp Accumulation

GTP pyrophosphokinases (*e.g.* RelA) are a family of enzymes that catalyse ppGpp formation at the onset of nutrient starvation. Within 10 minutes of activation, (p)ppGpp accumulates to a level higher than basal (~ 70 – 100% higher) – termed the apex level (Boer et al. 1976; Cashel 1969). The level then drops to a steady state that is about 10 to 20 fold higher than the basal – termed the adaptive level (Cashel 1969; Cashel & Kalbacher 1970; Cashel & Gallant 1969; Boer et al. 1976). **Figure 1.3** illustrates the accumulation of (p)ppGpp within a cell that is undergoing a shift from nutrient rich to nutrient poor. As a paradigm, the high level of (p)ppGpp is compared to the function of an emergency brake, where the high levels of (p)ppGpp rapidly halt growth related processes while concurrently activating and up-regulating any survival related processes, such as protein degradation and amino acid biosynthesis (Cashel 1969; Cashel & Kalbacher 1970; Kuroda 2006; Battesti & Bouveret 2006). This pattern of (p)ppGpp accumulation is commonly observed when monitoring (p)ppGpp synthesis *in vivo* (Cashel 1969; Boer et al. 1976; Lazzarini et al. 1971). The changes to the level of (p)ppGpp are thought to be due to changes in both the rate of synthesis and the rate of breakdown of (p)ppGpp during different stages of accumulation (*i.e.* from basal to apex or apex to adaptive) (Boer et al. 1976). A thorough investigation to purpose of these rate changes is still pending. Fiils et al. estimates that almost half of the GTP molecules are lost to pppGpp synthesis. This extensive depletion of GTP and ATP energy sources could make recovery from starvation more difficult (Fiil et al. 1972). Maintaining a high level of (p)ppGpp would therefore be less conducive to cell survival. One could speculate that these rate changes serve to conserve the levels of the two high energy molecules, adenosine triphosphate (ATP) and guanosine triphosphate (GTP), that are used in the synthesis of (p)ppGpp (**Figure 1.4**) (Cashel & Kalbacher 1970; Gallant et al. 1972)..

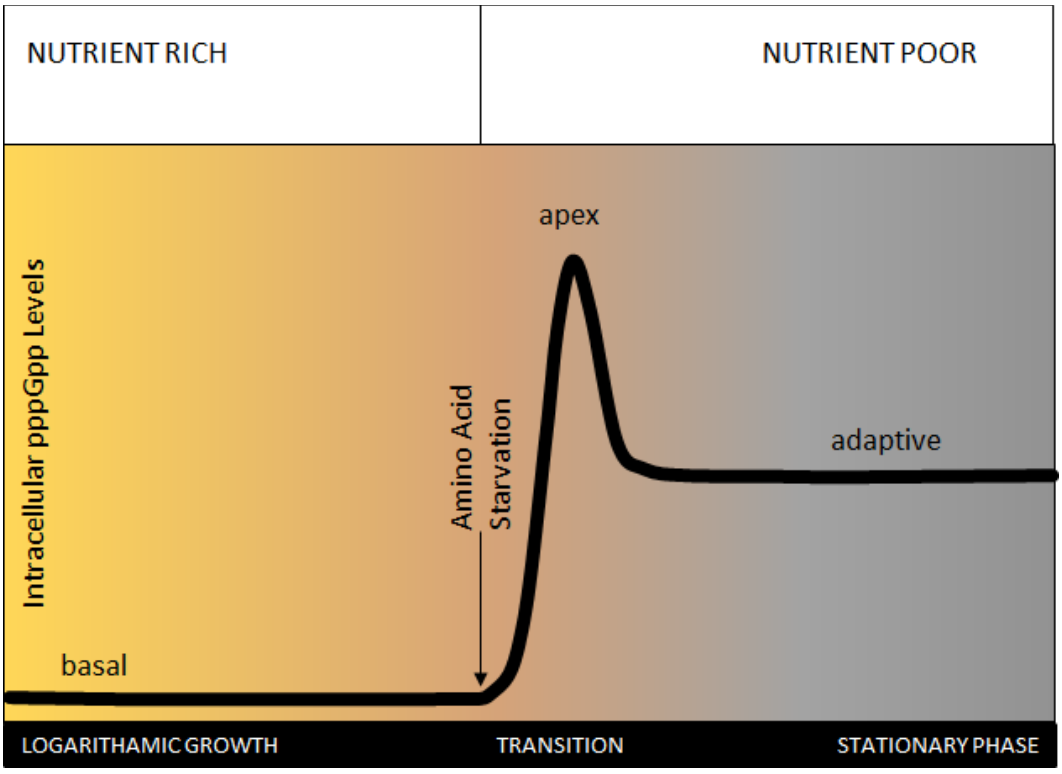


Figure 1.3: Accumulation of (p)ppGpp during amino acid starvation. Under nutrient rich conditions, the level of (p)ppGpp is maintained at a basal level. At the onset of amino acid starvation, the level of (p)ppGpp increases rapidly and within 10 minutes reaches a peak called the apex. (p)ppGpp level drops to an adaptive level during stationary phase, which is higher than basal level.

The signalling alarmone is first synthesised in the pentaphosphate form (pppGpp), which is then hydrolysed by a guanosine pentaphosphate phosphatase to yield the tetraphosphate form (ppGpp) (Kari et al. 1977). RelA, the GTP pyrophosphokinase in *E. coli*, transfers a pyrophosphate from ATP to the 3'–hydroxyl of a GTP and releases an AMP (adenosine monophosphate) and pppGpp (Cashel 1969). While the role of AMP as a signalling molecule in bacteria remains to be investigated, the effects of (p)ppGpp has been extensively studied for a number of different cellular processes.

Most of the metabolic changes that happened during the stringent response is mediated by ppGpp (Magnusson et al. 2005). As pppGpp has a transient appearance within the cell, its role in modulating cellular processes is unknown. Interestingly, it has been shown that pppGpp can substitute GTP in GTP dependent IF-2, EF-Tu, and EF-G in *in vitro* reactions (Mitkevich et al. 2010; Boer et al. 1976), suggesting the possibility that pppGpp can have an effect on GTPases within the cell.

The stringent response has been known for more than 50 years (Stent & Brenner 1961), and was thought to be present only in bacteria. In the past few years, enzymes involved in (p)ppGpp synthesis and hydrolysis have been identified and characterised in plants (*Arabidopsis*) (Biezen et al. 2000; Mizusawa et al. 2008; Braeken et al. 2006). Recently, an ortholog for ppGpp hydrolase SpoT has been identified in animals (*Drosophila*) (D. Sun et al. 2010). MESH1 or metazoan SpoT homolog, is a pppGpp hydrolase that is involved in nutrient dependent signalling in *Drosophila*.

1.5 (p)ppGpp the Global Regulator of Cellular Processes

In almost all instances of adaptation to stress there is an incentive to change the gene expression pattern. The decrease in rRNA (ribosomal RNA) transcription is the token hallmark feature for the stringent response. Through

the extensive studies that have been done to determine how ppGpp regulates gene expression (Magnusson et al. 2005; Chatterji et al. 1998; Srivatsan & Wang 2008; Artsimovitch et al. 2004), we know that (p)ppGpp regulates transcription via a direct and indirect mechanism (Barker et al. 2001; Lemke et al. 2011; Lange et al. 1995; J. Roberts 2009; Jishage et al. 2002; Condon et al. 1995; Chatterji et al. 1998; Magnusson et al. 2005).

1.5.1 (p)ppGpp Effects on Transcription

The major effect of (p)ppGpp is exerted at the point of transcription. In a study using a whole transcriptome shotgun sequencing technique called differential RNA sequencing, transcriptomic analysis of starved wildtype and mutant *Salmonella typhimurium* cells were compared to identified the number of genes that are either repressed or expressed in the presence of (p)ppGpp. ppGpp causes the redirection of transcription to genes found downstream of weak promoters (Ramachandran et al. 2012). These genes are critical for survival and are involved in amino acid biosynthesis (Paul et al. 2005), sporulation (Lopez et al. 1981; Ochi et al. 1982), virulence (Gaynor et al. 2005; Dalebroux et al. 2010), and long-term persistence (Primm et al. 2000). The genes that were repressed include those involved in fatty acid and lipid metabolism, peptidoglycan metabolism, translation related genes, pyrimidine and purine metabolism, and DNA/RNA interactions, and replication and metabolism (Ramachandran et al. 2012).

Control of transcription by ppGpp requires an interaction with the protein DksA, an RNAP binding transcriptional regulator (Paul et al. 2005; Brown et al. 2002). DksA and ppGpp can directly interact with RNAP β and β' subunits (*30*, *31*) and weaken the polymerase's affinity for proliferative promoters such as those found near rRNA and tRNA (transfer RNA) genes (Lemke et al. 2011; Magnusson et al. 2005; Bremer & Ehrenberg 1995). In addition, the DksA and ppGpp complex can indirectly alter gene expression by modulating expression

via sigma factor competition (Paul et al. 2005; Magnusson et al. 2005). High concentration of ppGpp inhibits RNAP binding to the housekeeping sigma factor σ^{70}, which is required to recognise promoters for rRNA and tRNA genes. RNAP interaction with other stress related sigma factors such as RpoS (general stress response sigma factor) is not affected in the presence of ppGpp (Battesti et al. 2011; Lange et al. 1995; Loewen et al. 1998; Merrikh et al. 2009; Brown et al. 2002; Jishage et al. 2002). The transcription bias of RNAP for the weaker promoters mediated by DskA and ppGpp ensures that expressions of survival related genes only takes places during nutrient poor conditions (Ramachandran et al. 2012).

Although the predominant effect of (p)ppGpp is at the level of transcription, (p)ppGpp functions both as a negative and positive regulatory on a number of different cellular processes (Magnusson et al. 2005; Szalewska-Pałasz & Potrykus 2011). With recent transcriptomic and proteomic studies, other proteins besides RNA polymerase have been identified as binding targets of (p)ppGpp (Traxler et al. 2008; Durfee et al. 2008; Magnusson et al. 2005; Kanjee et al. 2012).

1.5.2 (p)ppGpp Effects on Protein Synthesis and Degradation

(p)ppGpp exerts its effects on translation by directly interacting with translation factors (Boer et al. 1976; Hamel & Cashel 1973; Mitkevich et al. 2010). During stress condition, when ppGpp levels are equimolar to that of GTP, ppGpp interacts with IF2 in a similar way to GTP (Milon et al. 2006) and interferes with initiation complex formation. Translation elongation is also inhibited by (p)ppGpp binding to elongation factors EF-Tu and EF-G (Pingoud & W. Block 1981). The translation factors are suggested to be sensitive to the ratio of ppGpp:GTP rather than to the presence of (p)ppGpp alone. Activity of the translation factors are inhibited at high (p)ppGpp concentrations, while when ppGpp concentration is equal to GTP concentration, the translational factors are

able to regain partial activity (LaRiviere et al. 2001; Mitkevich et al. 2010; Rojas et al. 1984; Potrykus & Cashel 2008; Chaloner-Larsson & Yamazaki 1978). In addition, ppGpp mediates the formation of inactive ribosome (100S) dimers by inducing the expression of ribosome modulating factor (RMF) (Nomura et al. 1986). Overall, the down-regulation of protein synthesis during stress conditions serves to reduce translational errors and prevent the rapid depletion of energy sources and precursor molecules (Svitil et al. 1993).

pppGpp accumulation leads to degradation of stable RNAs and the respective ribosomal proteins (Sussman & Gilvarg 1969; Mandelstam 1958). High (p)ppGpp levels trigger the formation and accumulation of a polyphosphate (polyP) linear polymer, which interacts with the ATP-dependent Lon protease to mediate degradation of proteins (Kuroda 2006). PolyP is maintained at low levels during the exponential growth phase by the phosphatase activity of exopolyphosphatase (PPX). As high concentrations of pppGpp inhibit PPX activity, PolyP kinase catalyses the phosphotransfer of P_i from ATP to the growing chain of polyP.

1.5.3 (p)ppGpp Effects on DNA Replication

DNA replication and cell division are often attributed to nutrient rich environments. When nutrients are scarce these processes are shut down (Schreiber et al. 1995). Inhibition of replication initiation, reduction in cell size, reduction in DNA replication, and partial inhibition in septum formation are known to occur in *E. coli* undergoing the stringent response (Schreiber et al. 1995; Levine et al. 1995). Ferullo et al. report that RelA mediated ppGpp synthesis in concert with SeqA (a negative modulator of initiation of replication) and Dam methylase (a methylase of newly synthesised DNA) are necessary to bring about these reported changes to DNA replication and cell division during the stringent response (Ferullo & Lovett 2008). As neither SeqA nor Dam

methylase bind GTP or (p)ppGpp it is unclear how ppGpp, SeqA and Dam methylase cooperate to mediate the effects on DNA replication and cycle arrest.

1.6 *E. coli* (p)ppGpp Synthetase and Hydrolase

The level of (p)ppGpp is maintained by a family of proteins called the RSHs (RelA/SpoT homologs) (Atkinson & Hauryliuk 2012; Atkinson et al. 2011). This family is named after *E. coli* RelA and SpoT, the first two proteins to be identified and characterised (Metzger et al. 1988; Sarubbi et al. 1989). A phylogenetic and sequence search over 1000 genomes has identified a diverse distribution of these enzymes across prokaryotic and eukaryotic domains of life (D. Sun et al. 2010; Atkinson & Hauryliuk 2012; Mizusawa et al. 2008; Braeken et al. 2006; Atkinson et al. 2011).

This family of pppGpp synthases and hydrolases can be divided into two classes: mono-functional synthetases and bi-functional synthetases/hydrolases. In *E. coli,* the level of pppGpp is controlled by the mono-functional RelA and bi-functional SpoT (Atkinson et al. 2011; Metzger et al. 1988; Sarubbi et al. 1989). SpoT activity is required for maintaining both the basal level and adaptive levels of (p)ppGpp (Sarubbi et al. 1989; Xiao et al. 1991; V. Hernandez & Bremer 1991), while RelA activity is required to obtain the apex level.

While RelA mediated-(p)ppGpp synthesis is a ribosome dependent mechanism, SpoT mediated-(p)ppGpp synthesis is a ribosomes independent mechanism (Hara & Sy 1983). Although the catalytic mechanism of pppGpp synthesis has yet to be characterised, it is thought that both enzymes catalyse the synthesis of pppGpp by transferring a pyrophosphate from the donor ATP to an acceptor GTP (Hogg et al. 2004). pppGpp is hydrolysed by pppGpp phosphohydrolase to give ppGpp, which is the key effector of the stringent response (Hara & Sy 1983). Eventually, pppGpp is hydrolysed by SpoT to release GDP and inorganic pyrophosphate (Sarubbi et al. 1989). While RelA is activated

only during amino acid starvation conditions, SpoT is activated during other types of nutritional stresses (Potrykus & Cashel 2008). **Figure 1.4** briefly illustrates the cycle of (p)ppGpp within the cell.

Albeit having different activities, structural studies show that both RelA and SpoT have similar organization in their domains (Metzger, Sarubbi, et al. 1989). The activity of both proteins is localised to the N-terminal region while regulation is attributed to the C-terminal domain.

1.6.1 Domain Organisation in RelA and SpoT

RelA and SpoT contain similar domain architecture (Metzger, Sarubbi, et al. 1989) despite having low sequence homology (~36%) (Metzger, Sarubbi, et al. 1989).

RelA is thought to be a result of gene duplication of an ancestral *spoT* gene (Metzger, Sarubbi, et al. 1989). **Figure 1.5** shows the domain configuration for both RelA and SpoT as predicted using the Conserved Domain Database (Marchler-Bauer et al. 2009). **Figure 1.6** shows the nucleotide and amino acid sequences of RelA.

The two functionally distinct regions of RelA and SpoT were identified by progressively deleting regions in *relA* and *spoT* genes (Metzger et al. 1988; Sarubbi et al. 1989; Gropp et al. 2001; X. Yang & Ishiguro 2001). The N-terminal region, often associated with catalytic function, contains two domains the Histidine-Aspartate (HD) and Nucleotidyl-transferase (NT) domains. The C-terminal region, associated with the regulatory function, contains the TGS (acronym for threonyl-tRNA synthetase (ThrRS), GTPase, and guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase (SpoT)) and the ACT domain (named after the three proteins: aspartate kinase, chorismate mutase and TyrA) (Wolf et al. 1999; Potrykus & Cashel 2008; Chipman & Shaanan 2001).

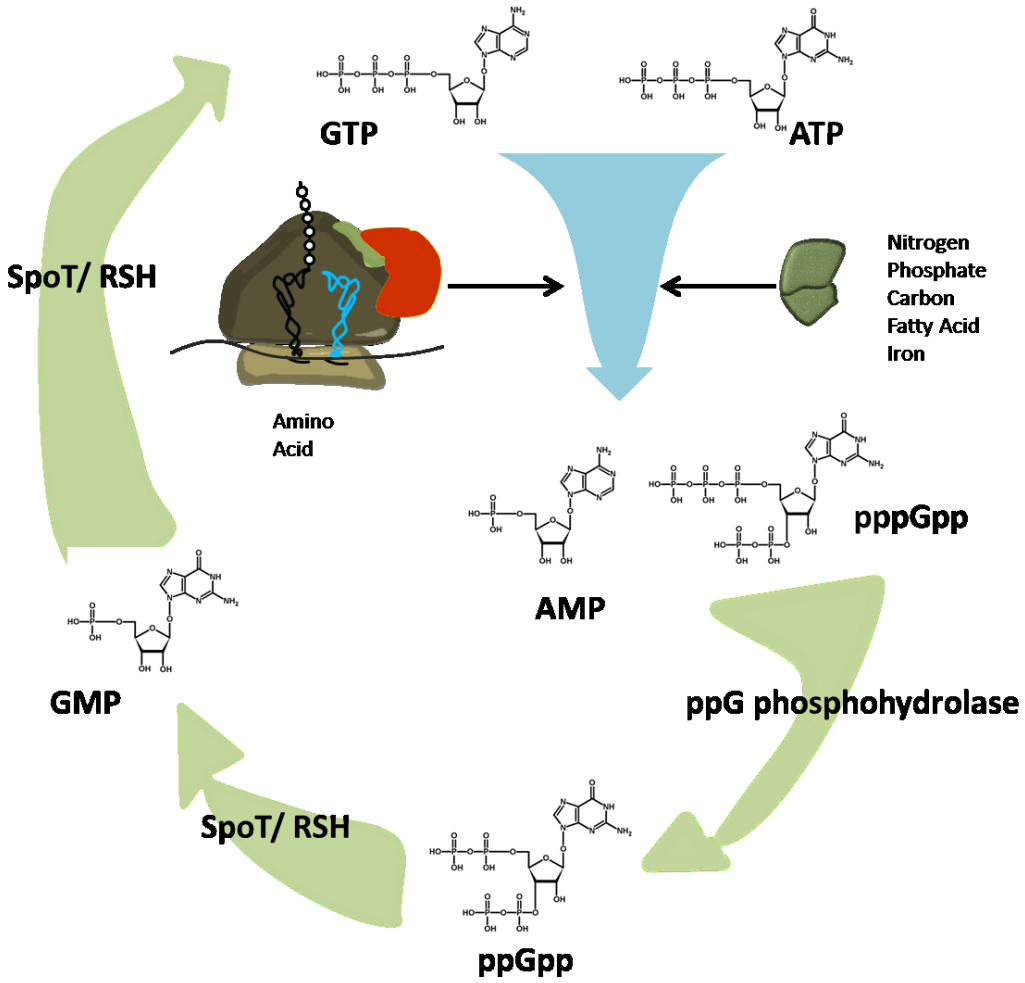


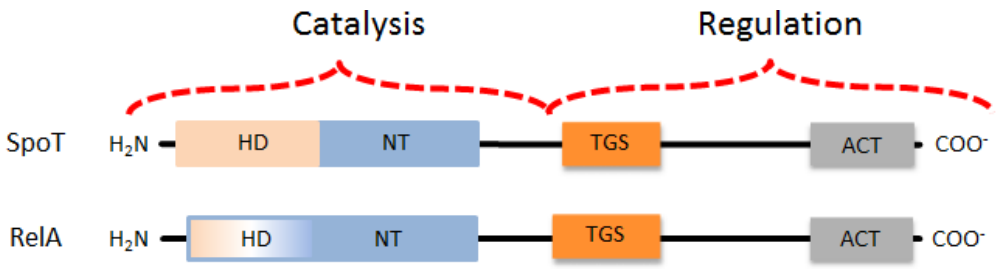
Figure 1.4: Synthesis and hydrolysis of (p)ppGpp by *E. coli* RelA and SpoT. In response to nutrient limitation (amino acid limitation for RelA and others for SpoT) RelA or SpoT catalyses the pyrophosphate transfer from ATP to 3‘-OH of GTP to produce pppGpp and AMP. pppGpp 5‘-phosphohydrolase (ppG) hydrolyses pppGpp to ppGpp, the key effector of the stringent response. SpoT further degrades ppGpp to GDP. Activation of RelA requires a cognate deacylated tRNA (blue) bound to the A-site of 70S ribosomes. Mechanism of SpoT activation is still unclear. RelA is represented by the orange protein and SpoT is represented by the green protein.

The HD domain is characteristic of enzymes involved in metal-dependent phosphohydrolytic activity (Aravind & Koonin 1998). This domain is found in SpoT and other RSH (p)ppGpp hydrolases. In *E. coli* RelA, the hydrolase activity is inactive due to the absence of a conserved HDXXED motif (Sajish et al. 2009; Hogg et al. 2004), making RelA an obligate synthetase.

The NT domain is the centre for catalytic transfer of the pyrophosphate from the donor ATP molecule to the acceptor GTP molecule (Gropp et al. 2001). In mono-functional enzymes, such as RelA, there is a conserved acidic triad (ExDD) in the synthetase catalytic site, while in bifunctional enzymes there is a charge reversal to conserved basic residues RXKD (Sajish et al. 2009).

The synthetase activity is regulated by the C-terminal region. The TGS domain has been proposed to be involved in nucleotide binding in light of the identity of the other proteins this domain has been identified in: ThrRS (threonine tRNA synthetase), GTPases, SpoT, (Dock-Bregeon et al. 2000). In ThrRS, this domain is associated with tRNA editing (Sankaranarayanan et al. 1999; Dock-Bregeon et al. 2000). In GTPases, the TGS domain it is involved in nucleotide binding (Wolf et al. 1999) and in SpoT, it binds to acyl carrier protein and mediates a conformational change that would favour either the synthetic or the hydrolytic activity of SpoT (Battesti & Bouveret 2006; Battesti & Bouveret 2009). The function of this domain in RelA is unknown.

The ACT domain is within a class of domains that bind to small ligands (Chipman & Shaanan 2001; Grant 2006) and is observed in several different amino acid biosynthetic proteins (Liberles et al. 2005; Grant 2006; Feller et al. 2006). Currently, the functional role of the ACT domain in RelA or in SpoT is still unclear. Some early studies have proposed that RelA activity could be regulated through oligomerization via the C-terminal domain (X. Yang & Ishiguro 2001; Gropp et al. 2001).



Domain	HD (Histidine – Aspartate)	Nucleotidyl-transferase	ThRS/GTPase/ SpoT	Aspartate kinase/ Chorismate mutase/ TyrA
General Function	Metal dependent phosphohydrolases	pppGpp synthetase	Nucleotide binding	Small ligand binding
Function in SpoT	Hydrolase	Synthetase	Acyl carrier protein binding	Unknown
Function in RelA	Inactive	Synthetase	Unknown	Unknown

