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Selective translation of leaderless mRNAs
under different stress conditions

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Sanda Pasc

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Betreuerin: Dr. Isabella Moll

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1. ABSTRACT

Upon infection of a host, bacteria experience changes in their environmental conditions, like variation of temperature and pH, nutrient or magnesium depletion as well as antibiotic treatment. Therefore they have evolved different mechanisms to modulate gene expression to allow fast adaptation to given conditions.

Recently, it was shown that treatment of *Escherichia coli* with the aminoglycoside antibiotic kasugamycin results in the formation of protein-depleted ribosomes. These particles selectively translate leaderless mRNAs, which contain a 5'-terminal AUG start codon and therefore lack other ribosome recruitment signals. Several lines of evidence indicate that the formation of a protein synthesis machinery, which is selective for leaderless mRNAs, might represent a novel stress response mechanism in bacteria. Therefore, one aim of this study was to determine conditions, which lead to selective or stimulated synthesis of leaderless mRNAs as a means to identify conditions affecting the protein complement of the ribosome. For this purpose, I tested a leaderless *gfp*-based reporter system, which would facilitate the screening of a variety of conditions. Moreover, to allow a direct comparison between leaderless and canonical mRNA translation, I constructed a bicistronic transcript, harbouring a leaderless *gfp* and a canonical *yfp* mRNA. The applicability of these constructs was validated in this study, employing different stress conditions.

The alteration of the transcriptional program represents a common mechanism for the stress response. The key molecules for the stimulation of alternative transcription are the guanosine tetra- and pentaphosphates (p)ppGpp, which accumulate fast upon nutritional stress by the activity of two enzymes, denominated RelA and SpoT, in a mechanism termed "stringent response". Recent studies

performed in the laboratory provide evidence for the accumulation of leaderless transcripts by alternative transcription upon kasugamycin treatment. Therefore, it was tempting to speculate that conditions, which induce the formation of leaderless mRNAs, might as well affect the protein synthesis machinery, making it selective for the leaderless mRNAs. As (p)ppGpp modulates transcription under stress conditions, the second aim of this study was to test whether (p)ppGpp is required for stimulation of leaderless mRNA translation. Employing pulse labeling assays, I was able to verify this hypothesis. Furthermore, several results indicate that the endonuclease MazF, which is part of the toxin-antitoxin system *mazEF*, is partially responsible for the formation of leaderless mRNAs via an mRNA processing mechanism. Since this toxin-antitoxin system can be triggered by increased levels of (p)ppGpp, I studied the effect of *mazF* overexpression on translation of a leaderless reporter mRNA. The results of the pulse labeling experiments revealed that overexpression of *mazF* concomitantly to formation of leaderless mRNAs affects the protein synthesis machinery and results in selective translation of this special class of mRNAs. This observation strongly supports the notion that under stress conditions the toxin-antitoxin system could be induced by increased levels of (p)ppGpp and consequently, the active MazF endonuclease might affect the selectivity of the ribosome.

Taken together, the formation of the leaderless mRNAs, which are selectively translated by modified ribosomes upon activation of MazF under stress could lead to the expression of genes, which are substantial for survival of bacteria under adverse conditions. Therefore, these observations point towards a novel post-transcriptional stress adaptation mechanism in bacteria to modulate gene expression.

2. ZUSAMMENFASSUNG

Bei der Infektion eines Wirtes werden Bakterien Änderungen in ihrer Umgebung ausgesetzt, wie z.B. Abweichungen der Temperatur und pH-Werte, Mangel an Nährstoffen oder Magnesium sowie die Behandlung mit Antibiotika. Darum haben sie unterschiedliche Mechanismen zur Regulation der Genexpression entwickelt, um eine schnelle Anpassung an die gegebenen Bedingungen zu ermöglichen.

Vor kurzem wurde gezeigt, dass die Behandlung von *Escherichia coli* mit dem Aminoglykosid-Antibiotikum Kasugamycin zur Bildung von protein-defizienten Ribosomen führt. Diese ribosomalen Partikel translatieren selektiv sogenannte „leaderless“ mRNAs, welche mit einem 5‘-terminalen AUG Startkodon beginnen und daher keine weiteren Translationsinitiationssignale aufweisen. Weitere Studien deuten darauf hin, dass die Ausbildung einer Proteinsynthesemaschinerie, die selektiv für „leaderless“ mRNAs ist, einen neuartigen Mechanismus in der Stressantwort von Bakterien representieren könnte. Daher war ein Ziel dieser Arbeit verschiedene Stressbedingungen auf eine selektive oder stimulierte Synthese von „leaderless“ mRNAs zu testen, um Bedingungen zu identifizieren die das Proteinkomplement von Ribosomen beeinflussen. Zu diesem Zweck habe ich ein „leaderless“ *gfp*-basiertes Reportersystem getestet, welches die einfache Untersuchung von unterschiedlichen Bedingungen ermöglicht. Um einen direkten Vergleich zwischen der Translation von „leaderless“ und kanonischen mRNAs zu ermöglichen, habe ich darüber hinaus ein bicistronisches Transkript konstruiert, das eine „leaderless“ *gfp* und eine kanonische *yfp* mRNA beinhaltet. Die Anwendbarkeit von diesem Konstrukt wurde in dieser Studie überprüft, indem unterschiedliche Stressbedingungen angewendet wurden.