Figure 1.5: Domain organisation of *E. coli* RelA and SpoT enzymes. RelA and SpoT are enzymes involved in maintaining (p)ppGpp levels. Mutational studies have shown that RelA and SpoT can be divided into two functional regions, the catalytic N-terminal region and the regulatory C-terminal region. Although RelA contains an HD domain, the hydrolase is inactive. At the N-terminal catalytic region there are two domains: HD and NT. At the C-terminal region there is the TGS and ACT. The table below lists some of the known functions of each domain.

```
atggttgcggtaagaagtgcacatatcaataaggctggtgaatttgatccggaaaaatgg        60
 M  V  A  V  R  S  A  H  I  N  K  A  G  E  F  D  P  E  K  W         20

atcgcaagtctgggtattaccagccagaagtcgtgtgagtgcttagccgaaacctgggcg        120
 I  A  S  L  G  I  T  S  Q  K  S  C  E  C  L  A  E  T  W  A         40

tattgtctgcaacagacgcaggggcatccggatgccagtctgttattgtggcgtggtgtt        180
 Y  C  L  Q  Q  T  Q  G  H  P  D  A  S  L  L  L  W  R  G  V         60

gagatggtggagatcctctcgacattaagtatggacattgacacgctgcgggcggcgctg        240
 E  M  V  E  I  L  S  T  L  S  M  D  I  D  T  L  R  A  A  L         80

cttttccctctggcggatgccaacgtagtcagcgaagatgtgctgcgtgagagcgtcggt        300
 L  F  P  L  A  D  A  N  V  V  S  E  D  V  L  R  E  S  V  G        100

aagtcggtcgttaaccttattcacggcgtgcgtgatatggcggcgatccgccagctgaaa        360
 K  S  V  V  N  L  I  H  G  V  R  D  M  A  A  I  R  Q  L  K        120

gcgacgcacactgattctgtttcctccgaacaggtcgataacgttcgccggatgttattg        420
 A  T  H  T  D  S  V  S  S  E  Q  V  D  N  V  R  R  M  L  L        140

cgatggtcgatgattttcgctgcgtagtcatcaaactggcggagcgtattgctcatcttg        480
 A  M  V  D  D  F  R  C  V  V  I  K  L  A  E  R  I  A  H  L        160

cgcgaagtaaaagatgcgccggaagatgaacgtgtactggcggcaaaagagtgtaccaac        540
 R  E  V  K  D  A  P  E  D  E  R  V  L  A  A  K  E  C  T  N        180

atctacgcaccgctggctaaccgtctcggaatcggacaactgaaatgggaactggaagat        600
 I  Y  A  P  L  A  N  R  L  G  I  G  Q  L  K  W  E  L  E  D        200

tactgcttccgttacctccatccaaccgaatacaaacgaattgccaaactgctgcatgaa        660
 Y  C  F  R  Y  L  H  P  T  E  Y  K  R  I  A  K  L  L  H  E        220

cggcgtctcgaccgcgaacactacatcgaagagttcgttggtcatctgcgcgctgagatg        720
 R  R  L  D  R  E  H  Y  I  E  E  F  V  G  H  L  R  A  E  M        240

aaagctgaaggcgttaaagcggaagtgtatggtcgtccgaaacacatctacagcatctgg        780
 K  A  E  G  V  K  A  E  V  Y  G  R  P  K  H  I  Y  S  I  W        260

cgtaaaatgcagaaaaagaacctcgcctttgatgagctgtttgatgtgcgtgcggtacgt        840
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```

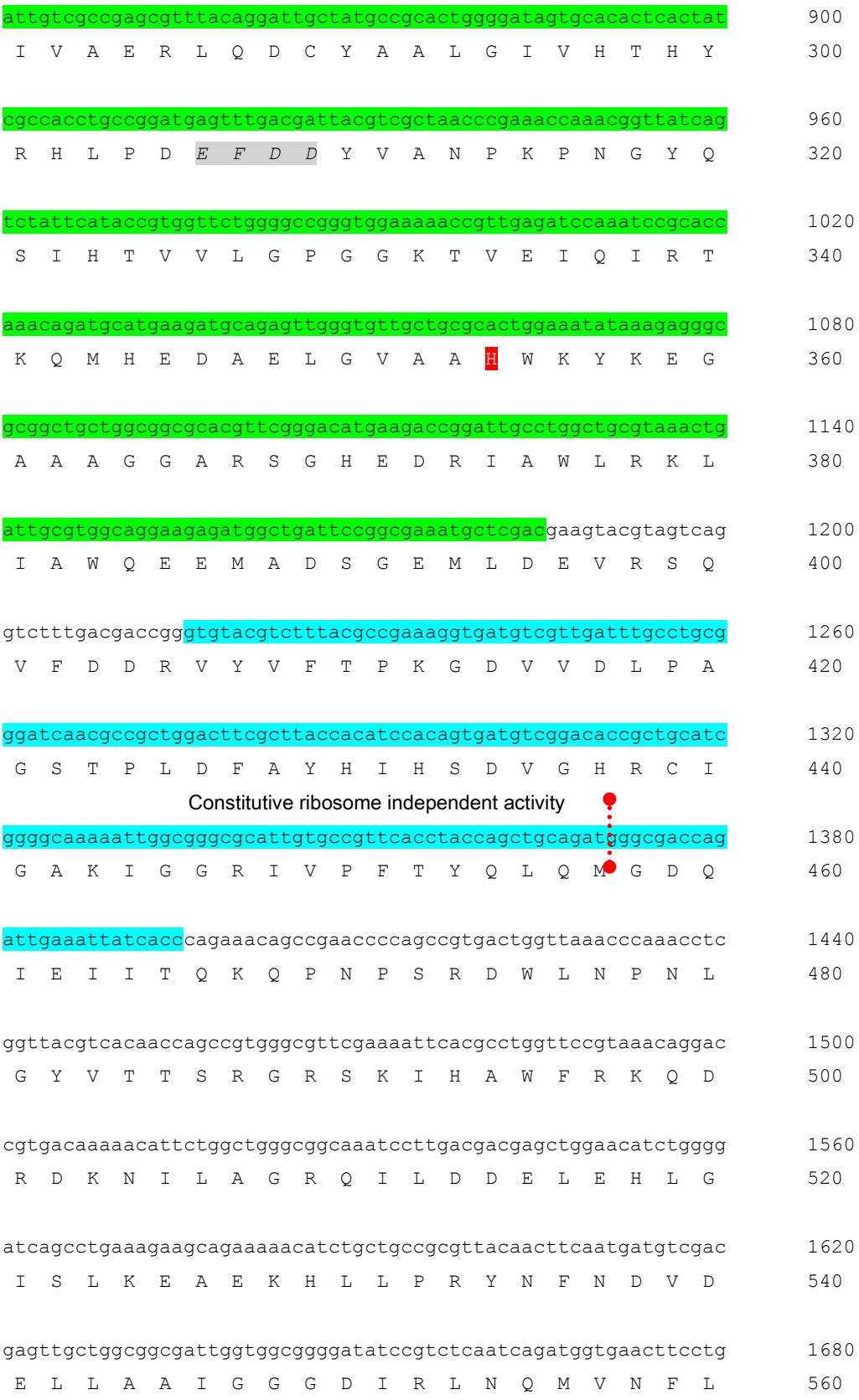
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I V A E R L Q D C Y A A L G I V H T H Y 300
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R H L P D E F D D Y V A N P K P N G Y Q 320
tctattcataccgtggttctggggccgggtggaaaaaccgttgagatccaaatccgcacc 1020
S I H T V V L G P G G K T V E I Q I R T 340
aaacagatgcatgaagatgcagagttgggtgttgctgcgcactggaaatataaagagggc 1080
K Q M H E D A E L G V A A H W K Y K E G 360
gcggctgctggcggcgcacgttcgggacatgaagaccggattgcctggctgcgtaaactg 1140
A A A G G A R S G H E D R I A W L R K L 380
attgcgtggcaggaagagatggctgattccggcgaaatgctcgacgaagtacgtagtcag 1200
I A W Q E E M A D S G E M L D E V R S Q 400
gtctttgacgaccgggtgtacgtctttacgccgaaaggtgatgtcgttgatttgcctgcg 1260
V F D D R V Y V F T P K G D V V D L P A 420
ggatcaacgccgctggacttcgcttaccacatccacagtgatgtcggacaccgctgcatc 1320
G S T P L D F A Y H I H S D V G H R C I 440
Constitutive ribosome independent activity
ggggcaaaaattggcgggcgcattgtgccgttcacctaccagctgcagatgggcgaccag 1380
G A K I G G R I V P F T Y Q L Q M G D Q 460
attgaaattatcaccagaaacagccgaaccccagccgtgactggttaaacccaaacctc 1440
I E I I T Q K Q P N P S R D W L N P N L 480
ggttacgtcacaaccagccgtgggcgttcgaaaattcacgcctggttccgtaaacaggac 1500
G Y V T T S R G R S K I H A W F R K Q D 500
cgtgacaaaaacattctggctgggcggcaaatccttgacgacgagctggaacatctgggg 1560
R D K N I L A G R Q I L D D E L E H L G 520
atcagcctgaaagaagcagaaaaacatctgctgccgcgttacaacttcaatgatgtcgac 1620
I S L K E A E K H L L P R Y N F N D V D 540
gagttgctggcggcgattggtggcggggatatccgtctcaatcagatggtgaacttcctg 1680
E L L A A I G G G D I R L N Q M V N F L 560

caatcgcaatttaataagccgagtgccgaagagcaggacgccgccgcgctgaagcaactt 1740
Q S Q F N K P S A E E Q D A A A L K Q L 580

cagcaaaaaagctacacgccgcaaaaccgcagtaaagataacggtcgcgtggtagtcgaa 1800
Q Q K S Y T P Q N R S K D N G R V V V E 600

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G V G N L M H H I A R C C Q P I P G D E 620

attgtcggcttcattacccaggggcgcggtatttcagtacaccgcgccgattgcgaacaa 1940
I V G F I T Q G R G I S V H R A D C E Q 640

ctggcggaactgcgctcccatgcgccagaacgcattgttgacgcggtatggggtgagagc 2000
L A E L R S H A P E R I V D A V W G E S 660

tactccgccggatattcgctggtggtccgcgtggtagctaatgatcgtagtgggttgtta 2060
Y S A G Y S L V V R V V A N D R S G L L 680

cgtgatatcacgaccattctcgccaacgagaaggtgaacgtgcttggcgttgccagccgt 2120
R D I T T I L A N E K V N V L G V A S R 700

agcgacaccaaacagcaactggcgaccatcgacatgaccattgagatttacaacctgcaa 2180
S D T K Q Q L A T I D M T I E I Y N L Q 720

gtgctggggcgcgtgctgggtaaactcaaccaggtgccggatgttatcgacgcgcgtcgg 2240
V L G R V L G K L N Q V P D V I D A R R 740

ttgcacgggagttag 2255
L H G S - 744

Figure 1.6: Nucleotide and amino acid sequence of relA gene and RelA protein. Nucleotide sequence is aligned with respective amino acid. Highlighted regions correspond to the Pfam (NCBI CDD) identified domains: HD domain (pink; residues 59-189), NT (green; 227 - 395), TGS (cyan; 406-465), and ACT (yellow; 667-731). The residues in red highlights (G251 and H354) are implicated in nucleotide binding. Residues highlighted in grey and italics indicate the catalytic core for the enzymes. The residues highlighted in black were identified to be involved in the oligomerisation of RelA. The red line indicated the minimal requirement to obtain constitutive RelA activity which is ribosome independent.

This could be due to the inherent property of the ACT domain to form various types of tertiary and quaternary structures (Feller et al. 2006; Liberles et al. 2005; Chipman & Shaanan 2001; Robin et al. 2010; Grant 2006). The presence of this regulatory domain in RelA and SpoT suggest that the catalytic regions may be regulated in response to an unidentified ligand.

1.6.2 SpoT Activity

E. coli SpoT is a bifunctional enzyme and its mechanism of regulation could be similar to that of the crystalized bifunctional RSH homolog of *Streptococcus dysgalactiae subspecies equisimilis*. Through SpoT crystal structure it was determined that the enzyme adopts two conformations: 1) Hydrolase-OFF/Synthetase-ON and 2) Hydrolase-ON/Synthetase-OFF (Hogg et al. 2004). The hydrolase and synthetase functions are regulated by the mechanism defined as conformational antagonism. In this mechanism, when the hydrolase is bound to ppGpp, the synthetase domain is inactive. Similarly, when the synthetase domain is bound to ATP and GTP, the hydrolase domain is inactive (Hogg et al. 2004; Mechold et al. 2002). Furthermore, aminoacylation states of the tRNA can regulate SpoT activity (Richter 1980). In *rel*$^{-}$strains, during amino acid deprivation and the presence of high concentrations of deacylated tRNAs, hydrolase activity of SpoT is inhibited and the synthetase (a weaker synthetase in comparison to RelA) is activated. Aside from the suggested role of tRNA and acyl carrier protein in the regulation of SpoT activity, the mechanism through which SpoT is activated during nutritional depletion is unknown (C. Fujita et al. 2002; Gentry & Cashel 1996; Sarubbi et al. 1988; V. Hernandez & Bremer 1991; Xiao et al. 1991; Sarubbi et al. 1989; Jiang et al. 2007; Gong et al. 2002; Murray & Bremer 1996; Ostling et al. 1996; Vinella et al. 2005; Richter 1980; Battesti & Bouveret 2006).

1.6.3 RelA Activity

The conditions necessary for RelA activation have been known since the 1970s. Unlike SpoT, which acts in a ribosome independent manner, RelA-mediated (p)ppGpp synthesis is a ribosome dependent reaction. The activation of RelA is dependent on the presence of deacylated tRNA bound to the A-site of 70S ribosomes. This requirement has been shown using both *in vivo* and *in vitro* assays (Haseltine & R. Block 1973; Jenvert 2007; Wendrich et al. 2002; Rojiani et al. 1989; Jenvert & Schiavone 2005). RelA interacts with the ribosome via its C-terminal region. This interaction is thought to cause a conformation change that leads to the activation of the N-terminal catalytic region. It is important to note that RelA is not only activated during instances of amino acid starvation but also when formation of aminoacylated tRNAs is affected. Temperature-sensitive tRNA synthetase mutants and tRNA synthetase inhibitors can also stimulate the stringent response by activating RelA-mediated (p)ppGpp synthesis (Cashel 1969; Cashel et al. 1996).

Monitoring nutrient usage is necessary for the cell to adapt rapidly to starvation. RelA serves as an indirect monitor for cellular levels of aminoacyl-tRNAs, which reflect the status of protein synthesis within the cell. In turn, the tRNAs serve as monitors for the intracellular amino acid pools. When amino acid pools are depleted to levels where most of the tRNAs are deacylated, protein synthesis is perturbed, (p)ppGpp is synthesised rapidly, and the appropriate regulatory actions are initiated. The current model for RelA-mediated (p)ppGpp synthesis is illustrated in **Figure 1.7**.

1.7 tRNAs - the Trigger for RelA Mediated (p)ppGpp Synthesis

Amino acid limitation is thought to be the preliminary response to another more serious starvation (Nyström 2004). As the expression of genetic information for all phenotypes is conferred through protein expression, a sufficient supply of amino acids is important.

Aside from their roles in translation, tRNAs can function as an intracellular sensor for amino acid levels (Banerjee et al. 2010). A constant supply of aminoacylated tRNA is maintained to cater to demands of protein synthesis. tRNAs that are utilised in translation can be found in two forms, aminoacylated (Neidhardt 1966) (charged) or deacylated (uncharged). When nutrients are abundant, most tRNAs are found in the aminoacylated form (Yegian et al. 1966; Blanquet et al. 2000). Aminoacylated tRNAs are often found in complex with the elongation factor EF-Tu and GTP in a tertiary complex ready for protein synthesis (Blanquet et al. 2000). As amino acids pools within the cell diminish, the respective tRNAs accumulate in the deacylated form (Haseltine & R. Block 1973). Unlike the aminoacylated tRNAs, deacylated tRNAs are free in the cytosol (LaRiviere et al. 2001; R. Block & Haseltine 1975). The rate of protein synthesis is estimated to be around 40 amino acid per second (Berg et al. 2002), therefore at the onset of amino acid limitation, there is a rapid accumulation of deacylated tRNAs (Haseltine & R. Block 1973; Rojiani et al. 1989).

Deacylated tRNAs will bind to the A-site only when present in excess of aminoacylated tRNAs (Rojiani et al. 1989). Deacylated tRNAs cannot compete with aminoacylated tRNAs for A-site binding. The association of aminoacylated tRNAs (in complex with EF-Tu and GTP) is 6 X $10^7 M^{-1}s^{-1}$ (Rodnina et al. 1996) which would be much faster than then passive or non-enzymatic association of deacylated tRNAs to the A-site. The only instances when deacylated tRNA can compete for the A-site are when about 80% of the tRNAs are present in the deacylated form (Haseltine & R. Block 1973) or when the ratio of deacylated:aminoacylated tRNA is between 5 to 10 (Rojiani et al. 1989).

With a deacylated tRNA bound at the A-site, the ribosome is trapped in a complex that is known as the RelA-Activating-Complex (RAC). The mechanism of how RelA detects, and is activated in the presence of, deacylated tRNA, is unclear.

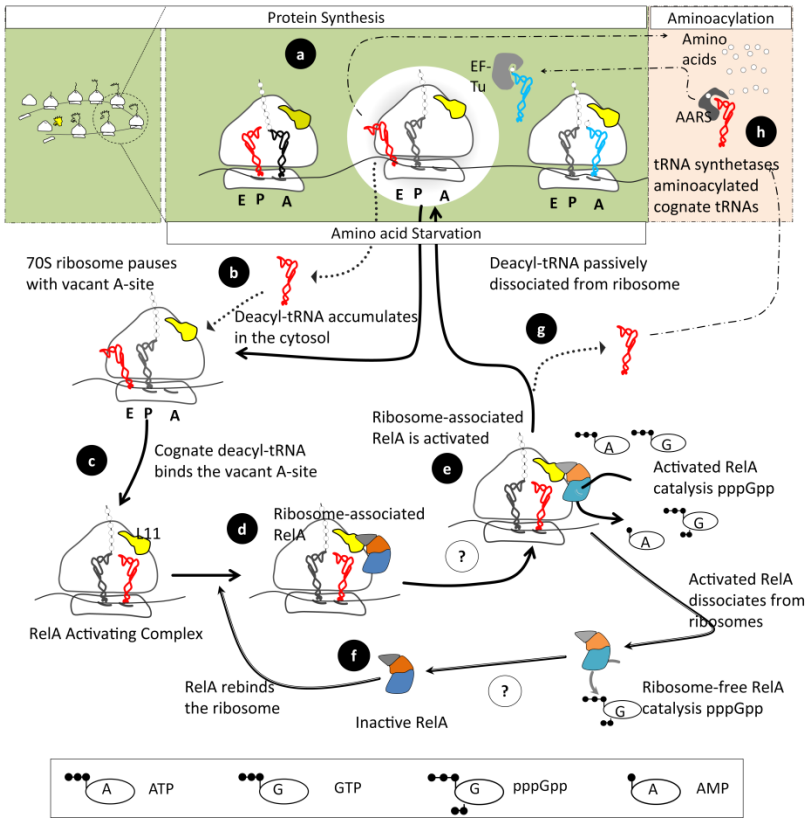

Figure 1.7: Model for RelA mediated (p)ppGpp synthesis. During nutrient rich conditions, most tRNA are involved in translation (a) are esterified with their respective amino acids and are in complex with translation elongation factor EF-Tu (h). When enzymatic aminoacylation is interrupted (*e.g.* amino acid starvation) deacylated tRNAs (red) accumulate in the cytosol (b) while 70S ribosomes pause at the respective codons for the limiting amino acid. A deacyl-tRNA passively binds the A-site (c) forming the RelA-Activating-Complex. The presence of the tRNA stimulates activation of RelA (d). Activated RelA catalyses the synthesis of pppGpp (e). RelA dissociates from the ribosome in its active form (f) and continues to synthesise pppGpp off the ribosomes for a period of time. RelA rebinds the ribosomes (d). The deacylated tRNA remains bound to A-site until it passively dissociates (g). If amino acid pools have been replenished, the deacyl-tRNA is aminoacylated (h) otherwise, the deacyl-tRNA will rebind at the A-site (b).

Once bound to the RAC and activated, RelA transfers the γ,β-phosphate from ATP to the 3' hydroxyl of a GTP or GDP molecule to form pppGpp and ppGpp respectively. (p)ppGpp is suggested to weaken the affinity of RelA for the ribosome (Wendrich et al. 2002). RelA dissociates from the RAC and remains active until it is inactivated, presumably by auto-regulation (English et al. 2011). For reactivation, RelA has to re-bind to the RAC. The dissociation of RelA from the RAC has no effect on the A-site bound uncharged tRNA as was initially proposed (Richter 1976). The dissociation of the deacylated tRNA is a passive process and is independent of RelA dissociation from the ribosome (Wendrich et al. 2002). The duration of the deacylated tRNA appears to correspond to the inherent stability of the tRNA to the A-site of the ribosomes (Fahlman et al. 2004).

1.8 Direct and Indirect Modulators of RelA Activity

Currently, three components have been identified as being involved in RelA activity: tRNA, ribosomal protein L11, and the C-terminal domain of RelA.

1.8.1 tRNAs - an Indirect Modulator of RelA Activity

Wendrich et al. (Wendrich et al. 2002) reported that the passive dissociation of theuncharged-tRNA from the ribosomal A-site is critical for the attenuation of the stringent response. The dissociation of the tRNA is not dependent on the presence of ppGpp and the dissociation of RelA from the ribosome. Dissociation rates, unlike the association rates, are different for individual deacylated tRNAs (Fahlman & Uhlenbeck 2004; Fahlman et al. 2004). The influence of these dissociation rates on the duration of the stringent response has never been previously examined. The number of rounds of activation RelA undergoes would be dependent on how long the RelA activating complex persists within the cell. In Chapter 2 of this thesis I present my study on how the identity of the deacylated tRNA bound to the A-site of the ribosome

influences the duration of (p)ppGpp synthesis *in vitro*. These results show that the attenuation of (p)ppGpp synthesis is difference is regards to tRNA used in the assembly of RAC.

1.8.2 C-terminal ACT domain – a Direct Modulator of RelA Activity

During exponential growth, cells maintain a basal level of (p)ppGpp, possibly to fine tune ribosome biosynthesis (Sokawa et al. 1975; Lazzarini et al. 1971). Maintenance of the basal (p)ppGpp is exclusively under the control of the bifunctional (p)ppGpp synthetase/ppGpp hydrolase SpoT (Sarubbi et al. 1989; Sarubbi et al. 1988). RelA is assumed to be inactive during exponential growth since *relA* knockout mutants sustain a basal (p)ppGpp level (Metzger, Schreiber, et al. 1989; Xiao et al. 1991). In the inactive state, RelA is found in association with the ribosome and once activated, RelA dissociates from the ribosomes. (Wendrich et al. 2002). This active RelA remains off the ribosome until it is inactivated. RelA has to re-associate with the ribosomes to go through another round of activation (English et al. 2011). The interaction of RelA with the ribosome is mediated via the C-terminal region. However, which of the two regions is involved was unknown until now.

The second mutation that was identified to confer the "relaxed" phenotype was a mutation to the *relC* gene. The gene *relC* was later mapped to the region of the genome that contained a cluster of ribosomal proteins and was later identified to be the gene for ribosomal protein L11 (*rplK*) (Cashel et al. 1996). In another independent assay, RelA was shown to localise to the 50S subunit of the ribosomes, the same subunit of L11 (Ramagopal & Davis 1974). Deletion of L11 does not affect cell viability during normal growth conditions but these cells exhibited the relaxed phenotype during amino acid limitation. L11 in addition to A-site bound tRNA is only necessary for activation of RelA and neither appears to affect binding of RelA to the ribosome (Jenvert & Schiavone 2007; Wendrich et al. 2002; Jenvert 2007). A detailed study done by Jenvert et al. has

shown that the N-terminus of the ribosomal protein L11, specifically the proline-rich region, is involved in the stimulation of RelA (Jenvert & Schiavone 2007). Deletion of the N-terminal region of L11 resulted in loss of RelA activity. However, activity was restored by the addition of the recombinant N-terminal domain into the *in vitro* assay (Jenvert & Schiavone 2007).

The C-terminal region of RelA is important for the ribosome dependent activation of RelA (Gropp et al. 2001; X. Yang & Ishiguro 2001). The involvement of, or even the joint contribution of, the TGS and ACT domains to this interaction are still unknown. In Chapter 3, I discuss the possibility of the ACT region being involved in the interaction with the ribosome.

The ACT domain is a small ligand binding domain often found in the regulatory region of biosynthesis enzymes (Liberles et al. 2005; Chipman & Shaanan 2001; Grant 2006). For most of these proteins, ligand binding had an inhibitory effect on protein activity. This prompted us to investigate the possible involvement of amino acids on the regulation of RelA activity. In Chapter 4, I discuss my work to determine the effects of amino acids on the (p)ppGpp synthesis.

1.9 Conclusion

The stringent response is an important survival response for all bacteria, particularly pathogenic bacteria. Further characterization of the mechanism may open up the possibility of the stringent response being used as a new target for antibacterial design. A recent study reports on the positive allosteric feedback regulation of ppGpp on RelA (Shyp et al. 2012). (p)ppGpp is proposed to bind to and enhance the activation of RelA. Wexselblatt et al. has reported the use of chemical analogues of (p)ppGpp that can bind and inhibit the activity of *E. coli* RelA and the Rel_{seq} (the catalytic domain of a bifunctional enzyme) (Wexselblatt et al. 2010). RelA-mediated (p)ppGpp synthesis functions are an early warning

system to impede starvation condition. By knocking out this response, the bacteria would remain "relaxed" or insensitive to dwindling nutrients condition and eventually succumb to starvation and death.

Generally, strains that are genetically defective for the stringent response ($ppGpp^0$) exhibit reduced virulence (Haralalka et al. 2003). Coincidently, these $ppGpp^0$ strains have been shown to have potential for use as vaccines (S. Park et al. 2010), tumour imaging and in combinational cancer therapy (V. Nguyen et al. 2010; Hyun et al. 2011; Zhao et al. 2007).

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Chapter 2

Dependence of RelA-Mediated (p)ppGpp Formation on tRNA Identity.

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2.1 Introduction

The stringent response is a bacterial adaptive response historically linked to amino acid limitations but is also involved in other nutrient limitations and cellular stresses (for recent reviews see references (Potrykus & Cashel 2008; Jain et al. 2006; Wu & Xie 2009)). The onset of the stringent response is marked by a rapid accumulation of the effector molecules, pentaphosphate guanosine (5'-triphosphate-3'-diphosphate guanosine or pppGpp) and tetraphosphate guanosine (5',3'-dibisphosphate guanosine or ppGpp), collectively referred to as (p)ppGpp (Haseltine et al. 1972; Cashel 1969). Despite the historically defined role as a negative regulator of ribosome biosynthesis, (Dennis & Nomura 1974), transcriptome analysis has revealed that (p)ppGpp affects the expression of a wide range of genes such as the amino acid biosynthetic genes (Durfee et al. 2008). Global changes in gene expression appears to include mechanisms involving alternative sigma factor stabilisation (Merrikh et al. 2009). As a result of modulating gene expression, (p)ppGpp facilitates cell survival by regulating a range of processes within the cell, such as protein degradation (Kuroda 2006), DNA replication (J. Wang et al. 2007), cell division (Ferullo & Lovett 2008), fatty acid biosynthesis (Polakis et al. 1973), and biofilm formation (Balzer & McLean 2002). It has also been reported that (p)ppGpp signalling is key to the pathogenicity of some infectious bacteria (Song et al. 2004; Dozot et al. 2006; Hammer & Swanson 1999). The regulatory effects of (p)ppGpp can be summarised as down-regulation of proliferation and growth phase processes and up-regulation of survival and stationary phase processes (Balzer & McLean 2002; Boehm et al. 2009).

Two proteins are central to the stringent response, RelA and SpoT, which catalyse the synthesis and hydrolysis of (p)ppGpp. In *Escherichia coli*, RelA catalyses the formation of the vast majority of (p)ppGpp synthesis during amino acid starvation (Friesen et al. 1978), while SpoT is responsible for basal level

(p)ppGpp formation and (p)ppGpp degradation upon cessation of the stringent response (Murray & Bremer 1996). The RelA/SpoT genes have been identified in most eubacterial (Masuda & Bauer 2004) and some archaea (Cellini et al. 2004) species. Homologues of RelA/SpoT have also been identified in plants (Biezen et al. 2000). Within the eubacterial kingdom there is an evolutionary dichotomy of RelA and SpoT genes. In some organisms, like *E. coli*, RelA and SpoT are expressed as two individual proteins (An et al. 1979; Friesen et al. 1978), and in others, like *M. tuberculosis*, there is a single RelA/SpoT fusion protein (Avarbock et al. 1999).

Transfer RNAs (tRNAs) are essential to the bacterial stringent response. The synthesis of (p)ppGpp by RelA is stimulated by ribosomes with an uncharged or deacylated tRNA (deacyl-tRNA) bound in the A-site. Under growth promoting conditions tRNAs are predominantly aminoacylated, are involved in active protein synthesis, and are rapidly re-aminoacylated by their respective aminoacyl-tRNA synthetases upon release from the ribosome. Under amino acids limiting conditions the deacylated forms of the respective tRNAs rapidly accumulate (Dittmar et al. 2005; Haseltine & Block 1973; Rojiani et al. 1989). The resulting ratio of deacyl-tRNA to aminoacyl-tRNA is considered to be an important parameter to the binding of deacyl-tRNAs to the ribosomal A-site (Haseltine & Block 1973; Rojiani et al. 1989). When the concentration of a particular aminoacyl-tRNA:EF-Tu ternary complex is sufficiently depleted, translating ribosomes begin to pause at codons corresponding to the limiting aminoacyl-tRNA. These translanslational pauses present the opportunity for a deacyl-tRNA to bind the vacant ribosomal A-site. Rapid delivery of aminoacyl-tRNAs to the ribosomsal A-site by aminoacyl-tRNA:EF-Tu ternary complexes otherwise exclude deacyl-tRNA binding. The binding of the deacyl-tRNA traps the ribosome in complex referred to as the RelA Activating Complex (RAC). RelA is activated upon binding to the RAC and catalyzes the transfer of the γ,β-pyrophosphate from ATP to the 3'-hydroxyl of either GTP or GDP to form

pppGpp and ppGpp, respectively (Haseltine & Block 1973). It has been suggested that the formation of (p)ppGpp weakens the affinity of RelA for the RAC and RelA dissociates from the complex (Wendrich et al. 2002). Additionally, it was reported that upon RelA dissociation from the ribosome, the deacyl-tRNA remains bound to the ribosomal A-site (Wendrich et al. 2002). Dissociation of the deacyl-tRNA is a passive and RelA independent process. The current tRNA-dependent model for RelA-mediated (p)ppGpp synthesis is illustrated in **Figure 2.1.**

As long as the deacyl-tRNA remains bound to the ribosomal A-site the cycle of RelA binding can continue, and protein synthesis remains stalled as a result of the A-site bound deacyl-tRNA blocking EF-Tu-dependent delivery of an aminoacylated tRNA (Pedersen et al. 1973). The passive dissociation of deacyl-tRNAs from the ribosomal A-site implies that the inherent stability of the RAC plays an important role, not only on the regulation RelA but also on the duration of its activity. The cessation of RelA stimulation results from the passive dissociation of the A-site bound deacyl-tRNA, a process that also releases the ribosome from the RAC to resume protein synthesis. We have previously demonstrated a significant variation in the rates of A-site dissociation by deacyl-tRNAs (Fahlman et al. 2004). In light of the variations in dissociation rates, we hypothesised that differences in tRNA binding would be reflected in RelA stimulation.

In this report we compare the *in vitro* stimulation of RelA by RACs formed with tRNAs that either rapidly ($tRNA^{Phe}$) or slowly ($tRNA^{Val}_{2A}$) dissociate from the ribosomal A-site. These two tRNAs have exhibit A-site dissociation rate constants that differ by approximately an order of magnitude (Fahlman et al. 2004). The differences in binding leads to a prediction that RelA-mediated synthesis of (p)ppGpp would persist at lower RAC concentrations for complexes containing the tightly binding $tRNA^{Val}_{2A,}$ in contrast to RACs containing $tRNA^{Phe}$.

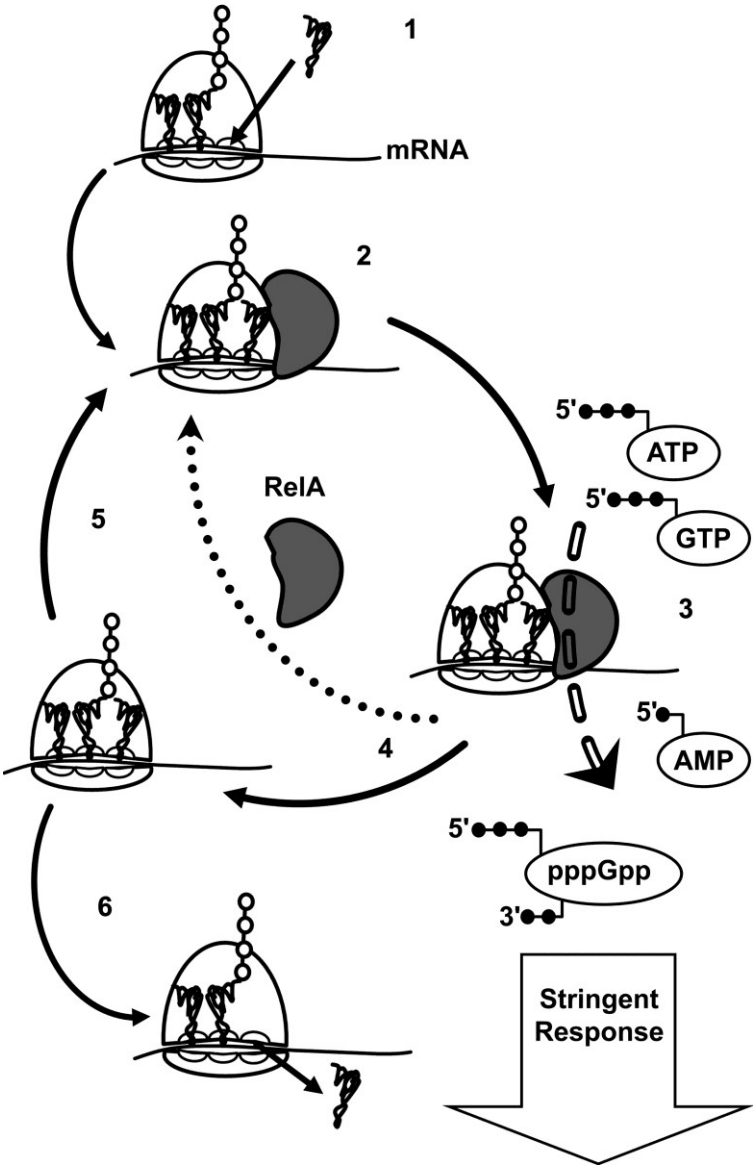

Figure 2.1: Model for (p)ppGpp synthesis and the stringent response. During amino acid starvation there is an accumulation of deacylated tRNAs in the cytosol (1). A deacylated tRNA binds to the cognate codon in the vacant A-site and stalls the ribosome (2). RelA binds a stalled ribosome and catalyses the formation of guanosine pentaphosphate (pppGpp) (3). After pppGpp synthesis RelA dissociates from the ribosome (4). The RelA can rebind or proceed to the next stalled ribosome (5). The cycle repeats until the deacylated tRNA passively dissociates from the ribosomal A-site, preventing further pppGpp synthesis (6).

2.2 Materials and Methods

2.2.1 Ribosome Purification

70S ribosomes from MRE600 cells were prepared according to previously described procedures (Spedding 1990). Final ribosome pellets were suspended in ribosomal storage buffer (50 mM Tris-HCl pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol (β-ME)) and aliquots stored at -80^{o}C.

2.2.2 Protein Purification

A hexahistidine-tagged clone (ORF: JW2755) of RelA was obtained in a pCA24N plasmid from the National BioResource Project (NIG, Japan):*E.coli* ASKA collection (Kitagawa et al. 2005). RelA was expressed and purified from BL21 cells cultured in Luria–Bertani broth medium containing 25 μg/mL of chloramphenicol. Protein expression was induced with 1 mM isopropyl thio-β-d-galactosidase (IPTG) when the culture reached an OD_{600} = 0.5. The culture was then grown for an additional 4 h at 37 °C. Cells were harvested by centrifugation (5000*g* for 10 min at 4 °C). The cell pellet was washed and suspended in Lysis buffer (25 mM $NaHPO_4$ (pH 7.5), 50 mM NaCl, 5 mM imidazole, 1 mM βME, and 1 mM PMSF) and then lysed by sonication. Cell debris was removed by centrifugation (15,000 rpm for 15 min at 4 °C); the cleared lysate was then loaded onto a 1 mL HiTrap FF column (GE Healthcare); the proteins were eluted with a stepwise gradient of binding buffer (lysis buffer without PMSF) to elution buffer (25 mM $NaHPO_4$ (pH 7.5), 50 mM NaCl, 1.5 M imidazole, and 1 mM *β*-ME). The elution process was as follows: after a 10 mL wash with binding buffer, the elution buffer concentration was increased to 50% for 10 mL; this was followed by 100% elution buffer for the final 10 mL. The elution fractions were collected as 1 mL fractions, and those containing the protein were treated as previously described (Jenvert & Schiavone 2005) with the following modifications. Fractions containing RelA were pooled and diluted to twice their original volume with imidazole-free binding buffer. This was done to suspend any RelA proteins

that had already precipitated in the fraction tubes. The diluted sample was then dialysed overnight against dialysis buffer A (10 mM Tris-HCl (pH 8.0), 14 mM $MgCl_2$, 60 mM KCl, 0.5 mM EDTA, 10% (v/v) glycerol, and 10 mM βME). Under these conditions RelA precipitates. The precipitate was then dissolved in suspension buffer (10 mM Tris-HCl (pH 8.0), 1 M KCl, 1 mM EDTA, 10% (v/v) glycerol, and 10 mM βME) and dialysed exhaustively against RelA storage buffer (30 mM HEPES-KOH (pH 8.0), 150 mM KCl, 20% (v/v) glycerol, and 10 mM *β*-ME). Aliquots of the protein were flash-frozen and stored at −80 °C.

2.2.3 Unmodified tRNAs and mRNA Preparation

Purified $tRNA^{Phe}$ from *E. coli* was purchased from Chemical Block (Russia). Unmodified tRNAs and mRNAs were prepared by *in vitro* transcription. The DNA templates for $tRNA^{Val}_{2A}$ and $tRNA^{Phe}$ transcripts were generated by primer extension of overlapping DNA oligonucleotides (IDT) and have been used in previous *in vitro* transcription reactions. The $mRNA^{Val}_{2A}$ (CAAGGAGGUAAAA**AUG**GUCGCACGU) and $mRNA^{Phe}$ (CAAGGAGGUAAAA**AUG**UUCGCACGU) sequences were generated from Milligan's transcription reactions (Milligan & Uhlenbeck 1989) using synthesised DNA oligonucleotide templates. For each of the sequences the AUG start codon for P-Site binding is shown in bold while the A-site codons are underlined. After transcription, all RNAs were purified by denaturing polyacrylamide gel electrophoresis.

2.2.4 End-Point in vitro pppGpp Synthesis Assay

In a standard (p)ppGpp synthesis assay RelA activating complexes were assembled in the following method. An aliquot containing 10 µM 70S ribosomes was heat activated by incubating at 42°C for 2 min then slowly cooled to 22°C. The 10 µM 70S ribosome stock was prepared in polyamine containing buffer (30 mM HEPES-KOH (pH 8.0), 15 mM $MgCl_2$, 15 mM KCl, 1 mM βME, 50 µM spermine, and 2 mM spermidine). A 9.6 µL sample of heat activated ribosomes

were programmed with mRNA (mRNA^{Val} or mRNA^{Phe}) by the addition 5.8 µL of 25 µM mRNA and incubated for 2 minutes at 22^{o}C. To fully occupy the ribosomal P-Site, the programmed ribosomes were incubated with 5.8 µL of 25 µM tRNA^{Met} for 10 minutes at 22^{o}C. Lastly, the appropriate A-site tRNA (5.8 µL of 25 µM tRNA$^{Val}_{2A}$ or tRNA^{Phe}) was added and the sample was incubated for 1 hr at 22^{o}C. For all the described steps, the added solutions all contained 1× reaction buffer (20 mM HEPES-KOH (pH 8), 15 mM $MgCl_2$, 15 mM KCl, and 1 mM β-ME). All added RNAs were in a 1.5-fold excess with respect to the 70S ribosomes.

Reactions were initiated by the addition of the RAC to mixtures containing 0.18 µM RelA, 1 mM ATP, 0.1 mM GTP, and α[^{32}P]-GTP in 1× reaction buffer. The substrates were added from a 10× substrate mix containing 10 mM ATP, 1 mM GTP, and α[^{32}P]-GTP (1 µL of labelled GTP to 20 µL of unlabelled GTP). The reaction tube was incubated for 1 hour (unless otherwise indicated) at 22^{o}C. Reactions were quenched by the addition of one volume of formic acid (88%) and then stored on dry ice until analysis to minimize hydrolysis of (p)ppGpp. From the quenched samples, 2 µL was removed and used for thin layer chromatography (TLC) analysis as previously described (Wendrich et al. 2002). After the chromatography run, the TLC plates were exposed to a phosphorimager screen which was then visualised and quantified using a phosphorimager (GE Healthcare). ImageQuant^{TM} and SigmaPlot were used for data analysis and curve fitting, respectively.

2.2.5 Time Course Analysis of (p)ppGpp Synthesis

The (p)ppGpp synthesis assay was carried out and visualised as described for the end-point (p)ppGpp synthesis assay with the exception that the total sample volume was increased to 50 µL to enable 3 µL aliquots to be taken over the course of the reaction.

2.2.6 3'-[^{32}P] Labelling of tRNAs.

$tRNA^{Phe}$, $tRNA^{Met}$, and $tRNA^{Val}_{2A}$ were 3'-labelled with [α-^{32}P]-ATP (PerkinElmer) as previously described (Ledoux & Uhlenbeck 2008).

2.2.7 Two-Layer Filter Binding

tRNA dissociation rate from ribosomes in the presence of active RelA was determined using a 96-well filtration protocol to quantify the amounts of ribosome-bound tRNA and unbound tRNA (Fahlman & Uhlenbeck 2004; Phelps et al. 2002). For these experiments the ribosome complexes were assembled in a similar manner to the ribosome complexes described in the (p)ppGpp synthesis assays, with the exception of using 3'-[α-^{32}P]-labelled tRNA for A-site binding at a 10-fold lower concentration than the ribosomes.

Dissociation experiments were initiated by diluting the samples 40-fold with 1× reaction buffer containing 200 nM unlabelled tRNA as a cold chase. At given time points, 30 µL of the diluted sample were filtered through the nitrocellulose-nylon double layer of membranes and washed with an equal volume of 1× reaction buffer. The membranes were then removed from the filter apparatus and then separated and exposed to a phosphorimager screen for quantification.

2.3 Results

The influence of tRNA identity on RelA activity was investigated using an *in vitro* enzymatic assay previously described by Wendrich et al. (Wendrich et al. 2002). To perform a comparative study between two tRNA species, the protocol was adjusted appropriately. The major alteration was the use of two 25 nucleotide "mini" mRNA sequences that only vary in sequence at the A-site codon. The A-site codons were either **G**UC for $tRNA_{2A}^{Val}$ binding or **U**UC for $tRNA^{Phe}$ binding. Each mRNA, being identically matched to the respective anticodons of each tRNA, facilitates the codon-specific occupation of the

respective tRNA into the ribosomal A-site. Since the mRNAs only differed by a single nucleotide at the first position of the valine and phenylalanine codons, their interaction with the ribosomes is unlikely to differ significantly. The mRNAs contain a Shine-Dalgarno (SD) sequence to facilitate tight 70S ribosome binding and A-site selectivity (Di Giacco et al. 2008). The mRNAs also contain a single AUG codon for positioning the mRNA into the correct reading frame when $tRNA^{Met}$ is bound in the ribosomal P-Site. We have extensively used these mRNA designs for several investigations of tRNA binding to the ribosomal P- and A-sites (Fahlman et al. 2004; Fahlman & Uhlenbeck 2004; Dale et al. 2009; Fahlman et al. 2006; Olejniczak et al. 2005).

The two tRNAs chosen to compare RelA stimulation are $tRNA^{Phe}$ and $tRNA^{Val}_{2A}$. Because of extensive use in other investigations of RelA activity, $tRNA^{Phe}$ was chosen as an appropriate control for our comparative study. The second tRNA, $tRNA^{Val}_{2A}$, was chosen for several reasons. $tRNA^{Val}_{2A}$ is known to bind the ribosomal A-site equally well whether it is deacylated or aminoacylated, in contrast to $tRNA^{Phe}$ (Fahlman et al. 2004). $tRNA^{Val}_{2A}$ is a low abundance isoacceptor and is present in *E coli* at amounts similar to that of $tRNA^{Phe}$ (Dong et al. 1996) Additionally, $tRNA^{Val}_{2A}$ lacks posttranscriptional modifications in its anticodon stem loop (Yaniv & Barrell 1971). While modifications in the anticodon stem loops of tRNAs often play significant roles in decoding and ribosome binding (Gustilo et al. 2008; Agris 2008), modifications within the body of the tRNA are generally for stabilizing tRNA folding (Motorin & Helm 2010). Our previous work demonstrates that $tRNA^{Val}_{2A}$ binds the ribosomal A-site equivalently regardless of the presence or absence of post-transcriptional modifications when appropriate buffer conditions are used (Fahlman et al. 2004). Others have clearly demonstrated that the post-transcriptional modifications of $tRNA^{Val}_{1}$ isoacceptor are important for tRNA folding and stability at low magnesium concentrations. The absence of post-transcriptional modifications can be accommodated during *in vitro* studies by having sufficient

millimolar concentrations of magnesium ions present in the sample (Derrick & Horowitz 1993; Kintanar et al. 1994). $tRNA^{Phe}$ only exhibits a marginal 2-fold reduction in A-site binding in the absence of post-transcriptional modifications (Fahlman et al. 2004). Unless noted otherwise, experiments were all performed with *in vitro* transcribed tRNAs and thus lacked all post-transcriptional modifications.

2.3.1 $tRNA^{Val}_{2A}$ Stimulation of RelA Activity

We verified the stimulation of RelA with transcribed $tRNA^{Val}_{2A}$ using the modified *in vitro* (p)ppGpp synthesis assay. The reaction consisted of tight-coupled 70S ribosomes, $mRNA^{Val}$, $tRNA^{Met}$, $tRNA^{Val}_{2A}$, RelA, ATP, and GTP (with [α-^{32}P]-GTP), which were added in a sequential order as described in the Materials and Methods section. The reaction was incubated in reaction buffer for 1 hr and then quenched prior to resolving the nucleotides by thin layer chromatography to observe the conversion of [α-^{32}P]-GTP to [α-^{32}P]-(p)ppGpp (**Figure 2.2**). In accordance with previous results with $tRNA^{Phe}$ (Wendrich et al. 2002), nearly complete conversion of [α-^{32}P]-GTP to [α-^{32}P]-(p)ppGpp can be observed when all of the components are present in the reaction mixture (lane 7). Similar results were obtained when $mRNA^{Phe}$ and $tRNA^{Phe}$ are used in lieu of $mRNA^{Val}$ and $tRNA^{Val}_{2A}$ (data not shown). Our data mimics previous investigations using polyU (Wendrich et al. 2002) as an mRNA template, which suggests that the codon context and SD sequence do not significantly alter the RelA reaction. Additionally, basal (p)ppGpp synthesis is observed when individual RNAs or ribosomes are omitted from the reactions (lanes 1-4). This basal activity is a result of the unstimulated activity of *in vitro* purified RelA, as activity is not observed in the absence of ATP (lane 6) or RelA (lane 5). This ribosome free stimulation of RelA has also been reported by other

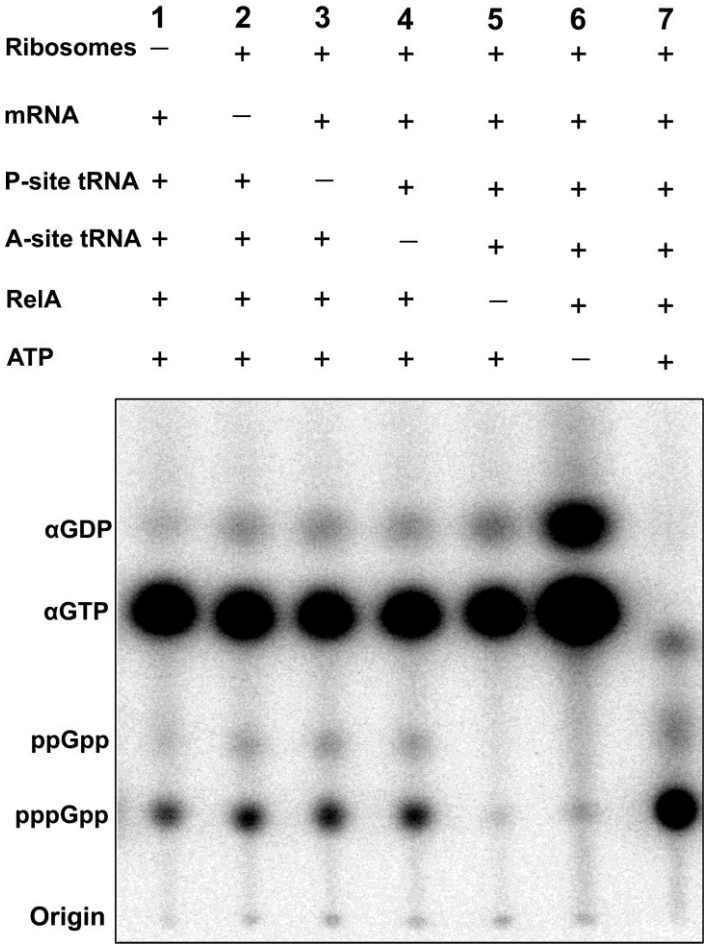

Figure 2.2: RelA dependent (p)ppGpp synthesis. (A) RelA mediated (p)ppGpp synthesis requires the presence of an uncharged $tRNA^{Val}$ in the A-site of a mRNA programmed ribosome. Thin layer chromatography is used to monitor (p)ppGpp synthesis after incubating the reaction mixtures for one hour. Samples contain either all reaction components (lane 7) or are missing individual components (lanes 1-6). Maximal (p)ppGpp synthesis was observed only when all components were present (lane 7). Lanes 1 – 4 reveal basal levels RelA mediated (p)ppGpp synthesis.

investigators (Jenvert & Schiavone 2005) and is assumed to be due to the poorly structured C-terminal domain.

2.3.2 Stimulation of RelA Activity by Unmodified tRNAs.

A potential effect with lack of tRNA post-transcriptional modifications on the RelA activity was investigated. We compared (p)ppGpp synthesis between reaction mixtures containing RAC formed with either fully modified $tRNA^{Phe}$, purified from *E. coli*, or unmodified $tRNA^{Phe}$, generated by *in vitro* transcription, bound to the ribosomal A-site. The data in **Figure 2.3** show (p)ppGpp formation for fully modified and unmodified $tRNA^{Phe}$, when used in independent RelA synthesis assays. The data indicate no significant difference in RelA stimulation by the RACs containing either between the modified or unmodified tRNA. Small observed differences in the absolute amounts of (p)ppGpp formation are a result of variations in TLC spotting. All quantification is by measuring the relative ratios of all the observed species in a sample so measurements are independent of spotting volumes.

2.3.3 Passive Dissociation of tRNAs

The mechanism of passive dissociation of the A-site bound deacylated tRNA as proposed by Wendrich *et al* (Wendrich et al. 2002), is the basis for our rationale for the investigation on the role of tRNA identity on the duration of RelA stimulation. This current model contradicts the earlier model where tRNA dissociation was either concurrent with or successive to RelA catalysis and dissociation from the RAC (Richter 1976). As filter binding has been routinely used to quantify tRNA binding to ribosomes (Fahlman et al. 2004; Fahlman & Uhlenbeck 2004; Fahlman et al. 2006; Olejniczak et al. 2005), we used a two-layer filter binding assay (Wong & Lohman 1993) to validate whether the tRNA release from the A-site was by passive dissociation or RelA-mediated active ejection after (p)ppGpp formation.

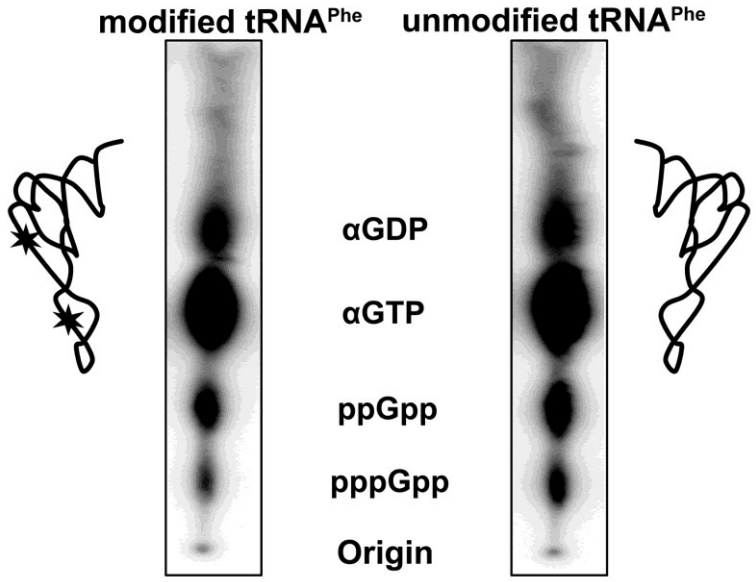

Figure 2.3: Dependence of tRNA post-transcriptional modifications on RelA activity. RelA mediated (p)pppGpp synthesis was examined using ribosome complexes form using either purified and post-transcriptionally modified $tRNA^{Phe}$ (left) or unmodified $tRNA^{Phe}$ generated by *in vitro* transcription (right). The reaction was allowed to proceed for 20 or 60 min prior to TLC analysis.

Reaction mixtures containing 70S ribosomes programmed with $mRNA^{Val}$ and $tRNA^{Met}$ in the P-site and 3'-[^{32}P]-labelled tRNA ($tRNA^{Val}_{2A}$ or $tRNA^{Phe}$) in the A-site were prepared as described in the Materials and Methods section. Dissociation was initiated by diluting the sample with an excess of unlabelled tRNA as a cold chase (Fahlman & Uhlenbeck 2004). Aliquots of the diluted sample were collected at different time points and filtered through a nitrocellulose membrane layered on top of a nylon membrane. Ribosomes and bound RNAs were trapped on a nitrocellulose filter, and unbound tRNAs were retained by the second positively charged nylon membrane. Potential RelA-dependent alterations to tRNA dissociation rates were investigated by the inclusion or exclusion of the ATP and GTP substrates. The data in **Figure 2.4** demonstrate that $tRNA^{Val}_{2A}$ dissociation rate from the ribosomal A-ite is unaffected by the presence or absence of the RelA and the triphosphate substrates. Data analysis reveals a dissociation rate constant of (2.8–3.1) × 10^{-3} min^{-1} from the ribosomal A-sSite. Identical results were observed in similar experiments comparing the presence and absence of RelA from the reaction mixture. Additionally, the dissociation of $tRNA^{Phe}$ from the ribosomal A-site is similarly unaffected by RelA and is significantly faster than the dissociation of $tRNA^{Val}_{2A}$. The summarised data from repeated analysis are reported in **Table 2.1**. As the dissociation of tRNAs from the ribosomal A-site is unaltered by the presence or absence of RelA activity, our results concur with the current model that tRNA release from the ribosomal A-site is independent of RelA dissociation.

2.3.4 Comparing tRNA Dependent (p)ppGpp Synthesis

At the onset of amino acid starvation, we assume that deacyl-tRNAs are present at saturating concentrations. For this reason, stalled ribosome complexes will equivalently stimulate RelA activity regardless of the identity of the A-site occupying tRNA.

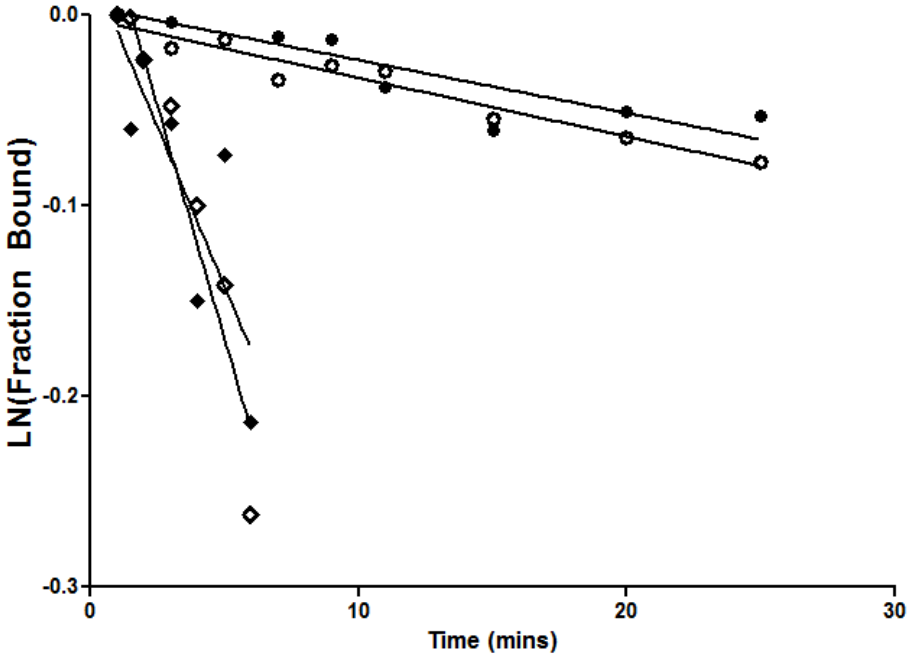

Figure 2.4: A-site dissociation of tRNAs in the presence of RelA activity. The dissociation of $tRNA^{Val}$ from the ribosomal A-site was measured using a double-filter binding assay in the presence (●) and absence (○) of RelA and its nucleotide substrates. The data reveal no measurable change in the dissociation rates. The data shown are from an individual experiment. For comparison, the dissociation of the weakly binding $tRNA^{Phe}$ is also shown in the presence (♦) and absence (◊) of RelA nucleotide substrates.

Table 2.1: Ribosomal A-site Dissociation Rates in the Presence of RelA.

tRNA	+ ATP and GTP	- ATP and GTP
$tRNA^{Val}$	2.8 (±0.5) $\times 10^{-3}$ min^{-1}	3.1 (±0.2) $\times 10^{-3}$ min^{-1}
$tRNA^{Phe}$	48.0 (±6) $\times 10^{-3}$ min^{-1}	21.0 (±12) $\times 10^{-3}$ min^{-1}

Dissociation rates of tRNAs from the ribosomal A-site in the presence of RelA in the presence or absence of the RelA nucleotide substrates.
Errors reflect the standard deviation from three independent experiments performed in triplicate.

We hypothesised that the tRNA identity may be influential in the recovery from the stringent state, *i. e*., during active amino acid biosynthesis and tRNA aminoacylation. As concentrations of aminoacyl-tRNA rise to levels that can support translation, protein synthesis will not resume until the A-site occupying deacyl-tRNA dissociates. Since different decay-tRNAs dissociate from the ribosomal A-site with rates that differ by over an order of magnitude (Fahlman et al. 2004), we propose that slowly dissociating or tight binding tRNAs will form stable RACs that may result in a slower attenuation of RelA-dependent (p)ppGpp synthesis. These tight binding tRNAs are also predicted to result in a greater accumulation of stalled ribosome complexes at lower concentrations of deacylated tRNA.

The stimulation of RelA activity by stalled ribosome complexes formed with either the weakly binding $tRNA^{Phe}$ or tightly binding $tRNA^{Val}_{2A}$ at the A-site were compared. For experimental comparison ribosomes were the limiting factor during RAC formation as opposed to the tRNAs. If tRNAs were the limiting factor during the association of ribosome complexes, differences in the folding efficiencies of the different tRNAs would be manifested as differences in RelA stimulation. Like many transcribed RNAs it is unlikely that the tRNAs will adopt the correct structure a 100% of the time, it would be expected to observe some quantity of misfolded RNAs in the sample. In addition, the extent of misfolding of the two tRNA sequences cannot be assumed to be equal, and any differences could impact the data. To avoid this challenge, the experiments were designed to be limiting in ribosomes such that the limiting factor is common between both samples.

Ribosome complexes formed with $tRNA^{Val}_{2A}$ stimulate RelA-dependent formation of (p)ppGpp at significantly lower concentrations than ribosome complexes formed with $tRNA^{Phe}$ (**Figure 2.5**). A fixed amount of RelA (0.18 μM) was titrated against a range of RAC concentrations.

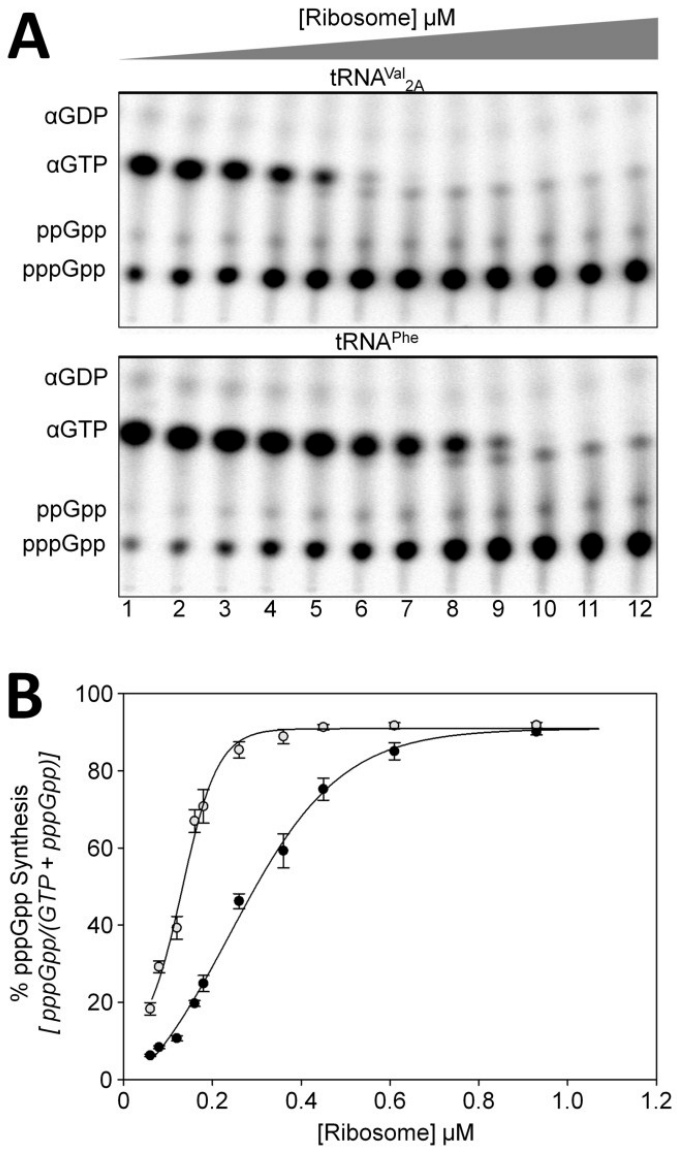

Figure 2.5: Differential (p)ppGpp synthesis. (A) The formation of (p)ppGpp was determined by thin layer chromatography using a range of concentrations (1 μM to 60 nM) of stalled ribosome complexes containing either $tRNA^{Val}$ (upper panel) or $tRNA^{Phe}$ (lower panel) in the ribosomal A-site. (B) Summarised data from five independent replicate experiments using ribosomal complexes with either (●) $tRNA^{Phe}$ or (○) $tRNA^{Val}$ in the ribosomal A-site.

Ribosome complexes were initially formed at high ribosome concentrations (2.6 μM) and then subsequently diluted with reaction mixtures containing RelA and nucleotide substrates (1 mM ATP, 0.1 mM GTP, and [α-^{32}P]-GTP). Formation of RAC at high concentrations ensures efficient ribosome complex formation. The subsequent dilution of the complex is analogous to when cells are recovering from stringent response, during which the amount of RelA activating complexes gradually decreases as the A-site tRNA dissociates. The sigmoidal nature of the resulting data is a result of both the background activity of RelA at low ribosome concentrations and the complete substrate depletion in samples containing high ribosome concentrations. Repeat analysis of five independent experiments consistently reveals a more than 2-fold difference in RAC concentrations that effectively stimulate RelA-dependent (p)ppGpp formation (**Figure 2.5B**). The difference is highlighted by comparing the ribosome concentrations that result in 50% conversion of [α-^{32}P]-GTP into (p)ppGpp. The RACs formed with $tRNA^{Val}_{2A}$ stimulated RelA activity at concentrations lower than that obtained with similar ribosome complexes formed with $tRNA^{Phe}$. These results are in agreement with our hypothesis that the tighter binding $tRNA^{Val}_{2A}$ forms a stable ribosome complex and can effectively stimulate RelA activity at significantly lower stalled ribosome concentrations.

Time course analysis of (p)ppGpp formation was also performed with samples containing different RAC concentrations (**Figure 2.6**). As with the experiment in **Figure 2.5**, the assembled RAC complexes were diluted prior to addition of RelA. Aliquots for analysis were removed from the samples at increasing time points after the addition of RelA. For RAC formed with either $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$ there was a large enhancement of (p)ppGpp formation in comparison to the no ribosome controls. All samples contained 0.18 μM RelA, and the maximal rates of (p)ppGpp formation were 0.12 μM/min in the absence of RACs and 2.1 and 3.3 μM/min for the samples containing 1.8 μM RACs formed with $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$, respectively.

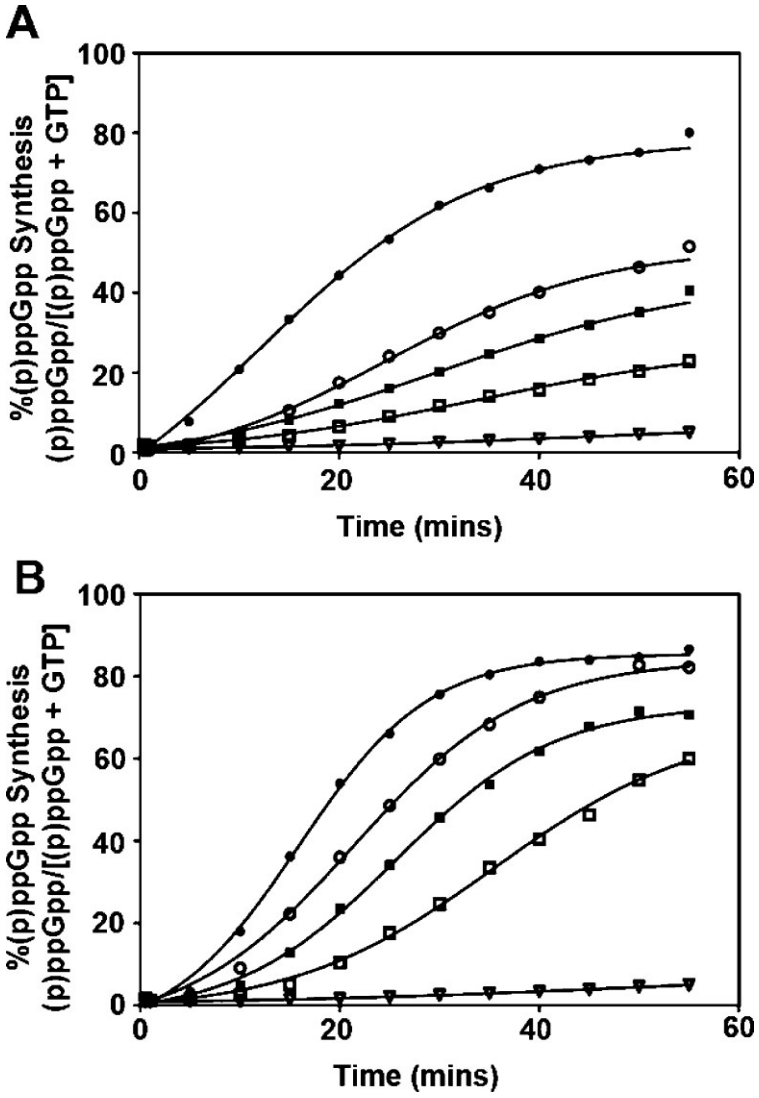

Figure 2.6: Rates of (p)ppGpp synthesis. RelA-dependent (p)ppGpp formation was measured in the presence of stalled ribosome complexes with either $tRNA^{Phe}$ (A) or $tRNA^{Val}$ (B) in the ribosomal A-site. Synthesis over time was measured using stalled ribosome complexes that have been diluted to 1.8 (●), 0.63 (○), 0. 45 (■), or 0.25 μM (□). Synthesis in the absence of ribosomes was also quantified (▽).

Comparison of the data from diluted RACs assembled with $tRNA^{Phe}$ (**Figure 2.6A**) and $tRNA^{Val}_{2A}$ (**Figure 2.6B**) reveals significant difference in (p)ppGpp formation. In all cases there appears to be a measurable delay in (p)ppGpp formation upon the addition of RelA; this may be a result of additional steps in RAC recognition by RelA prior to (p)ppGpp formation. As with the data from samples in **Figure 2.5**, the more tightly binding $tRNAVal_{2A}$ exhibits enhanced RelA-dependent (p)ppGpp formation.

2.4 Discussions

Our investigation into RelA stimulation by RACS with a tightly or weakly binding deacyl-tRNA in the ribosomal A-site was rationalized by two previous observations that pertain to the termination of RelA-mediated (p)ppGpp synthesis. The first was the current model for passive dissociation of deacyl-tRNAs from the ribosomal A-site for the attenuation of RelA activity (**Figure 2.1**). The second was the data demonstrating differences between the dissociation rates of different tRNA species from the ribosomal A-site. Our hypothesis was that ribosome complexes with slowly dissociating A-site bound deacyl-tRNAs would stimulate RelA activity at lower concentrations than similar ribosomal complexes formed with rapidly dissociating tRNAs. Presented data reveal that the inherent thermodynamic differences to ribosomal A-site binding by different deacyl-tRNAs are in fact reflected in RelA stimulation.

2.4.1 Stimulation of RelA Activity

Current models proposed that the binding of any deacyl-RNA to the ribosomal A-site was sufficient to stimulate RelA activity. Therefore, in our initial investigation we examined the ability of $tRNA^{Val}_{2A}$ to stimulate RelA activity, as earlier studies of RelA activity *in vitro* were done using $tRNA^{Phe}$ and polyU mRNA. As clearly demonstrated in **Figure 2.2**, all the components including ribosomes, mRNA, P-Site tRNA, and A-site $tRNA^{Val}_{2A}$, are required to maximally stimulate RelA-dependent (p)ppGpp formation, as was observed when polyU mRNA and

$tRNA^{Phe}$ were used instead (Wendrich et al. 2002). These data indicate that $tRNA^{Val}_{2A}$ bound to the A-site of a stalled ribosome complex is functionally equivalent to $tRNA^{Phe}$ with respect to RelA-dependent (p)ppGpp formation.

2.4.2 Unmodified versus Modified tRNA Stimulation of RelA Activity

When either fully modified or unmodified $tRNA^{Phe}$ occupied the ribosomal A-site, there was no significant difference in RelA activity, as shown in **Figure 2.3**. This outcome was predicted as previous results had reported only a marginal difference in A-site binding for modified and unmodified $tRNA^{Phe}$ (Fahlman et al. 2004). Although, the result suggests that the post-transcriptional modifications do not play a role in RelA activation, it does not rule out the possibility for the tRNAs where post-transcriptional modifications are critical for A-site binding (Yarian et al. 2000). If the modifications had a function beyond tRNA binding, we would expect a significantly reduced stimulation of RelA activity for the ribosome complexes formed with unmodified $tRNA^{Phe}$ than for similar complexes formed with fully modified $tRNA^{Phe}$. As previously mentioned, a possible role for the post-transcriptional modifications of $tRNA^{Val}_{2A}$ is unlikely as this tRNA isoacceptor is minimally modified and has no known modifications in the anticodon stem loop (Jühling et al. 2009) nor do the post-transcriptional modifications in the body of the tRNA affect ribosome binding (Fahlman et al. 2004).

2.4.3 tRNA Dissociation

Wendrich et al. had indirectly demonstrated, with $tRNA^{Phe}$, that the tRNA passively dissociates from the ribosomal A-site and RelA activity does not enhance or catalyse its dissociation. As deacyl-$tRNA^{Phe}$ weakly binds the ribosomal A-site, we investigated the dissociation of a tight binding tRNA in the presence and absence of active RelA. It was postulated that RelA could enhance the dissociation of tight binding tRNAs to match that of a rapidly dissociating tRNAs. The data presented in **Figure 2.4** and summarised in **Table 2.1,** reveal no

dependence of A-site dissociation by $tRNA^{Val}$ on RelA activity. These data demonstrate that RelA does not participate in the dissociation or destabilisation of tightly binding tRNAs as has been previously demonstrated for a weakly binding tRNA (Wendrich et al. 2002).

2.4.4 Attenuation of RelA Activity

Time scales for tRNA dissociation are on the order of magnitude pertinent to bacterial responses and growth. If the typical half-lives for tRNAs bound to the ribosomal A-site were on the order of seconds or milliseconds, differences between tRNAs would be insignificant with respect to cellular responses and growth. The reported half-lives for different tRNAs at the ribosomal A-site range from 13 to 210 min (Fahlman et al. 2004), which is a time scale relevant to bacterial growth. The longer half-lives surpass that of the doubling time of bacteria like *E. coli* during log phase growth in rich media and could be extremely detrimental if a significant number of ribosomes are trapped as stalled complexes. The toxicity of stalling a significant portion of ribosomes by deacyl-tRNA binding would be analogous to treatment with a ribosome targeting antibiotic such as tetracycline. Deacyl-tRNA dissociation rates *in vivo* may be significantly faster than the *in vitro* measured rates as a result of differences in ion concentrations. Nonetheless, even rates 2 orders of magnitude faster, for the slowly dissociating tRNAs, would result in significant stalling times of translating ribosomes. These slow tRNA dissociation rates were a major reason for the evaluation of whether RelA activity enhances tRNA dissociation rates.

The proposed model for RelA (Wendrich et al. 2002) suggests that during the recovery from the stringent response the tRNA passively dissociates from the ribosomal A-site to be aminoacylated by the respective aminoacyl-tRNA synthetase returning it into a substrate for active translation. During recovery from the stringent response there is a reintroduction of the limiting amino acids through the changes to the cellular metabolism and gene expression to support

amino acid biosynthesis. This results in a dwindling amount of stalled ribosome complexes (or RACs) as the complexes passively dissociate and the deacyl-tRNAs are returned to active translation by aminoacylation. To mimic this decreasing abundance of RACs in the recovery phase in cells *in vitro*, a dilution procedure was used to emulate the dissociation model. Ribosome complexes, or RelA activating complexes (RACs), were initially formed at high concentrations to support maximal association. At high concentrations, the RACs stimulate RelA activity regardless of the identity of the tRNA bound to the ribosomal A-site (**Figure 2.5**). The RACs are then diluted so the A-site bound deacyl-tRNA can dissociate as the A-site tRNA is generally the weakest binding component (Lill et al. 1986; Fahlman et al. 2004). Comparative analysis of the stimulation of RelA activity by ribosome complexes formed with either $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$ in the A-site was performed using this dilution method.

Comparison of RelA-mediated (p)ppGpp formation (**Figures 2.5 and 2.6**) reveals that RAC formed with $tRNA^{Val}_{2A}$ more robustly stimulated RelA activity after dilution than for RACs formed with $tRNA^{Phe}$. There is complete conversion of [^{32}P]-GTP to (p)ppGpp at lower ribosome concentrations when $tRNA^{Val}_{2A}$ occupies the ribosomal A-site (**Figure 2.5**). Moreover, $tRNA^{Val}_{2A}$ containing ribosome complexes resulted in higher levels of (p)ppGpp formation at all concentrations below 930 nM. Even at larger dilutions of ribosome complexes (to 60 nM), significant (~20%) conversion of GTP to (p)ppGpp is observed using ribosome complexes containing $tRNA^{Val}_{2A}$ in contrast to $tRNA^{Phe}$ where (p)ppGpp formation is essentially at background levels. Time course analysis (**Figure 2.6**) reveals reduced rates of (p)ppGpp synthesis after dilution of RACs formed with $tRNA^{Phe}$ in comparison to RACs forms with $tRNA^{Val}_{2A}$ in the ribosomal A-site. All comparisons clearly demonstrate that at reduced RAC concentrations the $tRNA^{Val}_{2A}$ containing ribosome complexes are more potent stimulators of RelA activity. This difference at the lower concentrations of RACS would be the most significant *in vivo*. During the onset of the conditions leading to the stringent

response there would be a gradual accumulation of the RACs until the initiation of the stringent response.

The absolute differences between the RelA stimulation by RACs assembled with $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$ cannot be interpreted as a direct measurement reflecting the thermodynamic parameters of binding. The RelA-mediated (p)ppGpp synthesis assay is a composite of molecular processes, such as tRNA dissociation, RelA binding and dissociation, and RelA-catalysed (p)ppGpp formation. The observed difference between the (p)ppGpp synthesis assays with the different tRNAs can therefore only be compared relatively. As consequence of being a multicomponent system, the absolute differences observed can be magnified or minimised by altering experimental parameters, such as the incubation time after ribosomal dilution and the addition of RelA. In light of these contributory factors, it is not unexpected that differences in binding affinity of the two tRNAs do not numerically match the differences between the RelA activity for reactions consisting of the different tRNAs.

2.5 Biological Implications

Differences in tRNA dissociation and subsequent stimulation of RelA activity have implications to the regulation of the metabolic pathways of *E. coli*. Our data predict that the absence or withdrawal of certain amino acid(s) will result in a more severe or long-lasting activation of the stringent response as the respective deacyl-tRNAs will dissociate with significantly slower rates from the ribosomal A-site. Our observations are adding to the developing complexity emerging with respect to the stringent response and individual tRNA sequences (Dittmar et al. 2005; Elf, Paulsson, et al. 2003; Elf, D. Nilsson, et al. 2003).

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Chapter 3

A Single Point Mutation in the C-terminal ACT Domain Impairs RelA-Mediated (p)ppGpp Synthesis.

3.1 Introduction

The RelA-mediated (p)ppGpp synthesis mechanism is dependent on the formation of the RelA activating complex (RAC). RAC is a 70S ribosome that contains an A-site bound deacylated transfer RNA (deacyl-tRNA) (Haseltine & Block 1973; Jenvert & Schiavone 2005; Wendrich et al. 2002; Payoe & Fahlman 2011). During amino acid starvation deacyl-tRNAs accumulate in the cytosol. The deacyl-tRNAs bind to the vacant cognate codon at the A-site of a stalled ribosome only when present in excess of the aminoacylated tRNAs (Rojiani et al. 1989). RelA is only activated in the presence of the bound deacyl-tRNA (Payoe & Fahlman 2011),the mechanism of activation is still unclear (Wendrich et al. 2002). In the current model for RelA mediated (p)ppGpp synthesis (**Figure 1.7**), RelA is thought to catalyse the formation of one molecule of pppGpp and dissociate from the stalled ribosome complex (Wendrich et al. 2002). RelA remains active in the cytosol for a finite amount of time, until inactivation (English et al. 2011). Reactivation of RelA requires the re-binding of RelA to the RAC (English et al. 2011; Wendrich et al. 2002). *In vivo,* RelA-mediated activation of the stringent response requires both the presence of the deacyl-tRNA and ribosomal protein L11 (Haseltine & Block 1973; Cashel 1975; Cashel et al. 1996; Yang & Ishiguro 2001b; Parker et al. 1976). Cells that carry L11 mutations are unable to activate the stringent response during amino acid starvation, a phenotype that is often described as being relaxed (Cashel et al. 1996). In these ribosomal rRNA, biosynthesis continues despite accumulation of (p)ppGpp (Cashel et al. 1996).

RelA localises to the 50S ribosomal subunit (Ramagopal & Davis 1974), possibly within the vicinity of ribosomal protein L11 (Schmidt et al. 1981). Through recombinant reconstitution assays, we know that the binding of RelA to the ribosomes is not compromised with the deletion of L11 (Jenvert & Schiavone 2007). Jenvert et al. have identified the proline-rich helix in the L11-NTD (N-

terminal domain) together with the flexibility of the L11-NTD contribute to the activation of RelA (Jenvert & Schiavone 2007).

The *E. coli* RelA protein can be viewed as having two functionally distinct domains (Metzger et al. 1989; Gropp et al. 2001; Friesen et al. 1978; Schreiber et al. 1991): the catalytic N-terminal region and the C-terminal regulatory region. By using the NCBI Conserved Domain Database (CDD), four structural domains are identified within these two regions (**Figure 1.5 and 1.6**) (Marchler-Bauer et al. 2009).

The N-terminal region of RelA houses the catalytic activity (Metzger et al. 1988) and contains a non-functional HD (Histidine–Aspartate) motif and a functional NT (Nucleotidyl-Transferase) domain. Generally, HD domains have nuclease or phosphohydrolase activity and are found in proteins that are typically metalloenzymes (Aravind & Koonin 1998). A common feature of these domains is the presence of specific combinations of metal-chelating residues: histidines and aspartates (Aravind & Koonin 1998). The nucleotidyl-tranferase domain catalyses the transfer of a pyrophosphate from a nucleotide triphosphates to a free hydroxyl group (Holm & Sander 1995).

The TGS and ACT domains are located within the C-terminal region of RelA. The TGS domain has been previously implicated in the regulation of SpoT (Battesti & Bouveret 2006) but the function in RelA has yet to be determined. SpoT is responsible for (p)ppGpp synthesis during other nutritional stress conditions, such as carbon, nitrogen, and iron limitation (Sarubbi et al. 1989; Ostling et al. 1996; Murray & Bremer 1996; V. Hernandez & Bremer 1991). The ACT domain is commonly a small regulatory domain found in a number of metabolic enzymes (Aravind & Koonin 1999; Ettema et al. 2002). Most ACT domains are 70-80 residues long and adopt the $\beta\alpha\beta\beta\alpha\beta$ fold (**Figure 3.1A**) (Aravind & Koonin 1999). In SpoT, the ACT domain is thought to bind to a ligand that modulates the synthetase and hydrolase activity of SpoT (Washio et al.

2011). The function of the ACT domain in RelA had not been studied until now. The ACT domain of RelA adopts the same secondary structure that is common to ACT domains as shown in **Figure 3.1B.** The structure was generated using the secondary structure prediction modelling program I-TASSER (Roy et al. 2010).

To date, there are only three reports regarding the characterisation of the RelA C-terminal region (Gropp et al. 2001; Schreiber et al. 1991; Yang & Ishiguro 2001a). The first 455 residues of RelA can be expressed to generate a ribosome-independent catalytic fragment (Schreiber et al. 1991), although this protein fragment is unstable *in vivo.* Wildtype RelA is reported to have an *in vivo* half-life of three hours, while the C-terminal truncated mutant (Δ455-744) has a half-life of 7.5 minutes (Schreiber et al. 1991). The current proposed mechanism of RelA regulation via the C-terminal region involves either a dimer or oligomer formed by RelA, which has a negative effect on (p)ppGpp synthesis (Yang & Ishiguro 2001a; Gropp et al. 2001). In other ACT domain containing proteins, dimer formation is required to mediate allosteric regulation of the enzyme (Robin et al. 2010; Feller et al. 2006). It is therefore possible that the regulatory mechanism entails RelA-RelA dimer formation. Gropp et al. identified three residues important for the oligomerisation of RelA: Cys-612, Asp-637, and Cys-638 (**Figure 1.6**). These residues are not present within either the TGS (406–465) or the ACT (667–731) domains. This led us to propose that perhaps, the ACT domain is involved in another function besides oligomerisation.

In this report we present our investigation on the RelA C-terminal domain. We report that full domain deletion drastically impacts RelA activity. We thus focused on the ACT domain of RelA as this domain has the potential to be a candidate regulator of RelA activity. We report that a single point mutation to a conserved region of the ACT domain significantly impairs RelA activity. Furthermore, contrary to previous reports on C-terminal deletion mutants (Jenvert & Schiavone 2007; Wendrich et al. 2002; Yang & Ishiguro 2001b).

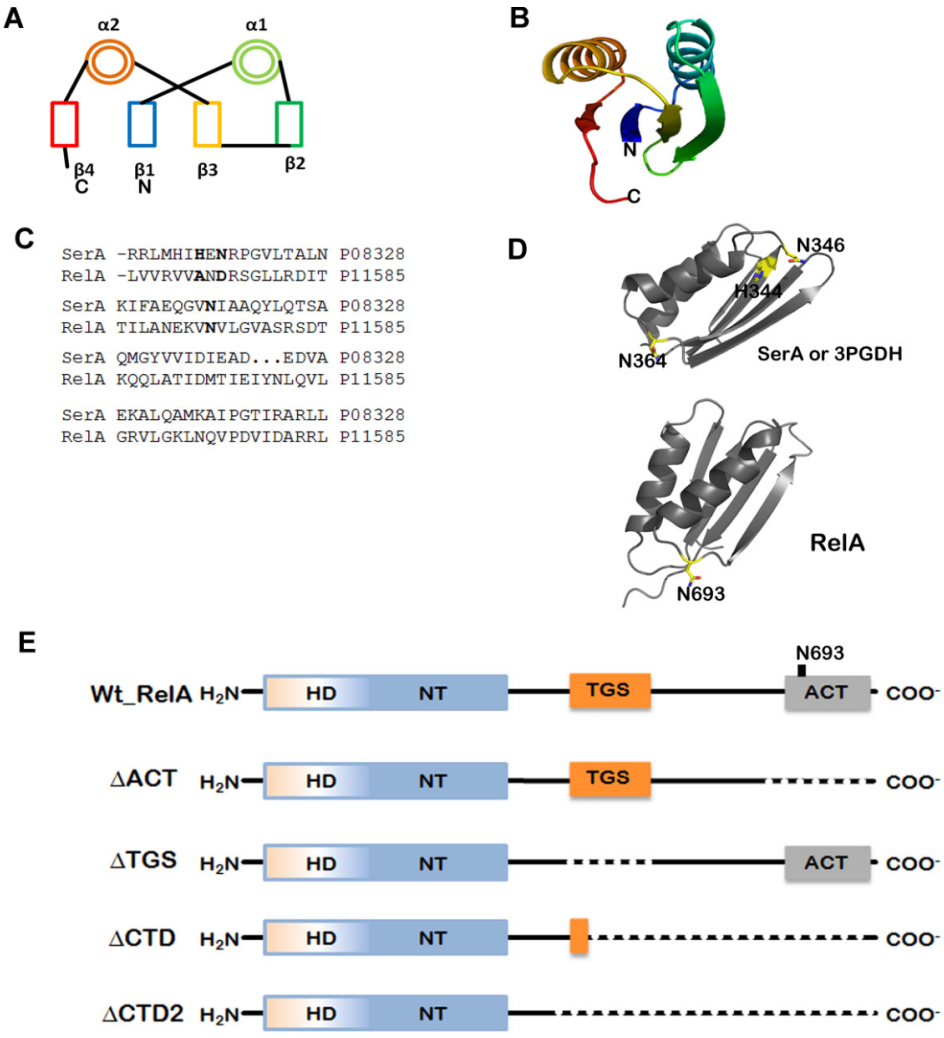

Figure 3.1: Structure and position of the RelA ACT domain. (A) ACT domain fold, N - N-terminus and C - C-terminus. (B) Predicted secondary structure for RelA ACT domain. (C) Sequence alignment of the ACT domain from *E. coli* SerA (3PGDH) and *E. coli* RelA. The bolded letters in SerA are the residues involved in serine interaction, the corresponding residues are also bolded in RelA. (D) Structure of the ACT domains from SerA and RelA indicating the positions of the residues bolded in C. N693 was the target for mutagenesis. (E) Schematic of all RelA constructs used in this study.

we report that we are still able to obtain (p)ppGpp synthesis *in vitro* in the absence of ribosomal protein L11

3.2 Materials and Methods

3.2.1 Expression Vectors and Mutagenesis

A clone of the wild-type *E. coli* RelA with a 6× histidine tag in a pCA24N expression vector was obtained from the ASKA strain from the National Institute of Genetics (Japan) (Kitagawa et al. 2005). Mutations to the wild-type RelA sequence were performed by site-directed mutagenesis (Chapnik et al. 2008; Winans et al. 1985). The DNA oligonucleotide pairs (IDT, USA) listed in **Table 3.1** were used to generate the mutants. All mutations were verified by DNA sequencing by the Medical Genetics Applied Genomics Centre (University of Alberta, Canada).

All the generated mutants used a standard site directed mutagenesis protocol. All enzymes used were purchased from Invitrogen. Template DNA (10 µg) was denatured for 30 minutes in 1M NaOH and 1 mM EDTA. Denatured DNA was purified by ethanol purification. Resuspended denatured DNA was then mutagenised with primers listed in **Table 3.1** via the following cycling conditions: 95^{o}C (1 min); 18 cycles of 94^{o}C (30 seconds), 55^{o}C (30 seconds), 68^{o}C (13.30 minutes); 94^{o}C (1min), 72^{o}C (10 minutes), 4^{o}C hold. PCR reaction was then subjected to *Dpn1* digestion to eliminate the template DNA.

To generate the ΔCTD mutant (Δ434-744) a *NotI* restriction site was introduced at position 1255 of the *relA* gene. This allowed us to use the internal 3'-*NotI* restriction site (located after the stop codon on 3' end of the plasmid) to generate the C-terminal truncated mutant. In order to generate the ACT deleted mutant, we introduced a stop codon at amino acid position 680 of the RelA protein. In order to generate the ΔΔTGS:ACT (or ΔCTD2) and ΔTGS, mutants two primers sets were used. First, primer set 4 was used to delete the TGS domain.

The primer was designed to anneal to the region adjacent to the TGS domain. After PCR amplification, in addition to having the TGS domain removed, the resulting construct also contained a frame-shift mutation that introduced a stop codon at amino acid position 437. The expression of this plasmid thus resulted in a protein that contained only the NT domain of the protein. Primer set 5 was used to reset the reading frame, resulting in a construct that had the catalytic N-terminal region and the ACT domain but not the TGS domain.

3.2.2 Protein Purification

Plasmids encoding the different RelA proteins were transformed into BL-21 DE3 cells. Enzymes were purified as previously described (Payoe & Fahlman 2011) and in Chapter 2 section 2.2.2.

3.2.3 Ribosome Purification

70S ribosomes were purified from MRE600 cells and ΔL11-70S ribosomes were purified from K12 *rplK:kan* cells. Ribosomes were prepared as follows. Cells were grown in LB media until OD of 0.5 at 600 nm. The culture flasks were incubated on ice for 30 minutes, before the cells were harvested by spinning at 5000 x *g* for 10 minutes at 4^{o}C. Cells were resuspended in lysis buffer [50 mM Tris-HCl, (pH 7.5); 1 M NH_4Cl; 10 mM $MgCl_2$; 0.5 mM EDTA; 6 mM βME]; 30 mL for every 5g of cells. The lysates were cleared by centrifugation at 20 000 rpm for 20 minutes at 4^{o}C in a Beckman-Coulter Avanti^{TM} J-20 Centrifuge. The cleared lysates were then centrifuged at 37,000 rpm for 16 hrs at 4^{o}C in a Beckman-Coulter Optima L-90K Ultracentrifuge. The dark brown pellet was resuspended in 4 mL of Buffer 1 [50 mM Tris-HCl, (pH 7.5); 1 M NH_4Cl; 10 mM $MgCl_2$; 0.5 mM EDTA; 6 mM βME]. The resuspended ribosome suspension was transferred to a chilled 1.5 mL centrifuge tube and spun at 14,000 rpm for 30 min at 4^{o}C. The supernatants were pooled and layered onto 10% sucrose cushion [10% Sucrose (v/v); 50 mM Tris-HCl (pH 7.5); 1 M NH_4Cl; 10 mM $MgCl_2$; 6 mM βME].

Table 3.1 Primers Used in the Design of RelA Mutants

Set	Construct	Primer Sequence
1	5' - ΔCTD	5'- CCA CAG TGA TGT CGG CGG CCG CTG CAT CGG GG-3'
	3' - ΔCTD	5'- CCC CGA TGC AGC GGC CGC CGA CAT CAC TGT GG-3'
2	5' - N693A	5'- CGA GAA GGT GGC CGT GCT TGG CGT TGC C -3'
	3' - N693A	5'- GGC AAC GCC AAC GAC GGC CAC CTT CTC G -3'
3	5' - ΔACT	5'- GAG CTA CTC CGC CGG A**TA A**AT TCG CTG GTG GTC CGC G -3'
	3' - ΔACT	5'- CGC GGA CCA CCA GCG A**AT T**TA TCC GGC GGA GTA GCT C- 5'
4	5' - ΔCTD2	5'- TCT TTG ACG ACC *GCA GAA ACA GCC GAA CCC C* -3'
	3' - ΔCTD2	5'- *CGG CTG TTT CTG* CGG TCG TCA AAG ACC TGA C -3'
5	5' - ΔTGS	5'- GGT CTT TGA CGA CCG GCA GAA ACA GCC GAA CC -3'
	3' - ΔTGS	5'- GGT TCG GCT GTT TCT GCC GGT CGT CAA AGA CC -3'

For primer set 1, the *NotI* restriction site is underlined. For primer set 3, the stop codon is bolded. Primer set 4 was designed to anneal to the region adjacent to the TGS domain. ΔCTD2 can also be referred to as ΔΔTGS:ACT.

The cushions were spun at 37,000 rpm for 16 hrs at 4^{o}C. The yellowish pellets are suspended in 4 mL of Buffer 1. The suspension was cleared again by centrifugation in 1.5 mL tubes spun at 14,000 rpm for 30 min at 4^{o}C. The pooled supernatants were layered onto a 30% sucrose cushion [30% Sucrose (v/v); 50 mM Tris-HCl (pH 7.5); 1 M NH_4Cl; 10 mM $MgCl_2$; 6 mM βME]. The ribosomes were pelleted by centrifugation at 37,000 rpm for 16 hrs at 4^{o}C. The cleared pellets were suspended in 1 mL of Buffer 2 [50 mM Tris-HCl, (pH 7.5); 100 mM NH_4Cl; 10 mM $MgCl_2$; 6 mM βME]. The pooled ribosomes were layered into a tube containing Buffer 2 and subjected to another centrifugations step at 37,000 rpm for 16 hrs at 4^{o}C. Final ribosome pellets were suspended in Ribosome Storage Buffer (50 mM Tris-HCl pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM βME) and aliquots stored at -80^{o}C.

3.2.4 Unmodified tRNAs and mRNA Preparation

Unmodified tRNAs and mRNAs were prepared by *in vitro* transcription. The protocol is described in (Payoe & Fahlman 2011) and section 2.2.3.

3.2.5 In vitro (p)ppGpp Synthesis Assay

In the standard (p)ppGpp synthesis assay, RelA activating complexes (RAC) were assembled by the following method. An aliquot containing 10 µM ribosomes (70S, ΔL11 (L11 deleted 70S ribosomes), depL7/L12 (L7/L12 depleted 70S ribosomes)) were first heat activated by incubating at 42^{o}C for 2 minutes, then slowly cooled to 22^{o}C. The 10 µM 70S ribosome stock was prepared in polyamine containing buffer or PA buffer (30 mM HEPES-KOH (pH 8.0), 15 mM $MgCl_2$, 15 mM KCl, 1 mM β-ME, 50 µM spermine, and 2 mM spermidine). A 9.8 µL sample of heat activated 10 µM ribosomes was programmed with mRNA ($mRNA^{Val}$ or $mRNA^{Phe}$) by the addition 3.7 µL of 50 µM $mRNA^{Phe}$ and incubating for 2 minutes at 22^{o}C. To fully occupy the ribosomal P-Site, the programmed ribosomes were incubated with 3.7 µL of 50 µM $tRNA^{Met}$ for 10 minutes at 22^{o}C. Lastly, the appropriate A-Site tRNA (3.7 µL of 50 µM $tRNA^{Phe}$) was added and the

sample was incubated for 1 hr at 22^{o}C. For all the described steps, each of the added solutions contained 1× reaction buffer (20 mM HEPES-KOH (pH 8), 15 mM $MgCl_2$, 15 mM KCl, and 1 mM β-ME). All added RNAs were in a 1.8-fold excess with respect to the 70S ribosomes. Concentration of components in the assembled RAC were: 4.7 µM ribosomes, 8.9 µM $mRNA^{Phe}$, $tRNA^{Met}$, and $tRNA^{Phe}$.

Each purified construct of RelA was diluted appropriately in PA buffer to prepare a 0.27 µM RelA Mix (final concentration 1X PA buffer). For example: 10.9 µL of 0.83 µM wildtype RelA was added to 3.4 µL of 10X PA buffer and diluted to a total volume of 34 µL.

A 30 µL of RelA mix was added to 10 µL of the assembled RAC mixture. After mixing by pipette, 18 µL of the RAC-RelA mix was transferred to a 0.6 µL eppendorf tube containing 2 µL of 10X substrate mix [10 mM ATP, 1 mM GTP, and α[^{32}P]-GTP (1 µL of labelled GTP to 20 µL of unlabelled GTP)]. Final concentrations in the reaction were as follows: 1 µM ribosomes; 2 µM $mRNA^{Phe}$, $tRNA^{Met}$, $tRNA^{Phe}$; 0.18 µM RelA; 1X substrate mix [1 mM ATP, 0.1 mM GTP].

The reaction tube was incubated for 1 hr (unless otherwise indicated) at 22^{o}C. Aliquots of the reaction were quenched at the following time points: 0.5, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 40, 50, and 60 minutes. Quenching was performed by adding 1 µL of reaction to 1 µL of formic acid (88%). Immediately after quenching, 1 µL of the quenched reaction was spotted onto a prepared PEI (polyethyleneimine) cellulose flexible back TLC plate (FisherScientific) to be used for thin layer chromatography (TLC) analysis as previously described (Payoe & Fahlman 2011). After the chromatography run, the TLC plates were exposed to a phosphorimager screen which was then visualised and quantified using a phosphorimager (GE Healthcare). ImageQuant^{TM} and GraphPad were used for data analysis and curve fitting, respectively.

3.2.6 70S Depletion of L7/L12 Ribosomal Proteins

This assay was performed in 4^{o}C room using pre-chilled tips and tubes. 450 µL of Extraction Buffer [20 mM Tris-HCl (pH 7.5); 600 mM NH_4Cl; 20 mM $MgCl_2$; 5 mM βME] was added to 50 µL of the 10 µM 70S ribosomes. The sample was mixed by pipette. After mixing 10 times, 250 µL of cold 95% ethanol was added to the sample. The sample was mixed by pipette and was allowed to stand for 5 minutes. After this step, another 250 µL of cold 95% ethanol was added. After another 5 min of incubation, the samples were centrifuged at 15 000 x g for 30 minutes at 4^{o} C to pellet the ribosomes.

The ribosome pellet was dissolved in 25 µL of Resolve Buffer A [20 mM Tris-HCl (pH 7.5); 30 mM KCl; 7 mM $MgCl_2$; 5 mM βME]. The pellet was stirred with a pipette to dissolve. Finally, 25 µL of Resolve Buffer B [20 mM Tris-HCl (pH 7.5); 30 mM KCl; 7 mM $MgCl_2$; 50% Glycerol (v/v); 5 mM βME] was added to the samples. Aliquots were flash-frozen and stored at -80^{o}C until use.

The supernatant was removed and subjected to acetone precipitation (4:1 ; acetone : sample). These samples were stored in -20^{o}C for 16 hrs. The supernatants were then centrifuged at 15,000 *x g* for 30 minutes at 4^{o} C to pellet proteins. The pellets were suspended in 30 µL of $TAKM_7$ buffer [50 mM Tris-HCl (pH 7.5); 70 mM NH_4Cl; 30 mM KCl; 7 mM $MgCl_2$]. The samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were run on 10% polyacrylamide gels to confirm removal of ribosomal protein L7/L12.

3.3 Results

3.3.1 Mutation to the C-terminal Domain of RelA.

Five different RelA constructs (**Figure 3.1E**) were generated using primers indicated in **Table 3.1**: the ΔCTD mutant (Δ435 – 744), the ΔTGS mutant (Δ406 – 461), the ΔACT mutant (Δ 668 – 744), the ΔCTD2 or ΔTGS:ΔACT mutant (Δ407-

744), and the N693A point mutant. The N693A point mutant was chosen because the residue was shown to be critical for ligand binding by SerA (**Figure 3.1C and D**) and the ACT domains of other proteins (Aravind & Koonin 1999; Chipman & Shaanan 2001; Grant 2006).

Using an *in vitro* assay the activity of each RelA protein was measured in the presence and absence of RACs assembled with wildtype 70S ribosomes. Tight-coupled 70S ribosomes were loaded with $mRNA^{Phe}$, $tRNA^{Met}$, and $tRNA^{Phe}$ to form the RelA activating complexes (RACs). Reactions were initiated by addition of nucleotide substrates. The reactions were quenched after an hour and aliquots were resolved by thin layer chromatography. Quantified results are shown in **Figure 3.2**. All of the mutants, except for the point mutant, showed a severe impairment in (p)ppGpp synthesis. A basal level of activity is observed in the absence of ribosomes for all RelA enzymes **Figure 3.2**. In the presence of ribosomes, only the wildtype and point mutant show an increase in (p)ppGpp synthesis. A previous report indicated that deletion of the C-terminal region of RelA resulted in a constitutively active ribosome-independent RelA (Gropp et al. 2001). In our study we show that ΔCTD (Δ434-744) had significantly reduced activity when used in equimolar concentration to wildtype RelA (**Figure 3.3**). Activity of ΔCTD (without RAC) is comparable to wildtype RelA (with RAC) only when present at an 8-fold higher concentration.

We next investigated (p)ppGpp activity for all RelA enzymes using RACs assembled with mutant ribosomes: ΔL11 70S ribosomes (henceforth referred to as ΔL11), and L7/L12 depleted 70S ribosomes (henceforth referred to as depL7). L11 is important for activation of RelA (Jenvert & Schiavone 2007) but not required for binding of RelA to the ribosome (Wendrich et al. 2002). L7/L12 has no known influence on RelA activity and is a protein required for factor recruitment during protein synthesis (Bocharov et al. 2004). Upon activation RelA resides as a ribosome-free ppGpp synthetase (**Figure 1.7**).

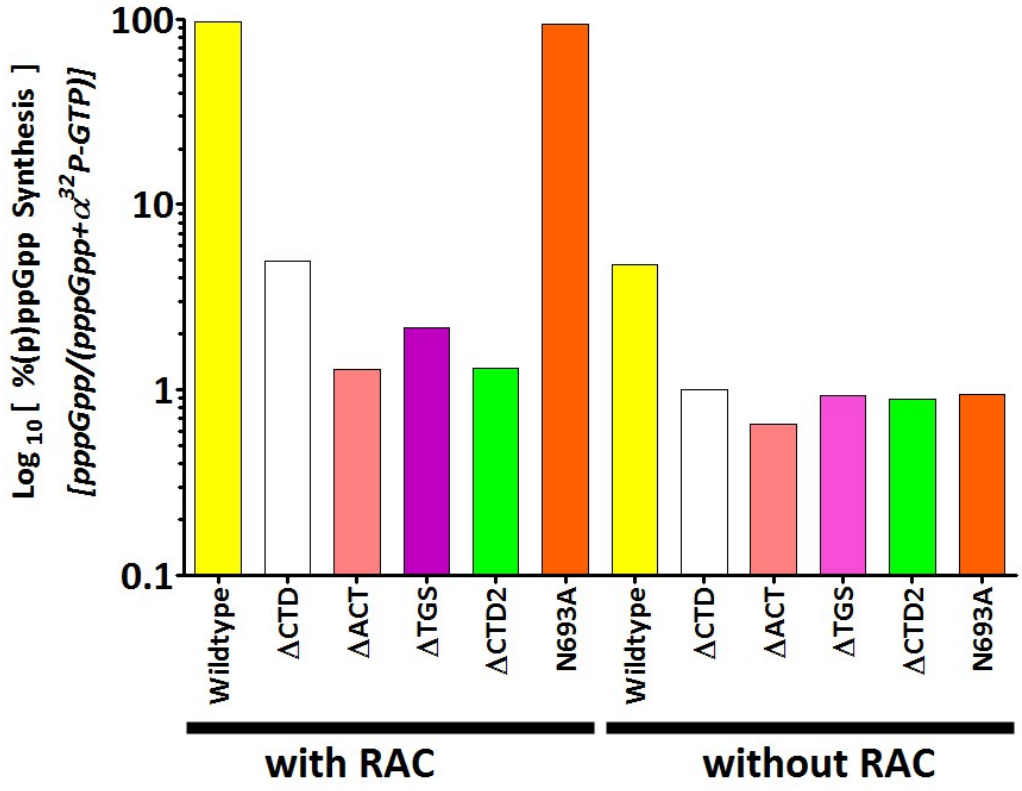

Figure 3.2: Endpoint pppGpp synthesis assay. The quantified results are from a single experiment. These results represents the relative amount of pppGpp formed for RelA (wildtype and mutants) in the presence and absence of RACs (RelA Activating Complexes). The RACs were assembled using wildtype 70S ribosomes. Assays were quenched after 1 hr and resolved by thin layer chromatography. Mutants correspond to the list shown in **Figure 3.1E**. The % synthesis is plotted as a $\log_{10}$ to show the weak basal activity of RelA.

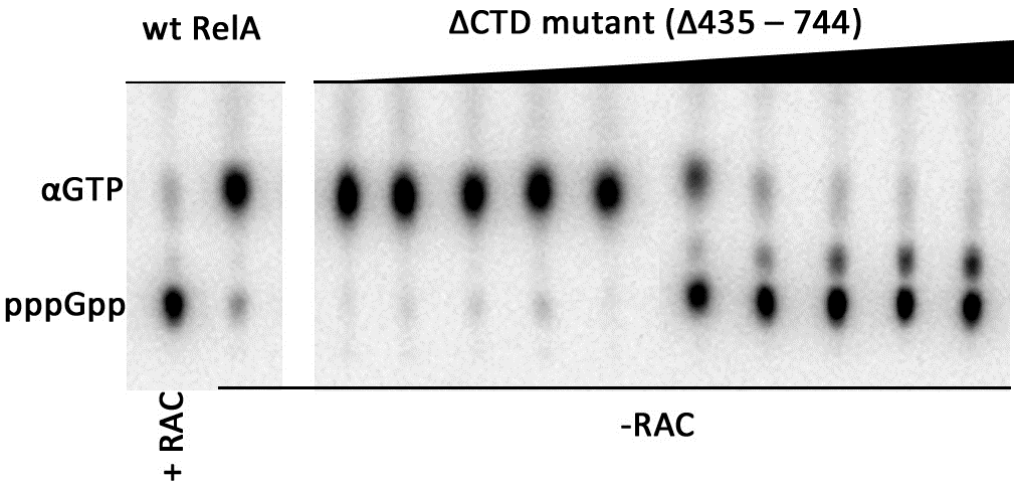

Figure 3.3: The ΔCTD mutant (Δ435 – 744) functions as a weak ribosome-independent pppGpp synthetase. Wildtype RelA and ΔCTD mutant RelA were assayed for the (p)ppGpp synthesis activity. Wildtype RelA was assayed at 0.18 μM concentration. ΔCTD mutant was assayed at: 0.07, 0.18, 0.40 , 0.60, 0.90, 1.5, 3.5, 7.7, 7.6 and 9.6 μM of ΔCTD. Wildtype ribosomes were used in the assembly of RACs (RelA Activating Complex). All ΔCTD assays were performed in the absence of ribosomes (RAC). The results of a 1 hr reaction are shown on this TLC plate.

After inactivation, inactive RelA needs to be recruited back onto the ribosome. We investigated the possibility of L7/L12 being involved in RelA recruitment to the ribosome. Both the ΔACT and ΔCTD2 retain a weak basal activity (ΔTGS was not text) (**Figure 3.4**). ΔCTD appears to have similar activity to wildtype RelA with mutant ribosome. The N693A mutant was inactive with ΔL11-RACs but not with depL7-RAC (**Figure 3.4**). While wildtype RelA had reduced activity with ΔL11-RACs, no effect was seen with depL7-RAC. The point mutant RelA (N693A) retained activity so it was used in further investigation to characterise the C-terminal domain of RelA. Further repeats of the assay are required to confirm if the observed results for the other RelA mutant enzymes and mutant ribosomes are valid.

3.3.2 Reduced Rates of (p)ppGpp Synthesis by the N693A Point Mutant.

For further investigation of the ACT domain's involvement in RelA activity, a time course study was done to see if the rate of (p)ppGpp accumulation had been altered by the point mutation. RelA and N693A were assayed using RACs assembled with wildtype 70S ribosomes (RAC). Reaction aliquots were taken at 2 minute intervals for 30 minutes and then 10 minute intervals to 60 minutes. Synthesis of (p)ppGpp was quantified as described in Materials and Methods and the results are shown in **Figure 3.5**. A clear difference can be seen in (p)ppGpp accumulation over time. Accumulation within the first 20 minutes is significantly reduced for the N693A (**Figure 3.5B**) compared to the wildtype RelA (**Figure 3.5A**). Each of the progress curve shows a delay in (p)ppGpp formation, probably implying a slow initial binding step. The delay is more pronounced in the case of the point mutant N693A.

If the delay in (p)ppGpp accumulation was due to a binding event, then pre-incubating RelA with the RAC prior to addition of substrate would decrease the lag in synthesis.

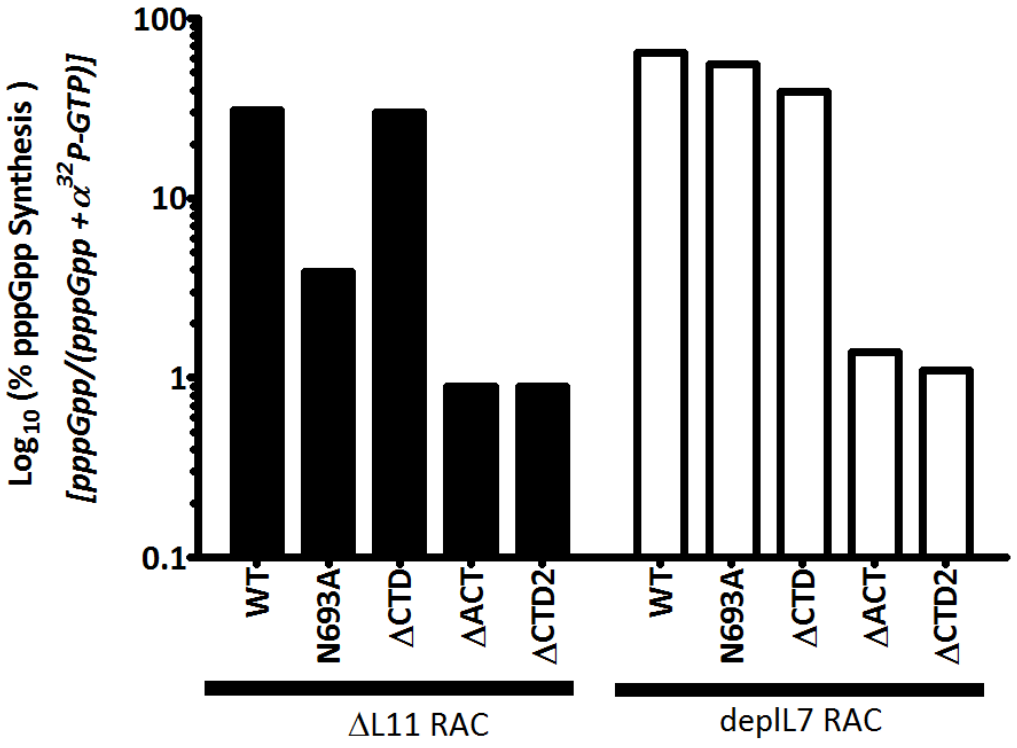

Figure 3.4: (p)ppGpp synthesis using RAC assembled with mutant ribosomes. RACs were either assembled with ΔL11 ribosomes (white) or depL7 ribosomes (black). These RACs were incubated with RelA and substrate. Reactions were quenched after 25 minutes and resolved by TLC. The figure is of the quantified results for a single experiment.

The time course assay was repeated with an additional incubation step where wildtype RelA or N693A was incubated with RAC for 30 minutes prior to the addition of substrate. As **Figure 3.5** shows, pre-incubation of RelA with the RACs significantly decreases the delay for both the wildtype and point mutant.

3.3.3 Point Mutation Appears to Affect Binding of RelA to Ribosomes.

The time course assay was repeated using the mutant ribosomes ΔL11-RAC and depL7RAC, with and without a RelA-RAC preincubation step. The results of this study are shown in **Figure 3.6**. Removal of L7/L12 had no effect on time course for either the wildtype or mutant. The (p)ppGpp synthesis curves are similar to that for wildtype ribosomes (compare **Figure 3.6B** with **Figure 3.5A)**. Deletion of L11, on the other hand, has a detrimental effect on both the point mutant and wildtype RelA enzymes (**Figure 3.6A**). The lag in (p)ppGpp accumulation could not be alleviated by preincubating RelA with the ribosomes. The most interesting finding was that the point mutant showed no activity both with and without RelA-ΔL11-RAC preincubation, even though wildtype RelA retained (p)ppGpp synthesis. The absence of ribosomal protein L11 decreased accumulation rates of (p)ppGpp to almost the same extent as the point mutation to the ACT domain. The results we see in **Figure 3.6A** (for the point mutant) is a combined effect of having both a point mutation and the absence of ribosomal protein L11.

3.4 Discussion.

The identification of an ACT domain at the C-terminal region of RelA prompted us to launch an investigation to gain further insight into the molecular mechanism of RelA regulation **(Figure 1.6 and 1.5**). We generated C-terminal mutants and tested their effects on (p)ppGpp accumulation using an *in vitro* assay (Wendrich et al. 2002; Payoe & Fahlman 2011). Our results show that the ACT domain may be involved in the interaction with the ribosomes. In addition, C-terminal mutation appears to affect the stimulation of RelA by the ribosomes.

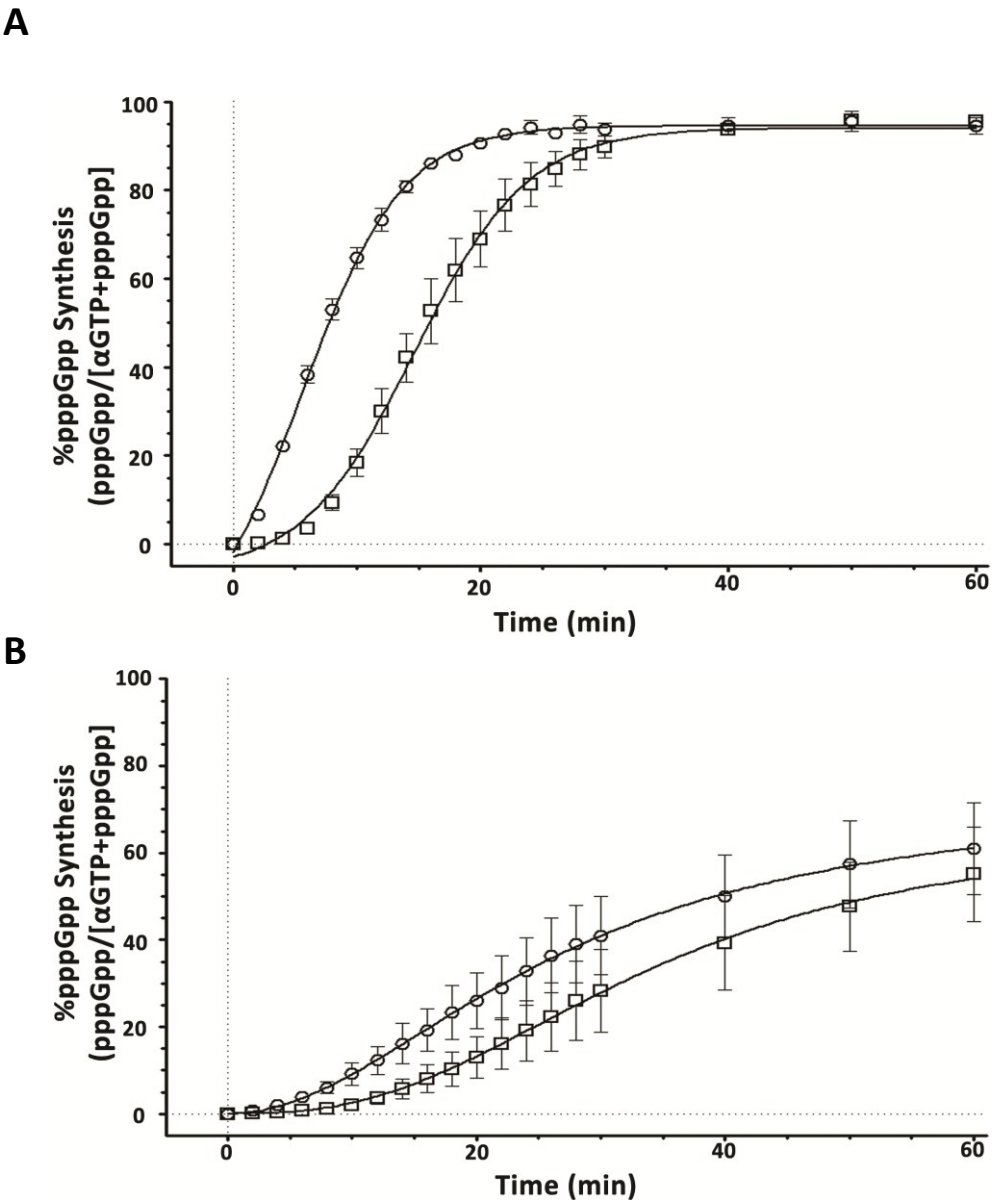

Figure 3.5: Rate of (p)ppGpp synthesis with and without a pre-incubation RelA and RAC. Wildtype RelA (A) or point-mutant N693A (B) and wildtype 70S ribosomes were used to measure the effect of RelA-RAC pre-incubation had on RelA mediated (p)ppGpp synthesis. Substrates were added either immediately after RelA was added to the RAC (○), or after 30 minutes of RelA-RAC pre-incubation (□). Aliquots were quenched at different times and spotted on TLC plates which resolved by thin layer chromatography. The curve represents the average of three independent assays.

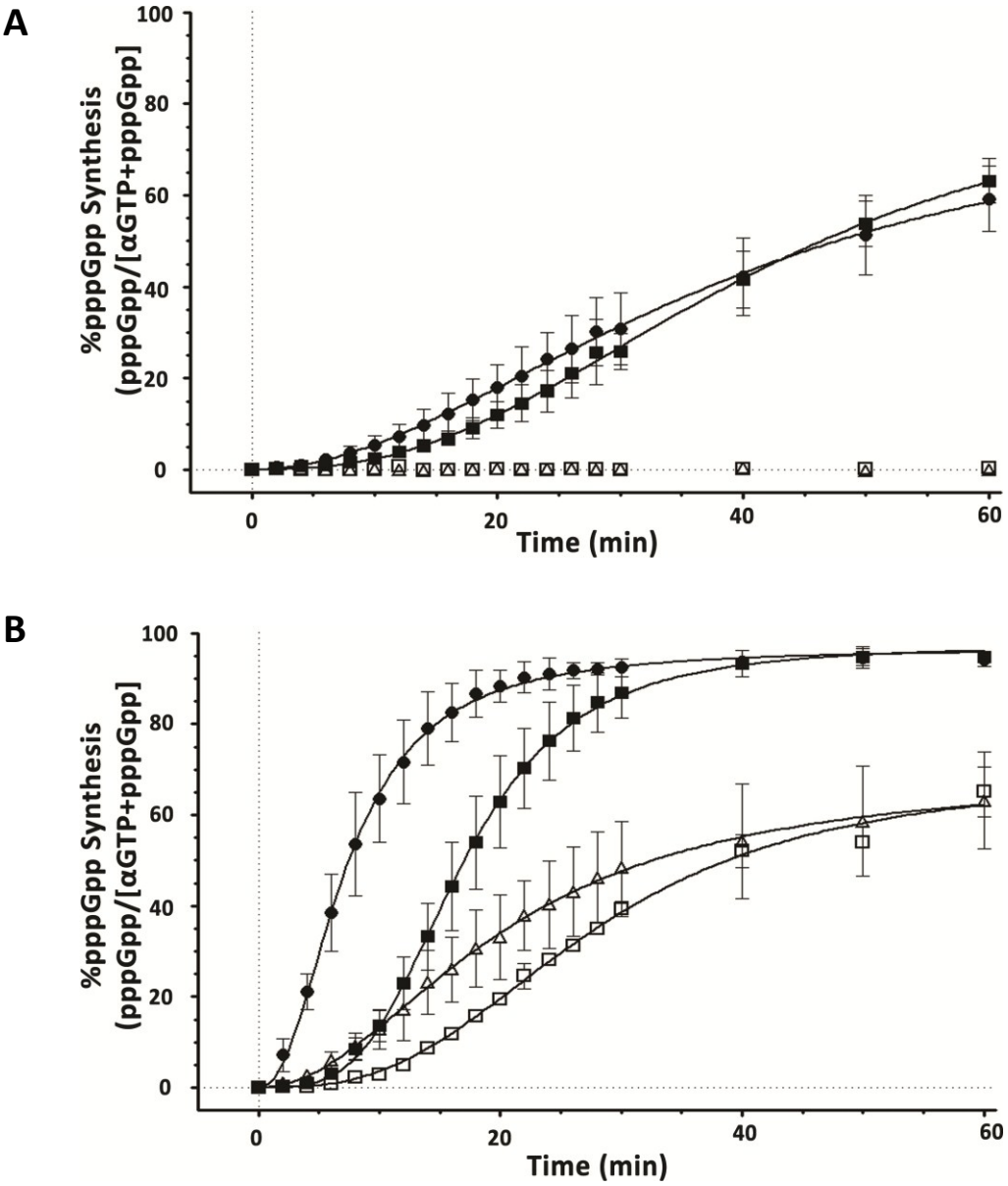

Figure 3.6: Rate of (p)ppGpp synthesis with and without a pre-incubation of RelA and mutant ribosomes assembled RAC. ΔL11 (A) and depL7 (B) ribosomes were in a time course assay. Substrates were added either immediately after RelA was added to the RAC (● for wildtype and Δ for N693A) or after 30 minutes of RelA-RAC pre-incubation (■ for wildtype and □ for N693A). Aliquots were quenched at different times and spotted on TLC plates which were resolved by thin layer chromatography. The curve represents the average of three independent assays.

3.4.1 C-Terminal Domain Mutants of RelA are Weaker Ribosome Independent Synthetases.

The minimal region required for ribosome independent (p)ppGpp synthesis consists of the first 455 residues of RelA (Schreiber et al. 1991). Looking at the sequence of RelA (**Figure 1.6**) this region ends within the TGS domain. In our attempt to generate a ribosome-independent mutant of RelA we constructed the two C-terminal domain deletion mutants: ΔCTD (Δ433-744) mutant and ΔCTD2 (or ΔTGS and ΔACT). The ΔCTD2 only had basal activity that could not be amplified with addition of the activating complex (ribosomes or RAC) (**Figures 3.2 and 3.4**). The ΔCTD mutant is a much weaker synthetase compared to wildtype RelA (**Figure 3.3**). The weakened activity can be compensated for by having ΔCTD present at 8-fold higher concentration to the wildtype RelA in order to obtain comparable amount of (p)ppGpp synthesis (**Figure 3.3**). Deletion of single domains (either TGS or the ACT) rendered the enzyme inactive. It is unclear, at this moment, if the binding of RelA to the ribosome or the stimulation of RelA activity had been altered for these mutants. Two hypothetical models can be made in light of these results. The first model consists of the deletion of the domains which impair the ribosome●deacylated-tRNA●L11 stimulation and the subsequent activation of RelA. This conclusion assumes that RelA-ribosome interaction is not impaired. The second model consists of mutations that have a direct effect on RelA binding to the ribosomes which in turn affects activation of RelA.

3.4.2 Conformational Switch for RelA Activation.

It is possible that by deleting these domains, the structural integrity of RelA is compromised and the result is a severely impaired synthetase that can still bind to the ribosomes but is unable to become activated. In bifunctional Rel/SpoT enzymes, the synthetase and hydrolase function like a dual switch. When one domain is “switched on”, the other is “switched off”. RelA, a mono-

functional (p)ppGpp synthetase, is thought to have arisen from a gene duplication from an ancestral bifunctional *spoT* gene which is identified in many other bacteria (Atkinson et al. 2011; Atkinson & Hauryliuk 2012). In their characterisation of the bifunctional enzyme of *Streptococcus equisimilis*, Mechold et al. proposed a switch model that achieves reciprocal regulation of synthetase and hydrolase activities of Rel_{seq} (Mechold et al. 2002). The synthetase region of the catalytic domain in Rel_{seq} is thought to exist in an inactive state through interactions with the regulatory domain. These two domains are linked through a flexible hinged? region (Mechold et al. 2002; Hogg et al. 2004). When the interaction between the C-terminal regulatory domain and the N-terminal catalytic domain is altered, a subsequent conformational change leads to the activation of the synthetase and inhibition of the hydrolase (Mechold et al. 2002). *E. coli* RelA has no hydrolase activity but instead, contains the remnants of this hydrolase domain (**Figure 1.5**), which can be identified using domain prediction databases, such as NCBI CDD (Marchler-Bauer et al. 2009). Although RelA has lost the hydrolase function, it is possible to maintain the mechanism of reciprocal regulation. A fully functional C-terminal domain may be required to bring about the necessary intermolecular configuration to achieve activity. Large deletions to the C-terminal domain may thus disrupt the structural integrity of RelA and thereby result in weakened synthetic activity. Further mutants of RelA need to be generated in order to confirm this model.

3.4.3 Mutations to C-Terminal Domain Interrupt the Interactions between RelA and the Ribosome.

We observed that large deletions to the C-terminal region resulted in almost complete loss in (p)ppGpp synthesis. A single point mutant was then generated to examine the function of a single C-terminal domain of RelA, the ACT domain. We were interested in this domain as it is a known regulatory domain found in many metabolic proteins (Chipman & Shaanan 2001; Aravind &

Koonin 1999; Grant 2006). Multiple sequence alignments of the ACT domain superfamily identified a conserved loop region between the first strand and the first helix (Aravind & Koonin 1999) (**Figure 3.1**). This region, as well as a conserved asparagine residue on the adjacent loop (the corresponding residue in RelA was mutated to alanine), were shown to interact with amino acid serine in D-3 phosphoglycerate dehydrogenese (Grant et al. 1996; Chipman & Shaanan 2001).

Both the point mutant and wildtype RelA were able to catalyse (p)ppGpp synthesis when wildtype ribosomes were used (**Figure 3.2**). However, the rate of (p)ppGpp accumulation is evidently slower with the point mutant (**Figure 3.5**). Both wildtype RelA and the point mutant progress curves for (p)ppGpp synthesis had a noticeable lag in the initial transient phase (first 5 minutes). We hypothesised that this was due to a slow initial binding step. *In vivo,* RelA is generally found bound to ribosomes during nutrient rich conditions (Block & Haseltine 1975). To mimic this we decided to incubate RelA with the RAC (RelA activating complex) prior to substrate addition. For both enzymes, we observed a significant increase in (p)ppGpp accumulation rates (**Figure 3.5**)**,** confirming our speculation. The pre-incubation affected the wildtype rates of (p)ppGpp more than it did the mutant, which implies that the ACT domain is involved in RelA binding to the ribosome. Our studies using mutant ribosome supports this idea (**Figure 3.6B**). With depL7-RAC, preincubation of the enzymes prior to substrate addition also increased the (p)ppGpp synthesis.

Deletion of ribosomal protein L11, which has been shown by different independent studies to be required for the activation of RelA (Jenvert & Schiavone 2007; Yang & Ishiguro 2001b; Wendrich et al. 2002; Parker et al. 1976), severely effects (p)ppGpp synthesis (**Figure 3.6**). Wildtype RelA activity is reduced almost by 5-fold (compare **Figure 3.5A** with **Figure 3.6A**)**.** N693A shows no activity, even with pre-incubation. As activity is still observed for the

wildtype-RelA and ΔL11-RAC, we propose L11 functions to enhance another stimulator of RelA activity. This alternative activator of RelA is still unknown at the moment. Cells that carry a mutation in L11 are slower than wildtype in responding to amino acid starvation (Cashel et al. 1996; Fiil & Friesen 1968). It is possible that without L11, cells lose the ability to promptly adjust to amino acid starvation as the rapid activation of RelA is lost.

The combined effect of the ΔL11 with the point mutant is most intriguing, suggesting that ACT domain may be involved at an interaction interface between RelA and the ribosomes. Stimulation of N693A is unaffected as we are able to measure (p)ppGpp synthesis when wildtype ribosomes are used to assemble the RACs. If binding were not impaired, pre-incubating N693A with ribosomes would have been sufficient to cause some (p)ppGpp synthesis with ΔL11-RACs. Unlike complete domain deletions, the single point mutation does not interfere with the intermolecular conformational changes that are needed to activate the synthetase. Further validation of these observations is required by performing binding assays to determine the effects on ribosomes. In our attempt to determine if binding is indeed affected we have used a combination of ultracentrifugation and size exclusion chromatography. These results are ambiguous.

These results have led to the expansion of the RelA activation model from **Figure 1.7** to **Figure 3.7**. The new model takes into consideration the two steps that are required in order to achieve RelA activation on the ribosomes. RelA initially starts as a ribosome bound protein. RelA is in proximity to ribosomal protein L11. When a deacylated-tRNA is bound at the A-site of the ribosomes, a switch is triggered within the C-terminal region of RelA. Stimulation of the C-terminal switch leads to a conformational change in the N-terminal region that activates the synthetase. In its new conformation, RelA's affinity for the ribosome decreases and RelA dissociates from the ribosomes.

RelA remains active and ribosome-free until inactivated and RelA is in a form that recognises and binds the ribosome via the C-terminal ACT domain.

The presence of the ACT domain in a protein not directly involved in biosynthesis is in itself a puzzling occurrence. Although previous studies have determined some properties of the C-terminal region of RelA, our investigation has added to this knowledge by focusing on a particular domain of RelA, the ACT domain. Furthermore, as the general domain organisation is shared amongst most members of the Rel/SpoT homolog superfamily, our result broadens the knowledge of GTP pyrophosphokinases regulation.

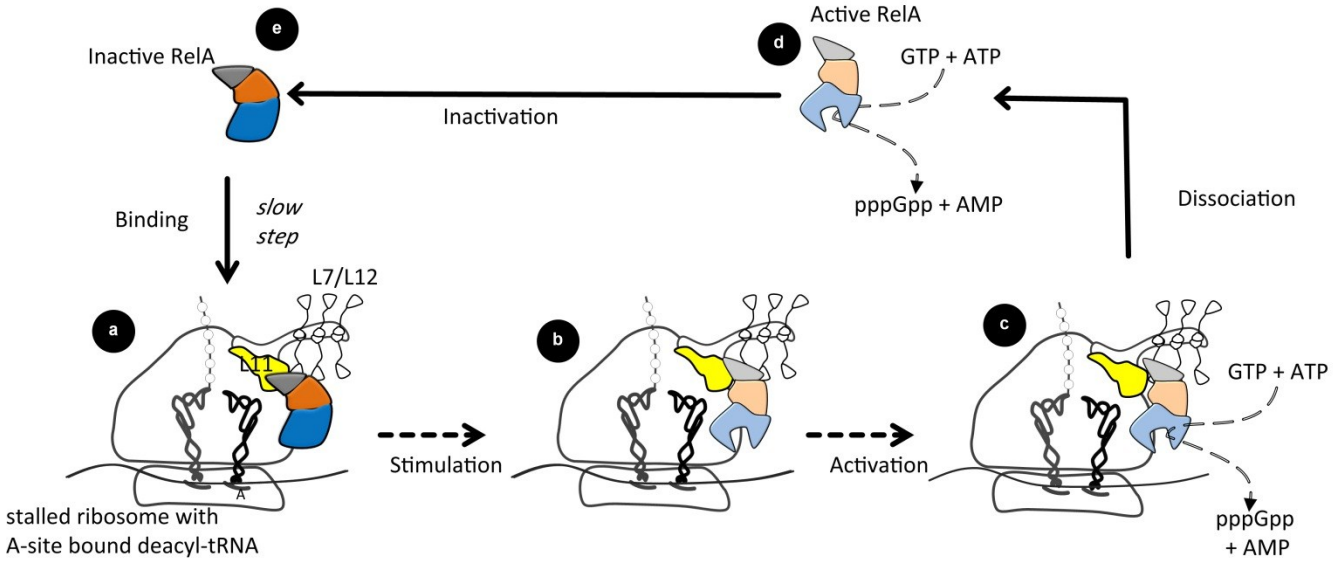

Figure 3.7: Model for RelA binding and activation on stalled ribosome complexes. Inactive RelA is thought to be bound to the ribosome (a). When a deacylated tRNA binds to the A-site during instances of amino acid starvation, RelA is stimulated to undergo a conformational change (b) that lead to activation. The activation mechanism of RelA is thought to involve an intermolecular domain reorganization that releases the N-terminal synthetase (dark and light blue) domain from the regulatory control of the C-terminal domain (c). This process is assumed to be a rapid process and is therefore indicated as dashed lines. When activated, RelA catalyses the formation of pppGpp using ATP and GTP. Activated RelA dissociates from the ribosome and remains active free in the cytosol (d) until it is inactivated (e). RelA has to rebind the ribosome to undergo another round (e). The binding of RelA to the ribosome is shown to be a slow step. This may be another method to regulate the number of times RelA is activated during amino acid starvation. The domains are indicated by the colours blue (catalytic region HD and NT), orange (TGS), and grey (ACT) and correlate to the organization map in **Figure 3.1**.

3.5 References

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Chapter 4

Effects of Amino Acids on RelA Activity.

4.1 Introduction

In addition to being building blocks of proteins, amino acids act as regulators of enzyme activity by interacting with specific domains within regulatory regions (Chipman & Shaanan 2001; Ettema et al. 2002; Avin-Wittenberg & Galili 2012). One such domain is the ACT domain which is the acronym for aspartate kinase, chorismate mutase and TyrA (prephenate dehydrogenase) (Aravind & Koonin 1999). Although the ACT domain has been identified in more than 100 proteins (Grant 2006), it has only been characterised (structurally) for ten different metabolic enzymes (Chipman & Shaanan 2001). There are still a number of proteins for which the function of the ACT domain has yet to be elucidated.

The ACT domain was first crystallised as part of the *Escherichia coli* (*E. coli)* D-3-phophoglycertate dehydrogenase (3PGDH or SerA) (Schuller et al. 1995), an enzyme involved in the catalysis of the first step in the serine biosynthesis pathway (Pizer 1963). In 3PGDH, serine is an allosteric inhibitor of 3PGDH, binding to the ACT domain and negatively regulating enzyme activity (Grant et al. 1996). Sequence alignments of the ACT domain superfamily revealed that the most conserved region is the loop between the first β strand and the first alpha helix (**Figure 3.1**) (Aravind & Koonin 1999). This region is also suggested to be critical for ligand binding (Aravind & Koonin 1999). In addition to this region, an asparagine residue located between the first helix and second β sheet is shown to interact directly with serine in SerA **(Figure 3.1D**) and mutations to any of these residues/region abolishes the inhibitory effect of serine on SerA (Grant et al. 1996).

RelA is amongst that list of proteins that do not have a specific function assigned to its ACT domain (Aravind & Koonin 1999; Chipman & Shaanan 2001). RelA is the enzyme required for the activation of the stringent response, during amino acid starvation, by catalysing the formation of the effector molecule

(p)ppGpp (Stent & Brenner 1961; Cashel 1975; Schreiber et al. 1991; Potrykus & Cashel 2008). *In vivo,* RelA is only activated on ribosomes with a deacylated tRNA bound to the A-site; these ribosomes are referred to as RACs or RelA activating complexes. In the current model for RelA mediated (p)ppGpp synthesis (**Figure 1.7**) after activation, RelA is a cytosolic protein. Details of the inactivation of RelA in the cytosol are unknown.

There are three hypothetical mechanisms through which RelA is inactivated. The first requires RelA to undergo an intermolecular conformational change where the C-terminal region inhibits the N-terminal catalytic domain (Hogg et al. 2004; English et al. 2011). English et al. propose an auto-inhibitory mechanism where after a number of rounds of (p)ppGpp synthesis, the N-terminal catalytic domain is inactivated by the C-terminal domain (English et al. 2011). The second requires RelA to form an inactive dimers as it comes across another RelA molecule in the cytosol (Gropp et al. 2001; Yang & Ishiguro 2001), whereas the third requires RelA to encounter a cytosolic factor or metabolite that inhibits activity by binding to one of its C-terminal domains (CTD). Given that the ACT domain serves as a regulatory domain for many metabolic proteins, via small ligand binding, it is possible that RelA activity is also regulated by the ACT domain. The regulation of RelA by small ligands has never been reported for RelA. However, if identified, it would suggest that RelA can also act as a sensor for a small metabolite.

The existence of an ACT domain in *E. coli* RelA led us to investigate the effect of amino acids on (p)ppGpp accumulation. Amino acids were chosen over other metabolites as they constituted almost half of the total amount of metabolites in *E. coli* (Bennett et al. 2009). Additionally, RelA activation is concomitant with amino acid starvation. The amino acid effect was also investigated on a point mutant we had generated for the study described in Chapter 3. The point mutation was at the asparagine residue, N693, that

corresponded to the asparagine residue involved in serine binding in 3PGDH (Chipman & Shaanan 2001; Schuller et al. 1995; Grant et al. 1996). Our results show that, *in vitro*, some amino acids are able to inhibit RelA mediated (p)ppGpp synthesis. The mechanism of inhibition did not appear to involve the RelA-ribosome interaction, implying that the amino acids do not affect RelA binding to the ribosomes by inhibiting RelA activity via an alternative mechanism.

4.2 Material and Methods

4.2.1 Primers and Plasmid

A hexahistidine tagged clone (ORF: JW2755) of RelA was obtained in a pCA24N plasmid from the National BioResource Project (NIG, Japan): *E.coli* ASKA collection (Kitagawa et al. 2005). Generation of point mutant N693A was done using standard site-directed mutagenesis (Winans et al. 1985). Primers used for site-directed mutagenesis are listed in **Table 3.1.** Chapter 3, section 3.2.1 contains the details for the generation of the point mutant N693A.

4.2.2 Protein Purification

Method is described in Chapter 3 Section 3.2.2.

4.2.3 Ribosome Purification

Method is described in Chapter 3 Section 3.2.3.

4.2.4 In vitro pppGpp Synthesis Assay

Concentration for assay components: Final concentration for each component in the reaction tubes was as follows: 70S ribosomes: 1 µM; poly-U mRNA: 1 mg/mL; $tRNA^{Phe}$: 5 µM; RelA: 0.15 µM; and 1X substrate mix. Substrate mix was prepared as a 10X solution containing: 10 mM ATP, 1 mM GTP, and $\alpha[^{32}P]$-GTP (1 µL of labelled GTP to 20 µL of unlabelled GTP)]. Substrates were added after 1 h RelA – RAC incubation.

RAC assembly for RelA enzymatic assay: The standard (p)ppGpp synthesis assay made use of pre-assembled RelA activating complexes (RAC). These were assembled using the following method. Ribosomes (10 µM) were heat activated by incubating at 42^{o}C for 2 minutes then slowly cooled to 22^{o}C. The 10 µM 70S ribosome stock was prepared in polyamine containing buffer or PA buffer (30 mM HEPES-KOH (pH 8.0), 15 mM $MgCl_2$, 15 mM KCl, 1 mM β-ME, 50 µM spermine, and 2 mM spermidine). Heat activated ribosomes were programmed with poly-U mRNA (Sigma-Aldrich) and incubated for 2 minutes at 22^{o}C. The programmed ribosomes were incubated with $tRNA^{Phe}$ for 1 hour at 22^{o}C. For each of the described steps, added solutions contained 1× reaction buffer (20 mM HEPES-KOH (pH 8), 15 mM $MgCl_2$, 15 mM KCl, and 1 mM β-ME).

Quenching and resolving assay aliquots: 1 µL reaction aliquots were quenched in 5 µL of formic acid (88%). Then, 1 µL of the quenched reaction was immediately spotted onto a prepared TLC plate to be used for thin layer chromatography (TLC) analysis as previously described (*28*). After the chromatography run, the TLC plates were exposed to a phosphorimager screen which was then visualised and quantified using a phosphorimager (GE Healthcare). ImageQuant^{TM} and GraphPad were used for data analysis and curve fitting, respectively.

Variation of protocol used for amino acid screening. Prior to substrate addition, amino acids were added either individually or as a tri-amino acid mix to final concentration of 1 mM. The reaction tube was incubated for 1 hour at 22^{o}C, reactions were quenched and treated as mentioned above.

Variation of assay protocol used measure to (p)ppGpp synthesis over time. Aliquots of the reaction were quenched at the following time points: 2, 5, 10, 15, 20, 25, 30, 45, and 60 minutes. As mentioned above, RelA (wildtype and point mutant N693A), ribosome and methionine were incubated an hour prior to substrate addition. (p)ppGpp synthesis was measure for five different

concentrations of methionine 0.5 mM, 2 mM, 5 mM, 10 mM and 20 mM. A subsequent assay was done wherein methionine was only added 15 minutes after the substrate addition.

4.2.5 In vitro Binding Assay and Western Analysis

Binding studies were performed as outlined in the following references (Wendrich et al. 2002; Shyp et al. 2012). Briefly: Illustra MicroSpin S-300 sephacryl columns (GE Healthcare) were pre-equilibrated by spinning the column with PA buffer at 700 x *g*. Experiments were done at a total volume of 100 µL. Reactions contained 0.5 µM ribosomes, 0.5 mg/mL poly-U mRNA, 2.5 µM $tRNA^{Phe}$, 0.15 µM RelA, 1 mM AMP-CPP (α,β-Methyleneadenosine 5'-triphosphate lithium salt) (Sigma Aldrich) and when present, methionine (Sigma-Aldrich), at final concentration of 20 mM.

Samples were loaded on the pre-equilibrated column and spun at 700 x g. After collecting the void volume, columns were washed five times with 100 µL of polyamine buffer. The void volume, which is reported to contain only ribosomes and any RelA bound ribosomes (Shyp et al. 2012), was analysed by silver stain and western blot for the presence of RelA. Equal quantities of protein were separated by SDS–PAGE (10% polyacrylamide gel) and transferred onto nitrocellulose membrane filters (Licor). Filters were incubated with anti-His antibodies at 1:1000 (Licor) and anti-S13 antibodies (1:500) (ATCC). Immunoblots were developed by using IRE-conjugated goat anti-mouse antibody as according to manufacture specification.

4.3 Results

4.3.1 Identification of amino acids that affected RelA activity

In *E. coli,* amino acids comprise almost 50% of the total metabolite concentration in the cytoplasm (Bennett et al. 2009). As there are no essential

amino acids in *E. coli,* we examined each amino acid for its potential to affect RelA activity (**Figure 4.1A**). From the individual amino acid tests with wildtype RelA, only methionine and glutamine resulted in a measurable effect on RelA activity. An additional test using three amino acid cocktails was also done to determine if the inhibitory effect could be reproduced (**Figure 4.1B and 4.1C**). Similarly, only cocktails that contained glutamine and methionine revealed reduced (p)ppGpp synthesis after an hour incubation. We also observed an inhibition with a methionine analogue, norleucine (Rowbury 1965) (**Figure 4.1**).

4.3.2 Effects of Amino Acid on (p)ppGpp Synthesis

We next investigated whether the presence of methionine affected the rate of (p)ppGpp accumulation. As both methionine and norleucine showed an effect on (p)ppGpp synthesis, we conducted the time course assay using methionine.

Wildtype RelA was incubated with RACs both/either in the absence and/or presence of increasing concentration of methionine (**Figure 4.2A**). Methionine did decrease the rate of (p)ppGpp synthesis and this inhibition of RelA activity was more severe at higher concentrations of methionine. Similar assays were done using the point mutant RelA (RelA N693A). The asparagine in this position was shown to be involved in amino acid binding in 3PGDH (Grant et al. 1996; Schuller et al. 1995). The presence of methionine affected (p)ppGpp synthesis to a lesser extent for the point mutant than for the wildtype (compare **Figure 4.2A** and **4.2B**). At 0.5 mM of methionine, almost no measurable effect on (p)ppGpp was observed. We begin to notice a significant lag in (p)ppGpp at 2 mM methionine for the wildtype (a 3 fold decrease in synthesis) and 10 mM for the point mutant (a 2-fold decrease in synthesis). At 5 mM, 10 mM and 20 mM, we observed the most pronounced inhibition to RelA activity for the wildtype RelA. Interestingly,

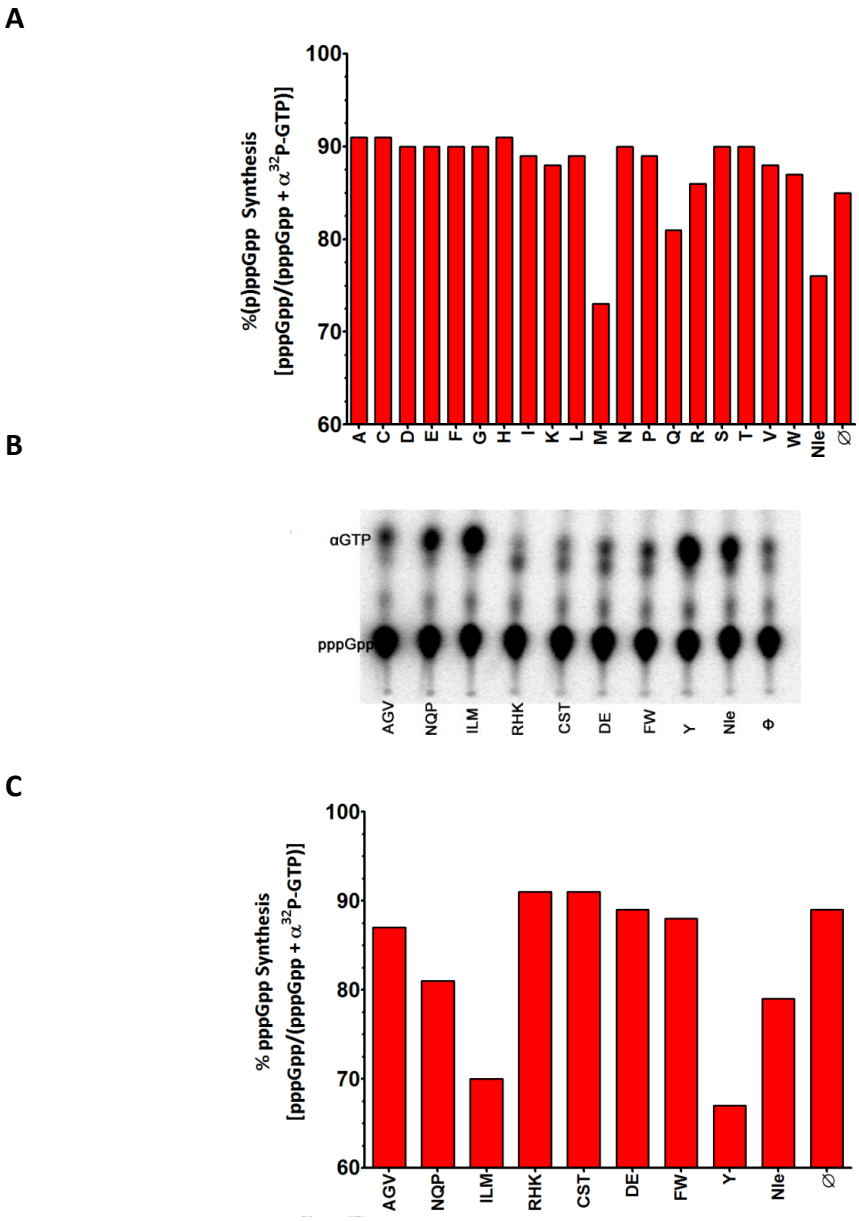
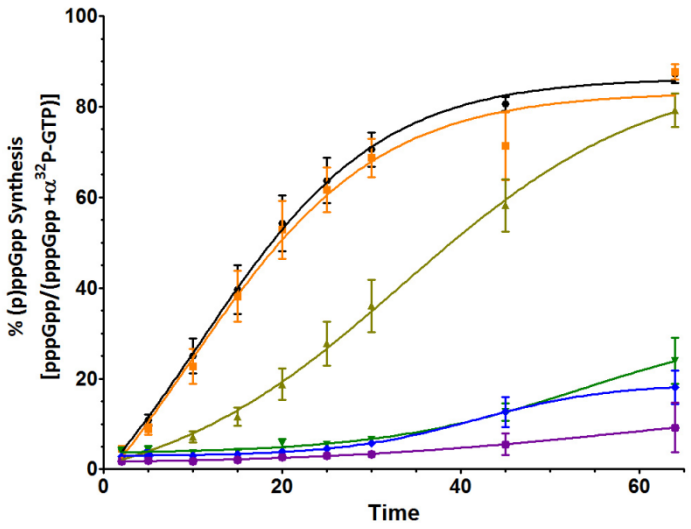


Figure 4.1: Screening amino acids for possible inhibition of RelA activity. pppGpp synthesis activity of wildtype RelA was examined in the presence of 1 mM amino acid. Assembled ribosomes were incubated with wildtype RelA, substrates and 1 mM of amino acid. After 1 hr, reactions were acid quenched and resolved by thin layer chromatography (B). The amino acids are represented via the single letter codes. We tested the amino acids individually (A) and as a tri-amino acid mix (B and C). Nle represents Norleucine, while Φ indicates no amino acids. Except for tyrosine (0.2 mM), amino acids concentrations were 1 mM .

A



B

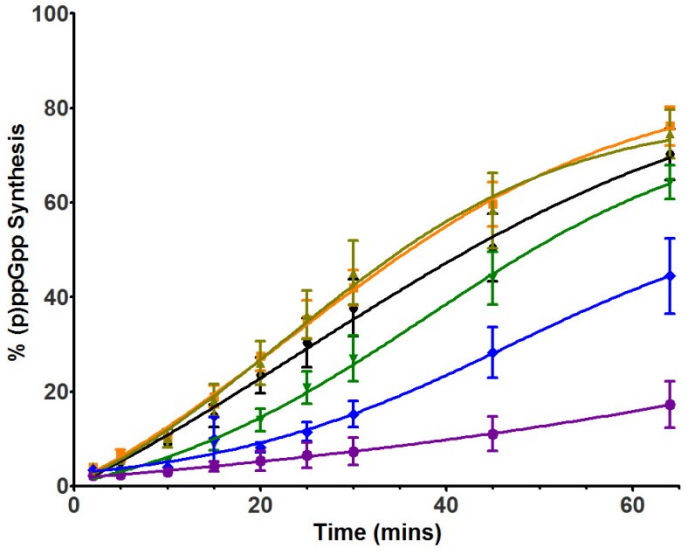


Figure 4.2: Time course analysis of amino acid effect on pppGpp synthesis. (p)ppGpp synthesis in the presence of amino acids were measured over time to determine if the amino acids affect RelA activity. Figure A (wildtype) and B (N693A) shows the (p)ppGpp formation when amino acids were preincubated with RelA and RAC prior to substrate addition. Concentration of methionine used are as follows: 0 mM (black ●), 0.5 mM (orange ■),2 mM (ochre ▲), 5 mM (green ▼), 10 mM (blue ◆), and 20 mM (purple ●). The data is the average of 3 to 5 different experiments.

when amino acids were added 15 minutes after the reactions were initiated, the inhibitory effect was less pronounced (**Figure 4.3A** and **4.3B**).

4.3.3 Effects of Amino Acid on RelA Binding the Ribosomes.

In a previous study (Chapter 3), the ACT domain was shown to interact with the ribosome. As this domain mediates regulation of enzyme activity through binding to a small ligand (Liberles et al. 2005), we investigated the effect amino acids had on RelA-ribosome interaction. RelA was incubated with RAC in the presence and absence of 20 mM methionine, which was the concentration at which we had observed the strongest inhibitory effect (**Figure 4.2** and **Figure 4.3**). The non-hydrolysable ATP analogue AMPCPP was used as a substrate. Western blot analysis reports that RelA binding to the ribosome is not affected by the presence of 20 mM methionine (**Figure 4.4**).

4.4 Discussion

4.4.1 RelA Mediated (p)ppGpp Synthesis is Dampened in Presence of Amino Acids

Activated RelA is a ribosome-free (p)ppGpp synthetase (Wendrich et al. 2002) (**Figure 1.7**). *English et al.* suggests that RelA eventually undergoes auto-inhibition via an unknown process involving the C-terminal domain (English et al. 2011). Part of the C-terminal region of RelA is the ACT domain, a regulatory motif known to bind small metabolites such as amino acids (Chipman & Shaanan 2001; Grant 2006). As there has been no investigation into the role metabolites play in the regulation of RelA activity, we chose to begin by examining if amino acids can affect RelA dependent (p)ppGpp synthesis by RelA.

We screened twenty amino acids and an amino acid analogue for the effects on (p)ppGpp synthesis. Of all the amino acids tested, only methionine, norleucine, and glutamine appeared to significantly inhibit RelA mediated

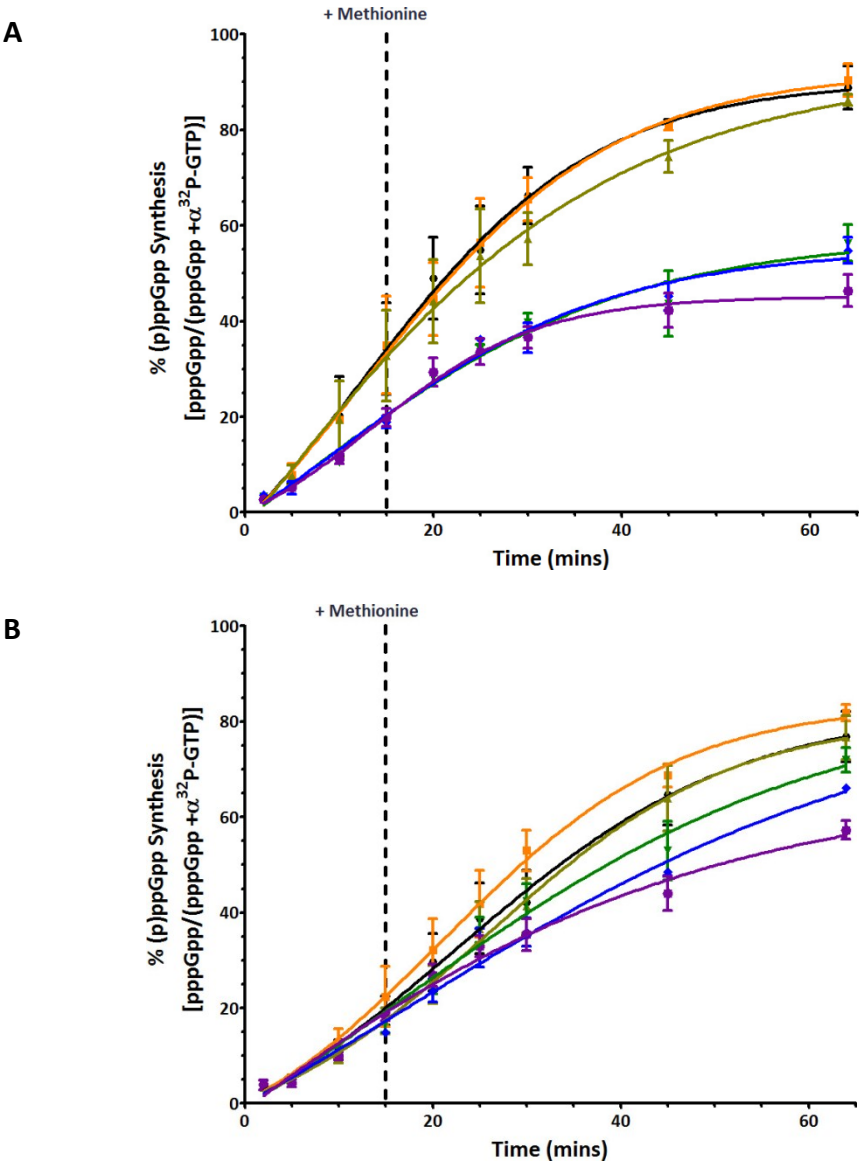


Figure 4.3: Time course analysis of amino acid effect on pppGpp synthesis, 15 minutes after substrate addition. Figures A (wildtype) and B (N693A) show the progress curve for (p)ppGpp synthesis when methionine was added 15 minutes into the reaction. Aliquots of reaction were taken at time intervals, quenched and resolved by TLC. The quantified results are presented on these graphs. Concentration of methionine used are as follows: 0 mM (black ●), 0.5 mM (orange ■), 2 mM (ochre ▲), 5 mM (green ▼), 10 mM (blue ◆), and 20 mM (purple ●). The data is the average of 3 to 5 different experiments.

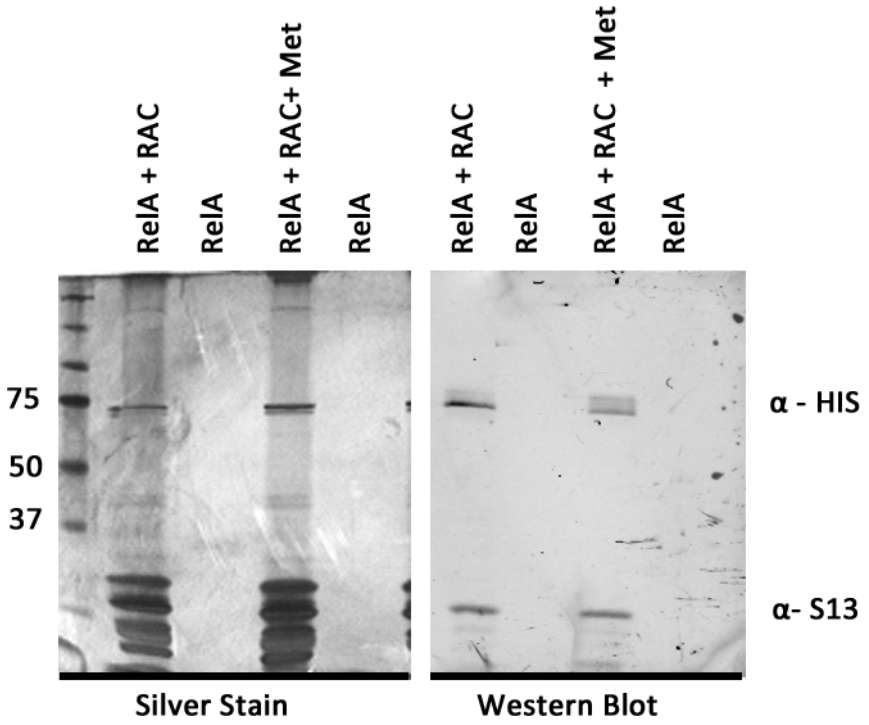


Figure 4.4: Methionine does not affect RelA binding to the ribosome. Fraction 1 for four different binding assays were analysed by silver stain (left) and western blot (right). Wildtype RelA was incubated with and without ribosome in the presence and absence of 20 mM Methionine. The sample was filtered through an S-300 sephacryl column. RelA bound ribosome can be detected in the void volume. RelA did not appear in the void volume unless ribosomes were present.

(p)ppGpp synthesis *in vitro* (**Figure 4.1**). A detailed time course study revealed that inhibition of (p)ppGpp synthesis was proportional to the concentration of methionine present in the reaction (**Figure 4.2**). The point mutation reduced the sensitivity of RelA to methionine (**Figure 4.2B**). For wildtype RelA, addition of methionine 15 minutes after the reaction commenced (that is, after substrates addition), did not appear to have the same potency as having methionine present prior to substrate addition (**Figure 4.3A**). The N693A point mutant however, showed a slight reduction in the rates of (p)ppGpp accumulation when methionine was added 15 minutes after substrate addition (**Figure 4.3B**). The results of the time course confirm that methionine is able to inhibit RelA activity. However, this mechanism of inhibition does not involve binding of RelA to the ribosomes (**Figure 4.4**) and RelA co-elutes with ribosome in the presence of 20 mM methionine (the concentration at which we observed the most inhibition). Regulation by methionine is likely mediated through the ACT domain since a mutation to this domain results in a decreased sensitivity to the presence of the methionine (**Figure 4.2A**).

These findings add a new complexity to the regulation of RelA activity. Activated RelA is thought to undergo an auto-inhibition mechanism after several rounds of (p)ppGpp synthesis. In that case, it would be interesting to further investigate whether the auto-inhibition is accelerated in the presence of methionine and thus results in the significantly reduced rate of (p)ppGpp synthesis.

Figure 4.5 is an illustration of a summary of our results. Our results in **Figure 4.2** clearly show that amino acid methionine can indeed inhibit RelA activity. From **Figure 4.4** we learn that the methionine does not inhibit RelA-ribosome interaction. This leaves two possible points in time at which methionine could inhibit RelA activity. Methionine could accelerate in the

inactivation of ribosome-free active RelA, otherwise methionine could affect the stimulation of RelA while on the ribosome.

Methionine and norleucine are structurally similar and both were discovered to inhibit (p)ppGpp synthesis. Norleucine is synthesised *in vivo* as a result of leucine and α-ketobutyrate accumulation (Bogosian et al. 1989; Traxler et al. 2008). Norleucine can be incorporated in place of methionine during protein synthesis (Bogosian et al. 1989). In light of this, it is possible that other metabolites having structural features similar to either methionine and glutamate may negatively influence RelA mediated (p)ppGpp synthesis.

With the rising need for new antibiotic drugs and new targets for drug design, this study and the potential identification of a metabolite that can inhibit RelA activity are very promising. The chemical compounds that can be used to attack the stringent response have been investigated before (Wexselblatt et al. 2010). Our results present an alternative angle through which the stringent response may be used to combat the rise in drug-resistant bacteria. Targeting the stringent response knocks out the bacteria's ability to sense nutrient conditions, leading to starvation and ultimately, death.

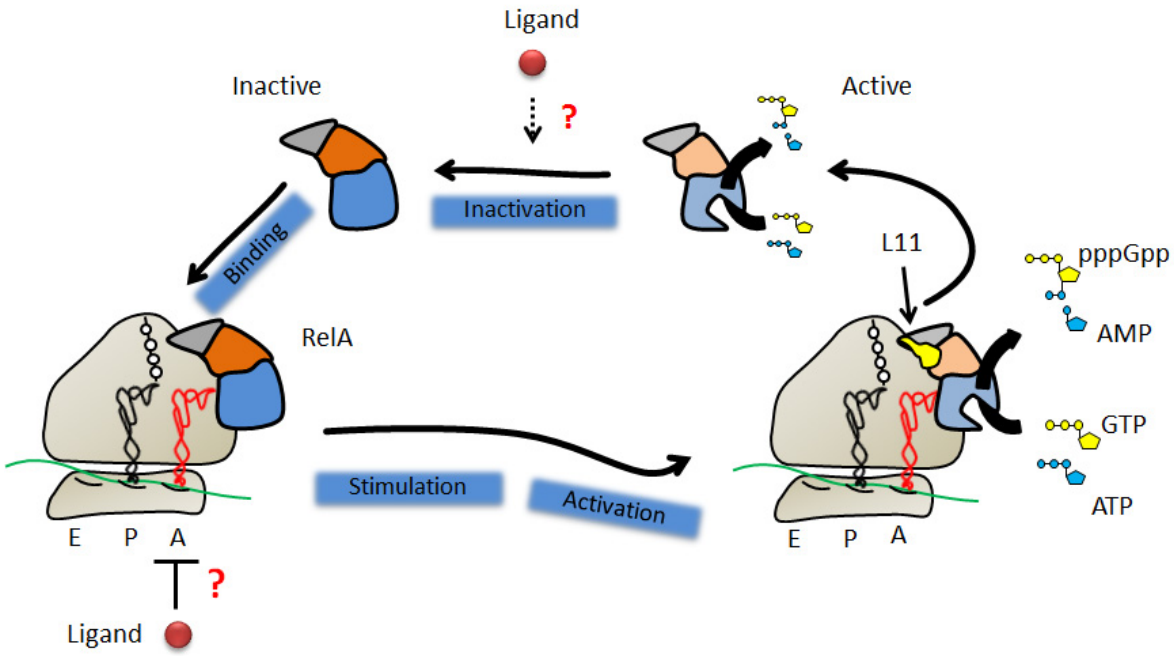


Figure 4.5: Model for small metabolite regulating active RelA. The stimulants for RelA activation are ribosomes with a deacylated tRNA (red) bound to the A-site and ribosomal protein L11 (yellow). RelA is thought to undergo a conformational change into its active form to synthesise pppGpp using ATP and GTP as substrates. Activated RelA dissociates from the ribosome and continues to synthesise pppGpp for a period of time before inactivation. RelA has to rebind the ribosome in order to become active. There are two possible sites that amino acid methionine could enforce its inhibitor effect. Methionine could either positively affect inactivation or negatively affect stimulation and activation of RelA.

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Chapter 5

Conclusions & Future Directions.

5.1 Overview

The stringent response is a regulatory response characterised by the rapid accumulation of two signalling alarmones, pppGpp and ppGpp (Cashel et al. 1996; Potrykus & Cashel 2008). Activation of the stringent response results in modulations to cellular physiology that serve to fortify the bacterial cell against the approaching nutrient stress (Braeken et al. 2006). In *B. subtilis*, the stringent response is necessary for the activation of the sporulation pathway (Ochi et al. 1982; Lopez et al. 1981). In *E. coli*, cell size is regulated in response to SpoT activation of stringent response (Yao et al. 2012). Entry into stationary phase is controlled by ppGpp in *L. pneumophila* (Hammer & Swanson 1999). The importance of the stringent response in bacterial pathogenesis (Godfrey et al. 2002; Dalebroux et al. 2010) has made it an attractive candidate for antibacterial drug design (Wexselblatt et al. 2010). In a recent report, a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) was discovered to contain a mutation in the *relA* gene that rendered constitutive activation of the stringent response (Gao et al. 2010). Four other gain-of-function mutations that resulted in antibiotic resistant *S. aureus* were also identified (Gao et al. 2010). In light of such reports, there is a need to increase our current knowledge in the regulation of this potent survival mechanism in order to design inhibitors.

The main effect of (p)ppGpp is the alteration of gene expression. Together with DksA, (p)ppGpp binds to RNA polymerase and represses transcription of growth related genes, while promoting expression of survival and virulence genes (Dalebroux & Swanson 2012; Magnusson et al. 2005). Experimentally determined protein targets of (p)ppGpp include translation factors (IF-2, EF-Tu, and EF-G), PNPase, DNA primase, LdcI, and Exopolyphosphotase (Gatewood & Jones 2010; Maciag et al. 2010; Guina et al. 2007; Srivatsan & Wang 2008; Kuroda 2006). In a thorough study on the experimentally reported ppGpp targets and those identified by sequence

alignment and *in silico* docking analysis, Kanjee et al. present a list of proteins that can be regulated by pppGpp. The list of targets can be divided into five different categories (Kanjee et al. 2012). These are 1) GTPases, 2) protein involved in nucleotide metabolism, 3) protein involved in lipid metabolism, 4) general metabolic proteins, and 5) basic aliphatic amino acid decarboxylases. In addition to these, (p)ppGpp is reported to be a positive activator of RelA in a positive feedback loop (Shyp et al. 2012; Hogg et al. 2004).

RelA/SpoT Homologue (RSH) proteins encompass the superfamily of enzymes that synthesise and/or hydrolyse (p)ppGpp in response to depleted nutrient conditions (Atkinson & Hauryliuk 2012; Atkinson et al. 2011). Most of our current understanding of these enzymes comes from investigations done on the *Escherichia coli* proteins RelA and SpoT (Schreiber et al. 1991; Sarubbi et al. 1989; Metzger et al. 1988). RelA is a mono-functional (p)ppGpp synthetase that acts as a sensor for amino acid starvation by monitoring tRNA aminoacylation states (Cashel et al. 1996). SpoT is a bifunctional (p)ppGpp synthetase/hydrolase enzyme that acts as a sensor to several other cues for nutrient starvation (Szalewska-Pałasz & Potrykus 2011). The details of the mechanism of regulation and activation for both proteins are still lacking. However, considerably more is known about RelA than about SpoT.

The main objective of this dissertation was to gain further insight into the RelA mediated (p)ppGpp synthesis mechanism. The results presented in Chapters 2, 3, and 4 have significantly contributed to the current literature as they pertain to aspects of the stringent response that have yet to be investigated. As a consequence of these discoveries, a number of new questions concerning the mechanism of RelA–dependent (p) ppGpp accumulation have emerged.

5.2 tRNA Identity and the Duration of the Stringent Response

My investigation into the role of tRNA identity on stringent response reveals a correlation between the inherent tRNA binding stability and the duration of RelA mediated pppGpp synthesis. I had compared the (p)ppGpp synthesis at various different concentrations of RAC (RelA activating complexes). RACs are ribosomes with an A-site bound deacylated tRNA. I chose to measure pppGpp synthesis for two tRNAs that are reported to have a five-fold difference in A-site dissociation rates (Fahlman et al. 2004). In the conditions used in my study the difference in dissociation rates was ~two-fold (**Table 2.1**). This fold difference is likely a result of the difference in the used buffer conditions. It is important to note that a two-fold difference pppGpp synthesis was also observed for the two tRNA ($tRNA^{Phe}$ and $tRNA^{Val}_{2A}$) used in the assay display in **Figure 2.5**.

RelA activation is dependent on the presence of a deacylated tRNA bound to ribosomal A-site. As a result of amino acid(s) starvation, charging (aminoacylation) levels of one or more tRNA species is affected. When the ratio of deacylated tRNA to aminoacylated tRNA is between 5 and 10 (Haseltine & Block 1973; Rojiani et al. 1989), the deacylated tRNA can compete for the A-site (Haseltine & Block 1973). Wendrich *et al* reported that the passive dissociation of the deacylated tRNA from the ribosomal A-site is critical for the attenuation of the stringent response (Wendrich et al. 2002). tRNA dissociation is independent of the presence of pppGpp and the dissociation of RelA from the ribosome (Wendrich et al. 2002).

Despite having similar tertiary structure, tRNAs are chemically unique and have various sequences, post-transcriptional modifications and esterified amino acids. Differences aside, the A-site association rates for all tRNAs are similar (Fahlman et al. 2004). This could explain why the rate of (p)ppGpp accumulation

in vivo is similar despite the identity of the limiting amino acid (Spadaro et al. 1981; Lazzarini et al. 1971; Lagosky & Chang 1980).

The A-site dissociation rates for individual deacylated tRNAs are quite different (Fahlman et al. 2004). The differences in binding stabilities of different tRNAs could be a consequence of multiple evolutionary factors. The control of ribosome biogenesis (Kaczanowska & Rydén-Aulin 2007), codon usage (Ikemura 1985; Elf et al. 2003), tRNA abundance (Dong et al. 1996), selective charging of the tRNA isoacceptors (Dittmar et al. 2005), and metabolic cost of the biosynthesis of different amino acids (Heizer et al. 2006), are some of the known contributors to this bias. Their influence on the stringent response has not been investigated.

5.2.1 Further Investigation into tRNAs and their Role in the Stringent Response.

As a follow-up to the report *in vitro* results, an *in vivo* study will be needed to ascertain if recovery from the stringent response can be influenced by identity of the tRNA. A tRNA is identified by the amino acid it generally decodes for. The relationship between tRNA identity and *in vivo* (p)ppGpp synthesis would reflect on the identity of the amino acid as well. This could mean that the potency of the stringent response (as a measure of duration) could vary depending on the amino acid that is limiting. This hypothesis will need further experimental verification. A standardised assay to measure pppGpp synthesis *in vivo* needs to be developed to limit the errors that are associated with data acquisition using cells in culture.

5.2.2 Future Investigation into Activity of SpoT

Recovery from the stringent response is linked to hydrolysis of (p)ppGpp to basal levels, which is mediated by the enzyme SpoT. SpoT hydrolase activity is reported to be inhibited in the presence of deacylated tRNAs (Richter 1980) and it is not clear if this mechanism involves a direct association of the tRNA with

SpoT. If there is a potential for tRNA interacting directly with SpoT to regulate the bi-functional activity, it would be interesting to see if the difference in tRNA features could also have an effect on SpoT activity.

5.3 Dual Function of the C-Terminal ACT domain.

My goal was to further characterise the role of the C-terminal domain in the regulation of RelA activity. My particular focus was on the ACT domain, which is part of the region of RelA reported to be involved in ribosome binding (Schreiber *et al* 1991) and dimerization (Gropp et al. 2001; Yang & Ishiguro 2001a). Although RelA has been known for more than 50 years, little is known about many molecular details on how RelA interacts with the ribosome and how RelA is activity regulated after its activation.

RelA localises to the 50S subunit (Ramagopal & B. Davis 1974) and fluorescent studies show that RelA diffusion in cell is similar to that of the ribosomes (English et al. 2011) for nutrient rich condition. RelA is tightly associated with the ribosomes and binds at the region in proximity to the ribosomal protein L11 (Jenvert & Schiavone 2007; Yang & Ishiguro 2001b). This interaction between RelA and ribosomes is proposed to be mediated through the C-terminal domain of RelA (Potrykus & Cashel 2008). RelA is also assumed to bind near to the acceptor site or the factor binding site, as the presence of a deacylated tRNA is a criterion for RelA activation (Payoe & Fahlman 2011; Richter 1976). Translation factors are present at significantly higher concentrations than RelA with the cell, e. g. EF-G is at ~ 20μM and RelA is at ~15nM (Hirokawa et al. 2005; Block & Haseltine 1975). The influence of factor binding on RelA binding is therefore of interest. It is important to note that only EF-G inhibits RelA-mediated (p)ppGpp synthesis (Wagner & Kurland 1980). The addition of fusidic acid (an inhibitor of EF-G) is shown to decrease (p)ppGpp synthesis while the removal of ribosomal protein L7/L12 (protein important for

factor recruitment) diminishes the inhibition of EF-G in (p)ppGpp synthesis. The ribosome sites associated with the interaction with these two factors are thought to either partially overlap or are the same regions for both proteins (Wagner & Kurland 1980). RelA mediated pppGpp synthesis can also be observed with RACs assembled with 80S ribosomes (Pollard & Parker 1977). This observation may provide some clues to identify the region of the ribosomes that is involved in RelA interaction with ribosome.

The TGS and the ACT domains are two domains identified within the C-terminal region of RelA. Both of these domains are involved in the regulation of enzyme activity (Battesti & Bouveret 2006; Grant 2006). However, their functions in RelA have not been specifically verified. By using site-directed mutagenesis, I identified the ACT domain as the region of RelA that is involved in the ribosome-RelA interaction. A single point mutation in the ACT domain, in combination with the deletion of ribosomal protein L11, results in loss of activity. Although activity was observed with wildtype RACS (these were assembled with wildtype ribosomes), it was significantly (~ eight-fold) lower to wildtype RelA. Additionally, this same domain appears to regulate activity via binding to amino acids. The results in chapters 3 and 4 present a novel mode of RelA regulation involving a domain in RelA that had not been previously characterised. An extensive examination is required to determine the details and the mechanism by which the ACT domain is involved in RelA activity.

5.3.1 Future Investigation into the Function of RelA ACT Domain

Additional mutations to the conserved region with the ACT, particularly between the first beta sheet and alpha helix, would need to be generated and assayed to determine the effects on both the ribosome interaction and amino acid inhibition. The effects on (p)ppGpp with various different L11 mutants, such as those used in Jenvert et al. 2007 (Jenvert & Schiavone 2007), could be used in

conjunction with RelA mutants to determine if the ACT domain is involved in the transduction of the activation signal from the ribosome to RelA.

Additional future work includes 1) functional studies on the TGS domains in RelA and its possible role in the sensing of tRNA at the A-site; 2) footprinting assays to determine the location of RelA interaction with the ribosomes and understand the necessity for the A-site bound tRNA for RelA activation; and 3) a detailed screen of non-amino acid metabolites for ability to inhibit or activate RelA activity.

5.4 Closing Remarks

The induction of the stringent response allows a cell to survive or to adapt to nutritional stress. Successful survival to nutrient stress entails a prompt response prior to the complete exhaustion of nutrients. Expanding our understanding of mechanisms that assist in the survival of bacteria, we open up new doors into therapeutic approaches against antibiotic resistant strains of bacteria.

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Appendix 1

Binding Partners of Stringent Response Protein RelA.

Note: Results in these sections were obtained by undergraduate students: Shawna La (Biochem 398), Daesom Kang (Biochemistry 499) and Braden Milan (Biochemistry 398).

A1.1 Introduction

In almost all of the *in vitro* studies done on RelA, a hexahistidine tag fusion construct was used to purify RelA (Wendrich et al. 2002; Jenvert 2007; Jenvert & Schiavone 2005). As this tag is small, it does not interfere with RelA activity or ribosome association. Ni-NTA columns are known to provide large yields of protein, at the cost of purity (Lichty et al. 2005). However, in some instances, this property has an advantage since protein-protein interactions can be preserved under native buffer conditions (Schmitt et al. 1993).

As an auxiliary observation made on different RelA preparations, we noticed that purity of the recombinant RelA decreased as IPTG induction progressed for more than 4 hours. A delayed harvest time resulted in an increase in the number of proteins that co-purified with RelA (**Figure A1.1**). We have identified some of these proteins using liquid chromatography mass spectroscopy (LC-MS/MS) (Schieltz et al. 2006) and MASCOT (Koenig et al. 2008) and are listed in **Table A1.1**.

We initially thought these contaminants would not interfere with RelA activity. **Figure A1.2** shows the results of the activity assay for the three different RelA preparations: $RelA_{0.8}$, $RelA_{1.5}$, and $RelA_x$. The subscripts indicate the optical density of the culture at the point of harvest. The subscript x is used to designate the RelA preparation that was obtained after an overnight induction. Although equimolar amounts of protein were used in each of the reactions, $RelA_{1.5}$ showed an eight-fold decrease in RelA activity when compared to either the $RelA_{0.8}$ and $RelA_x$. Examination of the elution profiles for each of the preparation showed that $RelA_{1.5}$ was not nearly as pure as the other two preparations. This observation prompted us to ask the question: is one of the contaminating proteins a putative regulator of RelA or is the decrease in RelA activity merely an artifact of the picomolar differences of RelA in the reactions?

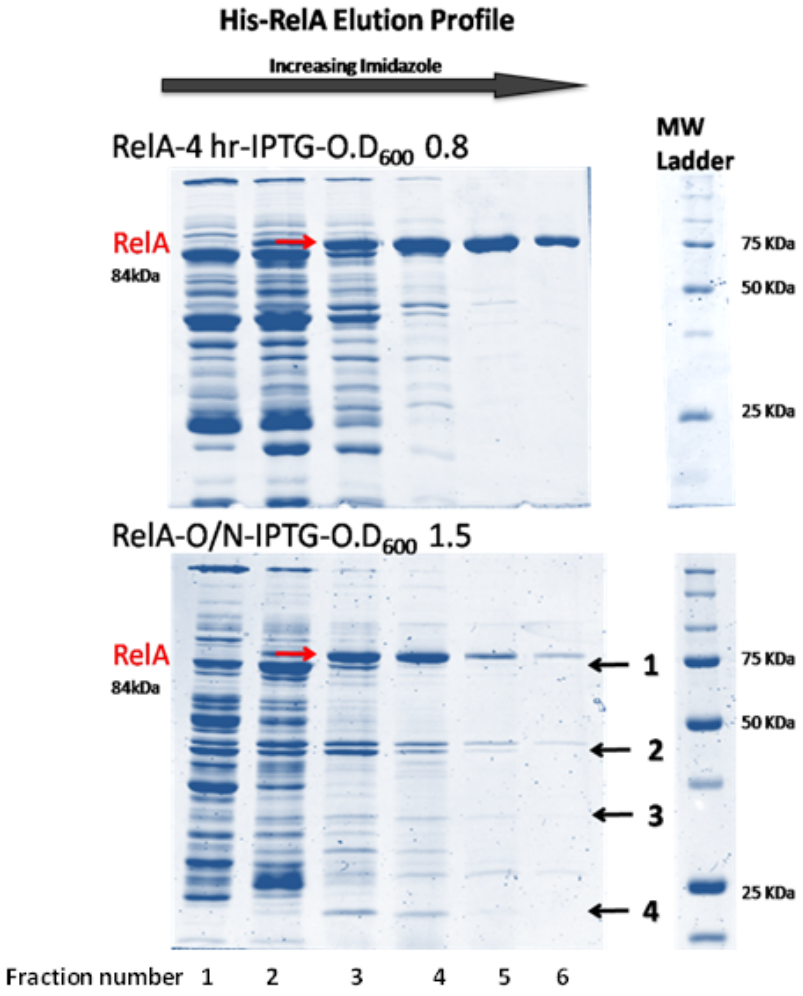

Figure A1.1: Elution profiles of two RelA preparations. The elution profiles of two different RelA preparations. $RelA_{0.8}$ was purified from cells harvested 4 hours post-IPTG addition with an OD_{600} = 0.8. $RelA_{1.5}$ was purified from cells presumably at late log phase growth or stationary phase growth? (OD_{600} =1.5 after one overnight culture post-IPTG induction). The numbered arrows correspond to the protein identified by tandem mass spectroscopy. The identities are listed on Table 1. RelA (84 KDa) is the protein band identified by the red arrow.

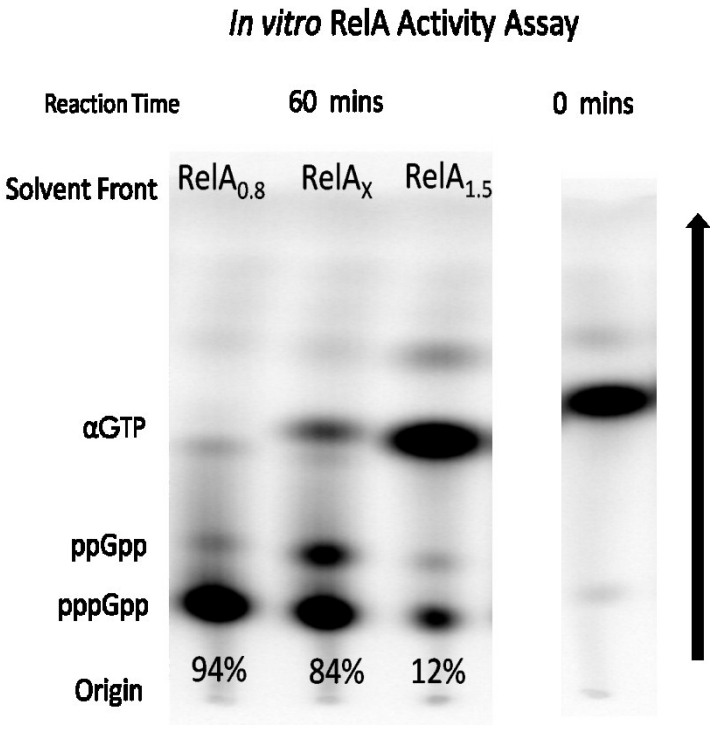

Figure A1.2: Comparison of (p)ppGpp synthesis activity with RelA from three different preparations. In vitro assay was conducted with 12:1 ribosome to RelA ratio and were analysed by TLC. $RelA_{1.5}$ had an eight-fold reduction in activity compared to $RelA_{0.8}$. The activity is recorded as a percent conversion of αGTP to (p)ppGpp and are reported on the figure. The $RelA_x$ prep was obtained similarly to $RelA_{1.5}$ but the OD of the cell culture was not measured prior to lysis, and thus was designated $RelA_x$.

Table A1.1: Identities of Some Protein that Co-elute with RelA

*Stringent response inhibits peptidoglycan synthesis

**Hypothesised to interact with C-terminal domain of RelA

Band	Full Name	Code Name	Function
1	bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase	AraN**	peptidoglycan biosynthesis *
2	bifunctional succinylornithine transaminase/acetylornithine transaminase	ArgD	Arginine Catabolism
3	cAMP-Regulatory protein	CRP	RNA polymerase promoter recognition
4	transcription termination factor Rho	Rho	Transcription termination

Given the magnitude of this decrease, we were dubious if concentration of RelA was the only reason for this difference in activity.

There is almost nothing known about the proteins that can interact with RelA or if there are proteins involved in the regulation of RelA activity. We therefore began a preliminary investigation into putative interactive partners of RelA. We approached this study by first conducting a comparison of elution fractions from Ni-NTA and GST columns. GST was chosen as the tag because it had less non-specific binding while still maintaining yields (Lichty et al. 2005). Our approach has generated a putative list of RelA-interacting proteins.

A1.2 Material and Methods

A1.2.1 Construction of GST-tag RelA Fusion Protein.

The *relA* gene was cloned from the pCA24N (N-terminal hexshistidine tag) to pGEX6p1 (N-terminal GST tagged) vector. The following primer set: 5'-BamHI-RelA *(+)* primer 5'- GCC CCC TGG GAT CCA TGG TTG CGG TAA GAA GTG CAC-3' and 3'-NotI-RelA (-) primer 5'- TCA GTC ACG ATT TAG CGG CCG CAT AGG CCA CTC CCG-3' was used to PCR amplify the wildtype *relA* gene. A PCR reaction was carried out with the following components at a final concentration of: 1 X *pfu* PCR buffer, 0.5 mM dNTPs, 1uM primers, 100 ng DNA template, and 2.5 units of *pfu* polymerase (Invitrogen). Cycling conditions were: 94℃ 2min, 30 cycles of 94℃ 30sec, 55℃ 30sec, 68℃ 5min, 1 cycle of 94℃ 1min, 55℃ 1min, 72℃ 10min, and final 4^{o}C hold. The PCR product (2214bp) was confirmed by 0.8% agarose gel electrophoresis, as shown in **Figure A1.3A**. After gel extraction (Qiagen), the purified gene and the pGEX6P1 vector were digested with *Bam*HI (Invitrogen) in a 50 µL reaction that was incubated at 37℃, 2 hours. After a cleaning up using a PCR cleanup kit (Truin Science) both the vector and insert were digested with *Not*I (Invitrogen). The reaction condition was the same as is

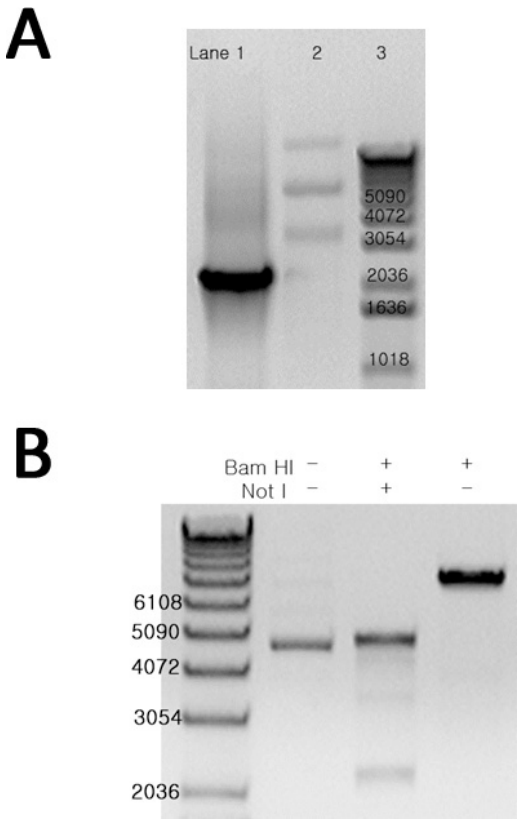

Figure A1.3: Construction of pGEX6P1-RelA plasmid. A. Agarose gel electrophoresis of *relA* cloned by PCR reaction. The PCR amplified *relA* gene was run on a 0.8% agarose gel to confirm success of PCR reaction. White arrow shows the band at 2214 base pairs (as expected) which corresponds to *relA.* Lane 1: 1.3µg of PCR-amplified relA. Lane 2: 6µl PCR reaction control (no enzyme). Lane 3: 10ul 1Kb DNA ladder. B. Agarose gel electrophoresis of undigested, single-digested, and double-digested pGEX-*relA*. pGEX6P1 vector is 4492 bp. *relA* gene is 2214 bp. pGEX-*relA* is 7198 bp.

mentioned above. Again, after another cleaned up (PCR cleanup kit (Truin Science)) the digested vector was treated with CIAP (Invitrogen). The vector was subjected to another clean up and then ligated to the insert in a ligation reaction by T4 ligase (Invitrogen). Ratio of insert to vector was kept at 1.5:1. The standard plasmid preparation protocol was used to amplify and purify the newly generated plasmid.

A1.2.2 Expression and purification of GST-tag RelA and His-tag RelA.

The GST-tagged RelA and His-tag RelA were expressed and purified in BL21 cells cultures in LB broth medium containing 25 µm/mL of ampicillin and chloramphenicol, respectively. Protein expression was induced with 1 mM isopropyl thio-β-D-galactosidase (IPTG) when cultures reached OD_{600} = 0.5. Induced cells were harvested after 4hrs and 16hrs by centrifugation (5000 g for 10 min at 4^{o}C). The cells were divided into two and each half was resuspended in either GST lysis buffer (PBS, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3, and 1 mM PMSF) or His lysis buffer (25 mM $NaHPO_4$ (pH 7.5), 50 mM NaCl, 5 mM Imidazole, 1 mM β-ME, and 1 mM PMSF).

The cells were then lysed by sonication. Cell debris was removed by centrifugation (15 000 rpm for 15 min at 4^{o}C) and the cleared lysates were then loaded manually (using a syringe) onto a 1 mL GSTrap FF column or 1 mL HisTrap FF column(GE Healthcare), depending on the lysis buffer the cells were resuspended in.

The column was washed with lysis buffer (without PMSF). The proteins were then eluted with 10 mL of GST elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) or His elution buffer (25 mM $NaHPO_4$ (pH 7.5), 50 mM NaCl, 1.5 M Imidazole, and 1 mM β-ME). The elution fractions were collected as 1 mL fractions which were resolved by gel electrophoresis.

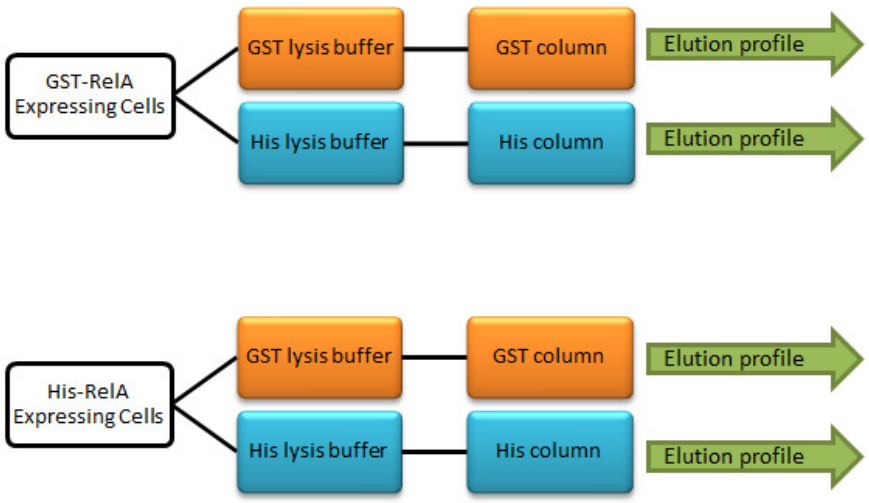

Figure A1.4. Experiment flowchart. GST-RelA and His-RelA expressing cells will be induced with IPTG. At the point of harvest, the cells will be divided into two. Each cell batch will be resuspended with either GST-lysis buffer or His-lysis buffer. Cleared lysates will be passed through either a GST column or a His column. The subsequent elution fraction will be resolved by SDS-PAGE to generate an elution profile.

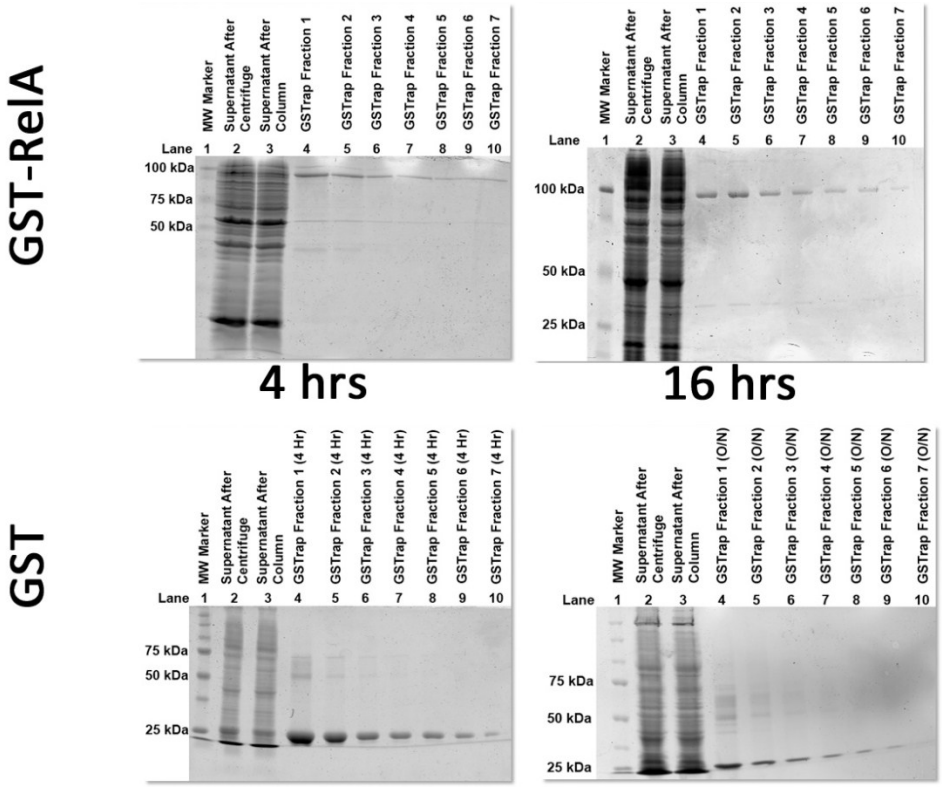

Figure A1.5: SDS-PAGE gels of GST and GST-RelA purified at 4hrs and 16hrs post IPTG addition. The lanes of first elution fractions were digested and submitted for protein identification

The flowchart in **Figure A1.4** illustrates the method. Elution fractions were resolved by SDS-PAGE (**Figure A1.5**).

A1.2.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

His-RelA on GSTrap FF™, GST-LF on HisTrap FF™, and GST on GSTrap FF™ and HisTrap FF™ served as negative controls for proteins which bind non-specifically to each column. Eluted proteins were subjected to SDS-PAGE analysis (**Figure 4**). A list of proteins found in each elution profile was compiled following MS/MS protein identification. Proteins identified in the negative control column were removed from the experimental data set.

A1.3 Results

E. coli cells transformed with plasmids containing glutathione-S-transferase-tagged RelA transferase (GST-RelA) or His-tagged RelA (His-RelA) were lysed after inducing expression for either 4 hours or 16 hours (overnight) (Figure 6A). Lysate from each culture was equally distributed across either GSTrap FF™ or HisTrap FF™ columns as illustrated in **Figure A1.5.**

The Venn diagram in **Figure A1.6** (**Figure 1.6B**) summarises the results for GST and GST RelA alone. The results of His-RelA and His alone are still pending. The total numbers of protein identified for RelA-4hr, RelA-16hr, GST-4hr, and GST-16hr are 103, 43, 33, and 180, respectively. Proteins that were only unique to both RelA-4hrs and RelA-16hrs were compared to distinguish between proteins that potentially interacted with RelA only during log phase growth from those that interacted with RelA during stationary phase growth. The proteins were categorised according to their cellular function and the results are presented in **Figure 1.6C.** There is a significant difference in the number of proteins that co-elute with RelA before and during stationary phase.

The lanes were excised into 1mm x 1mm gel slices for in-gel trypsin digestion. The digested proteins were eluted into a reverse phase C18 column for separation. The peptides were eluted based on hydrophobicity using a 40% gradient from buffer A (95% water, 5% acetonitrile and 0.2% formic acid) to buffer B (100% acetonitrile 0.2%, formic acid) over 60 minutes. The peptides were then fed into the electrospray ionization mass spectrometer (Orbitrap LTQ XL, Thermos Scientific) and the proteins were identified and quantified by the in-house SEQUEST server.

A1.4 Discussion

Cells harvested after 4 hours of IPTG induction were measured to have an OD_{600} at approximately 0.8. We presume the cells have yet had to activate the stringent response and therefore it is the reason we identified a number of ribosomal proteins in these elution fractions. RelA is known to be associated to the ribosomes when inactive (Ramagopal & Davis 1974; Wendrich et al. 2002; Cashel et al. 1996). The absence of ribosomal protein after 16 hours is not entirely a surprise as at this point in time, cells have already undergone the stringent response, and therefore RelA is active and ribosomes are free (English et al. 2011). Our data, although preliminary, is in agreement with the current model of RelA activation as illustrated in **Figure 1.6**. At this moment, we are unclear if the significant reduction in the number of proteins that interact with RelA at stationary phase is due to any handling error or if it indeed reflects to some extent what occurs *in vivo* for RelA. The experiments are therefore currently being repeated.

Through analysis of the list generated for 4 hour induction, we were able to determine that AraN or UDP-N-glucosamine 1-carboxyvinyltransferase is not an interacting partner of RelA as also present in the control samples. In contrast, cyclic AMP receptor protein was only found in the RelA-4hr sample and not in the GST-4hrs, making it an attractive target for further validation.

The location of RelA on the ribosome is still unknown. By examining the list of putative interacting proteins after 4hrs we see that ~20% of the identified proteins are ribosomal proteins. Although the locations of these ribosomal proteins on the ribosomes are quite dispersed, we believe that with a sufficient amount of data, we may be able to narrow down the region where RelA and the ribosomes interact.

Our results are still at the preliminary stage and will need to be further characterised before any firm conclusions about the data are made.

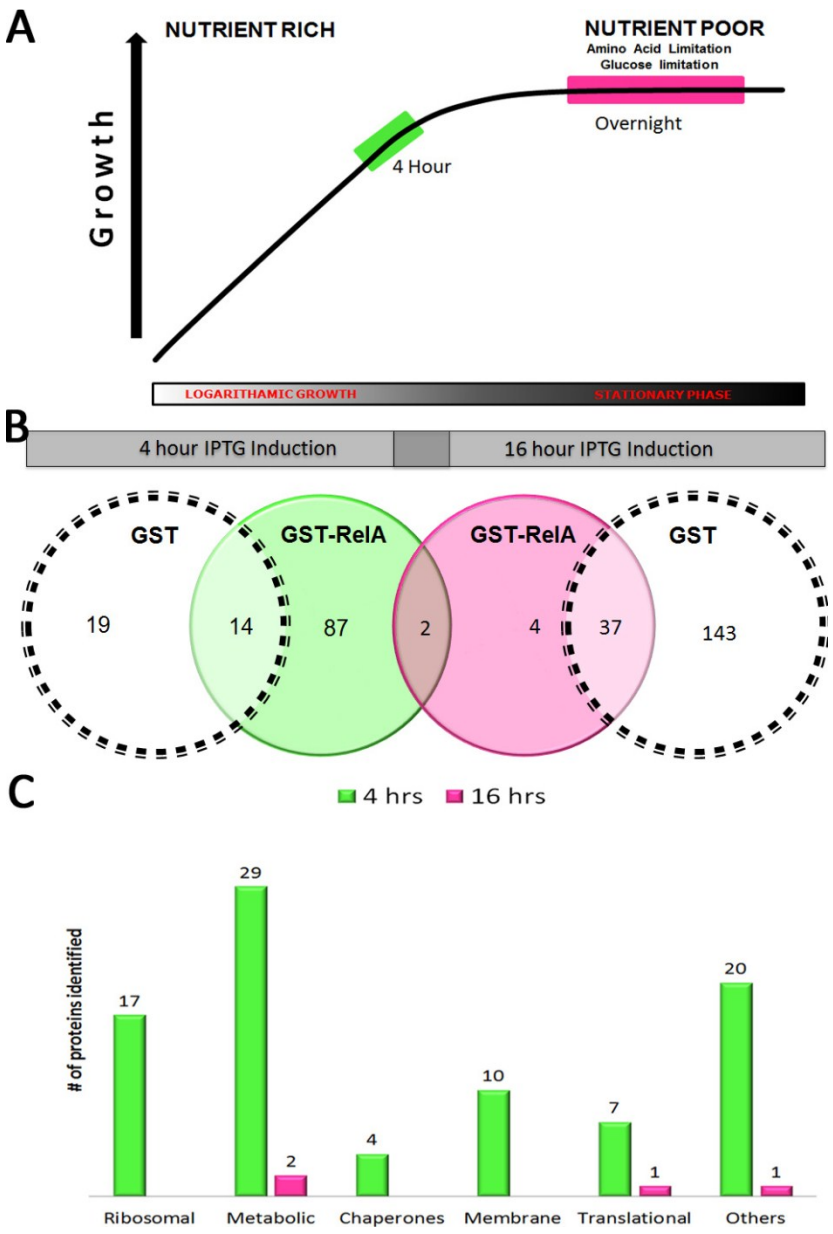

Figure A1.6: Comparison of elution profiles from 4hr induction and 16 hr induction. (A) Cells were harvested at either lag phase (OD_{600} =0.4) or during stationary phase (OD_{600} = 1) (B)The protein lists for GST-RelA 4hr and GST-RelA 16hrs (overnight) were compared after eliminating those proteins that were also identified in control GST elution profiles. Graph below displays the categories of these identified proteins according to cellular function (C)

Table A1.2: List of proteins identified for GST-RelA after a 4 hour induction	
30S ribosomal protein S2	heat shock protein 90
30S ribosomal protein S4	hypothetical protein b1604
30S ribosomal subunit protein S1	hypothetical protein b2513
30S ribosomal subunit protein S10	hypothetical protein Z4556
30S ribosomal subunit protein S11	hypothetical protein Z5038
30S ribosomal subunit protein S12	lipoprotein-34
3-oxoacyl-(acyl carrier protein) synthase	membrane protein
3-oxoacyl-[acyl-carrier-protein] reductase	membrane-bound ATP synthase, F0 sector, subunit b
50S ribosomal protein L1	membrane-bound ATP synthase, F1 sector, alpha-subunit
50S ribosomal protein L11	membrane-bound ATP synthase, F1 sector, beta-subunit
50S ribosomal protein L4	membrane-bound ATP synthase, F1 sector, gamma-subunit
50S ribosomal subunit protein L13	NADH dehydrogenase I chain B
50S ribosomal subunit protein L14	NADH:ubiquinone oxidoreductase, chain C,D
50S ribosomal subunit protein L2	orf, conserved hypothetical protein
50S ribosomal subunit protein L27	outer membrane protein 3a (II*;G;d)
50S ribosomal subunit protein L3	outer membrane protein X
50S ribosomal subunit protein L7/L12	PEP-protein phosphotransferase of PTS system (enzyme I)
50S ribosomal subunit protein L9	peptidoglycan-associated lipoprotein
acetyl CoA carboxylase, carboxytransferase component, alpha subunit	peptidyl-prolyl isomerase
acetyl-CoA carboxylase beta subunit	Polyribonucleotide nucleotidyltransferase (Polynucleotide phosphorylase) (PNPase)
ATP synthase delta subunit [Escherichia coli]	predicted lipoprotein
beta-D-galactosidase	predicted transporter
cell division inhibitor	protease specific for phage lambda cII repressor
cell division protein FtsZ	protein assembly complex, lipoprotein component
cell wall structural complex MreBCD, actin-like component MreB	protein chain elongation factor EF-Ts
Chain length determinant protein (Polysaccharide antigen chain	protein disaggregation chaperone

regulator)	
conserved inner membrane protein	PTS enzyme IIAB
cyclic AMP receptor protein	PTS enzyme IID, mannose-specific
cysteine synthase A, O-acetylserine sulfhydrolase A	PTS system galactitol-specific enzyme IIC
cytochrome d terminal oxidase, subunit I	putative ATP-binding component of a transport system
cytochrome o ubiquinol oxidase subunit II	putative GTP-binding factor
dihydrolipoamide acetyltransferase	putative outer membrane protein
DL-methionine transporter subunit	ribose-phosphate pyrophosphokinase
DNA gyrase subunit B, type II topoisomerase	RNA polymerase, beta prime subunit
DNA-binding response regulator in two-component regulatory system with BasS	succinate dehydrogenase flavoprotein subunit
elongation factor EF-2	succinate dehydrogenase, FeS subunit
fused DNA-binding transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	succinyl-CoA synthetase alpha subunit
fused mannitol-specific PTS enzymes: IIA components/IIB components/IIC components	translation initiation factor IF-2
fused ribonucleaseE: endoribonuclease/RNA-binding protein/RNA degradosome binding protein	trigger factor
galactitol-1-phosphate dehydrogenase	undecaprenyl phosphate-L-Ara4FN transferase
gluconate transporter, high-affinity GNT I system	universal stress protein
glyceraldehyde-3-phosphate dehydrogenase A	uracil phosphoribosyltransferase
GTP-binding elongation factor	Zn metallo-peptidase, integral membrane cell division protein Z

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Appendix 2

Generation of Fluorescent Mammalian Ribosomes.

A2.1 Introduction

In both prokaryotic and eukaryotic cells, ribosome biogenesis is the major biosynthetic process. In a nutrient rich environment, most of the transcription machinery is entailed in ribosome biogenesis. This mechanism would have to be curtailed in order to accommodate nutrient availability and growth factors. While in the prokaryotic system, regulation of ribosome biogenesis redirecting transcription (Kaczanowska & Rydén-Aulin 2007). In eukaryotes, in addition to the regulation of ribosomal components (rRNA and ribosomal proteins), regulation of ribosome biogenesis involves other proteins and noncoding RNAs that participate in processing, modification, assembly, and nuclear transport of the ribosome (Lempiäinen & Shore 2009).

To study ribosomes under different stress conditions we constructed a green fluorescents protein (GFP) – tagged version of ribosomal protein L11. This protein was chosen due to its high conservation between kingdoms of life and its location in a position on the ribosomes where we predict that GFP fusion would not impede ribosome function.

A2.2 Materials and Methods

A2.2.1 Generation of C-Terminal GFP Labelled Ribosomal Protein L11.

pEGFP-N1 plasmid was used as a vector (donation from Michalak lab). We cloned *rpl11* gene into the vector, which was amplified using the following primers 5'BglII- TTT AGA TCT CACC ATG GCGGATCAAG GTGAAAAG and 3'EcoRI - TTT GAA TTC GTT TGC CAG GAA GGA TGA TCC C.

A2.2.2 Antibodies.

Primary rabbit Anti-GFP antibody was obtained from Dr. Berthiaume lab. Secondary antibodies for Western blot analysis (goat anti-rabbit) coupled to IRDyes® were purchased from LI-COR.

A2.2.3 Sucrose Gradient Fractionation of Ribosomal Subunits.

Stable cells with GFP-L11 were lysed using a non-denaturing lysis buffer (20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA). The lysates were loaded ontoa 30% sucrose cushion w/w (15 mM Tris-HCL pH 7.5, 6 mM $MgCl_2$, 0.5 M KCL) to pellet crude ribosomes. The cushions were run on Ti-70 rotor for 39,000 rpm for 16hrs at 4^oC. The crude ribosomes were resuspended in 500 µL of a Low Mg Buffer (20 mM Tris-HCl –pH 7.5, 150 mM KCL, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT). The crude ribosomes were then loaded onto 10%-30% (w/w) sucrose gradient (prepared in buffer containing 20 mM Tris-HCL –pH 7.5, 150 mM KCL, 1mM $MgCl_2$, 0.1 mM EDTA). After a high-speed centrifugation (20,000 rpm 16hrs at 4^oC) in a swinging bucket rotor (SW-41 Ti, Beckman Coulter), the gradients were eluted on a gradient fractionator (Brandel) and monitored by UV at 254 nm. 0.5 mL fractions were collected. rRNA was extracted from 100 µL aliquots of fraction by phenol-chloroform extraction and ethanol precipitation. Samples were run on a 0.5% agarose gel. 20 µL of the fractions were analyzed by SDS-PAGE. After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (LI-COR Biosciences). The membranes were blocked with Odyssey blocking buffer (LI-COR), probed with primary (rabbit anti-GFP 1:3500) and secondary antibodies, and imaged with an Odyssey® Infrared Imaging System using the manufacturer's recommended procedures (LI-COR).

A2.2.4 Fluorescence Imaging of GFP Labelled Ribosomes.

Stably transfected HeLa cells were seeded on coverslips in 12 well plates (2.5×10^3 cells/well) and grown for 48 hours. Cells were then washed with PBS++

(PBS containing 1mM $MgCl_2$ and 1mM $CaCl_2$) and fixed in 4% paraformaldehyde for 15 minutes at 22^{o}C. The cells were washed with PBS++. After washing, the cells were stained with? DAPI nuclear counterstain (1:1000 dilution). After a single wash step, the coverslips were mounted on slides using Dako Fluorescence mounting medium and were allowed to set overnight prior to imaging.

Fluorescence images were obtained with an Axiocam on an Axio Observer microscope (Carl Zeiss, Jena, Germany) using a 100X plan-Aprochromat Lens. The images were deconvolved using the Axiovision 4 software.

A2.3 Results and Discussion

Whole cell lysates were fractionated by sucrose gradient centrifugation. The fractions were analysed by agarose gel, SDS-PAGE and western blot. The results are shown in **Figure A3.1**. GFP was only detected in the fractions that contained the 28S rRNA confirming that the labeled L11 proteins were incorporated into 80S ribosomes.

Immunofluorescence studies **(Figure A3.2)** showed the location of GFP at the nucleosome (the site of ribosome assembly) and the cytosol (the site of ribosome function.

We have managed to construct functional labeled ribosomes that can be used as a tool in future studies.

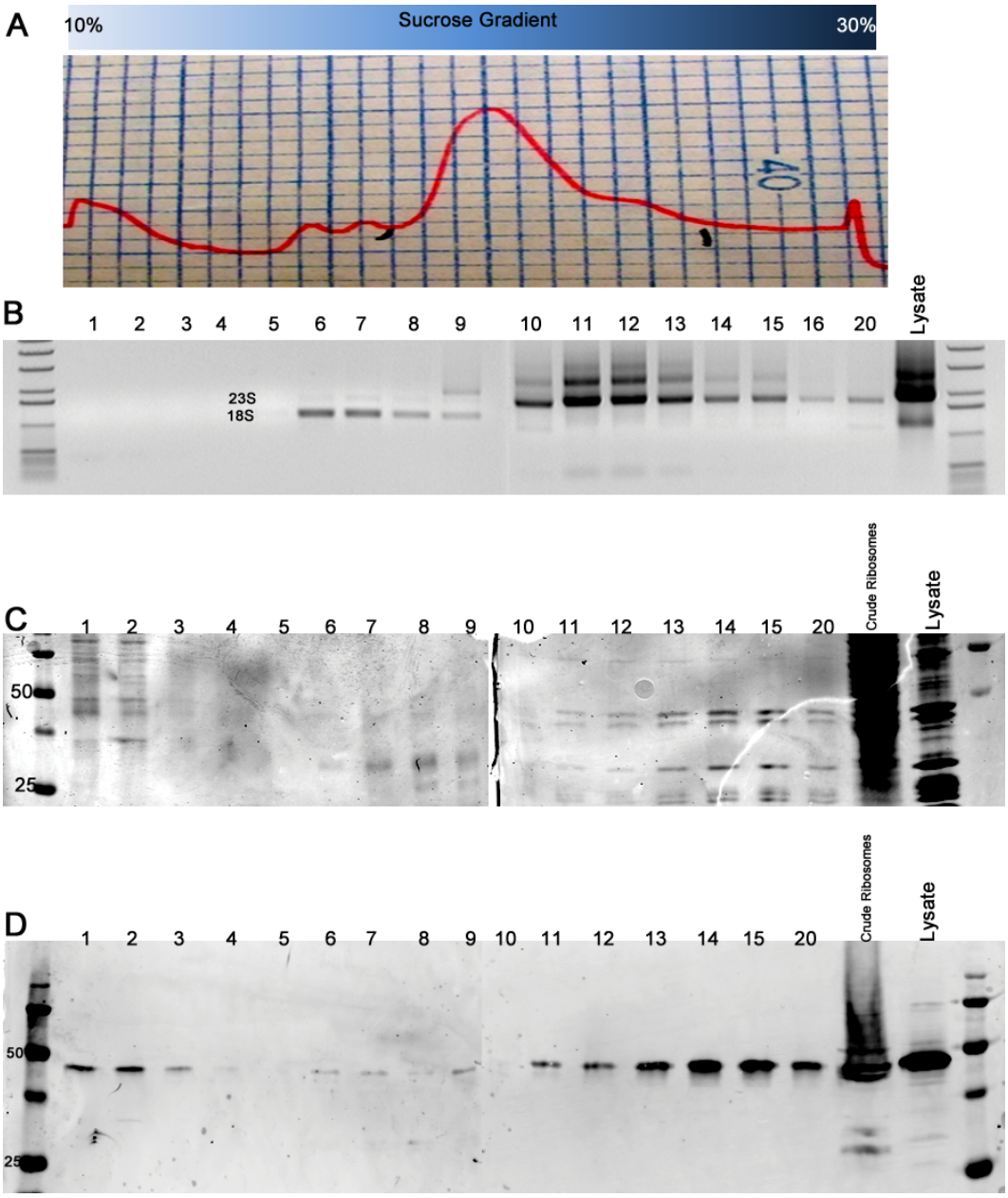

Figure A2.1: Sucrose gradient purification of ribosomal subunits. A) Fractionation trace for sucrose gradient. 0.5 mL aliqouts were collected and analysed by agarose gel (phenol chloroform extracted rRNA) (B), SDS-PAGE (C), and western blot (anti-GFP) (D). L11 is present at the earlier fractions which contain non-assembled ribosomal proteins and only in the fraction that contained the 60S subunit, those fractions corresponding to the 28S ribosomal RNA.

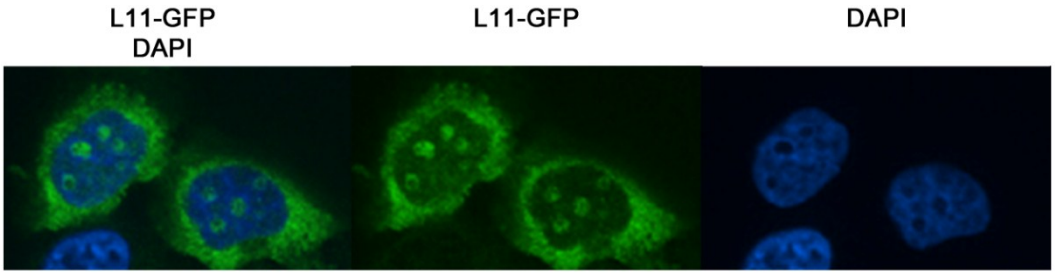

Figure A2.2: Distribution of GFP tagged ribosomal protein L11. The L11-GFP constructs were expressed in HeLa cells. The autofluoresecense of GFP and the DNA stain DAPI was observed with confocal microscopy. Ribosomal protein L11 is observed in the nucleolus, the region of ribosome biogenesis, and in the cytosol, the region of translation.

A.2.4 References

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