Die Änderung des Transkriptionsprogramms stellt einen allgemeinen Mechanismus für eine Stressantwort in Bakterien dar. Die Schlüsselmoleküle für die Stimulierung der alternativen Transkription sind Guanosintetra- und pentaphosphat, (p)ppGpp, welche bei Nährstoffmangel durch die Aktivität zweier Enzyme, RelA und SpoT, in einem als „stringente Kontrolle“ bezeichneten Mechanismus schnell angereichert werden. Aktuelle Studien im Labor weisen auf die Bildung von „leaderless“ mRNAs durch alternative Transkription nach Behandlung mit Kasugamycin hin. Daher wäre es denkbar, dass Bedingungen, welche die Ausbildung von „leaderless“ mRNAs induzieren, sich auch auf die Ribosomen auswirken könnten und diese selektiv für „leaderless“ mRNAs machen. Da (p)ppGpp die Transkription unter Stressbedingungen moduliert, war das zweite Ziel dieser Arbeit zu testen, ob (p)ppGpp die Translation von „leaderless“ mRNAs stimulieren könnte. Mit Hilfe von „Pulse-labeling“ Analysen, konnte ich diese Hypothese bestätigen. Weitere Studien weisen auch auf einen Einfluss der Endonuklease MazF, welche einen Teil des Toxin-Antitoxin Systems *mazEF* darstellt, auf die Bildung von „leaderless“ mRNAs durch einen mRNA-abbauenden Mechanismus hin. Da dieses Toxin-Antitoxin System auch durch erhöhte Konzentration von (p)ppGpp aktiviert werden kann, habe ich den Effekt der Überexpression von *mazF* auf die Translation einer „leaderless“ Reporter-mRNA untersucht. Die Ergebnisse der „Pulse-labeling“ Experimente zeigten, dass Überexpression von *mazF* gleichzeitig die Ausbildung von „leaderless“ mRNAs und die Proteinsynthesemaschinerie beeinflusst und weiters zu selektiver Translation dieser speziellen Klasse von mRNAs führt. Diese Beobachtung unterstützt die Hypothese, dass das Toxin-Antitoxin System durch eine erhöhte Konzentration an

(p)ppGpp unter Stressbedingungen aktiviert wird und die aktive MazF Endonuklease die Selektivität des Ribosoms beeinflussen könnte.

Somit könnte unter Stress MazF sowohl zur Bildung von „leaderless“ mRNAs als auch zur Modifikation von Ribosomen führen. Die resultierende selektive Translation könnte wesentlich für das Überleben von Bakterien unter ungünstigen Bedingungen sein. Zusammengefasst, weisen diese Ergebnisse auf einen neuen Mechanismus in der Stressantwort hin, der es Bakterien ermöglicht, rasch auf sich ändernde Bedingungen zu reagieren.

3. PREFACE

It is commonly assumed that life originated from RNA instead of DNA and protein. An increasing number of experiments support this hypothesis of a primordial RNA world (Mojzsis et al., 1996; Joyce and Orgel, 1999; White, 1976; Green and Noller, 1997; Bartel and Unrau, 1999; Poole et al., 1998; Cech, 2009) in showing that RNA plays a key role in gene regulation and cellular processes which depicts its biochemical complexity and evolutionary occurrence.

In theory, RNA molecules catalysed biochemical reactions and stored genetic information in the early evolution of life (Gilbert, 1986). Presumably, catalytic RNAs served as genes, facilitated by the linearity of its sequence, and were amplified easier than proteins (Bartel and Unrau, 1999). Eventually, RNA was gradually replaced by proteins as the main biological catalyst. One general assumption is the transfer of catalysis from RNA → ribonuleoprotein (RNP) → protein (Poole et al., 1998). Recently, further investigations on RNPs illustrate the similarity of the enzymatic activity of contemporary RNPs and proteins (Cech, 2009). The author argues that in a primordial RNA world the self-replication of RNA and ribozyme catalysis had to cope with peptides of mixed chirality and random sequence in their environment. In short, even though RNA is able to catalyse reactions alone, the presence of peptides and other small molecules might have prevented them from the opportunity to operate as a pure ribozyme. During evolution DNA replaced RNA as genetic polymer and the protein replaced RNA as major biocatalyst. However, conversion to protein catalysis is believed to be incomplete and therefore RNA maintains a key role in protein synthesis (Green and Noller, 1997).

More than 50 years ago the Central Dogma of molecular biology was first proposed (Crick, 1958; Crick, 1970). Mainly, it states that transfer of sequential information takes place from DNA → RNA → protein. However, different observations challenged the Central Dogma. First, the discovery of the reverse transcriptase, an enzyme able to catalyse DNA synthesis based on an RNA template, extended the Central Dogma (Baltimore, 1970; Temin and Mizutani, 1970). Consequently, the informational transfer from RNA → DNA was evidenced and awarded with the Nobel Prize in 1975. Finally, new phenomena like epigenetic modifications of DNA, RNA interference, RNA splicing and editing (Jukes, 1990; Thieffry and Sarkar, 1998) appearing in the informational transfer from DNA → proteins challenged the Central Dogma.

Discussion about the existence of a pre-RNA world preceding the DNA world is going on (Orgel, 1986; Schwartz et al., 1987; Joyce, 1989; Orgel, 1989; De Duve, 1993; Piccirilli, 1995; Miller, 1997). Alternative hypothesis are a simultaneous appearance of RNA and DNA (Oro and Stephen-Sherwood, 1974) as well as DNA before proteins (Benner et al., 1987; Benner et al., 1989; Benner et al., 1993). An increasing number of experiments suggest the development of protein synthesis in the RNA world as summarized by Kumar and Yarus (2001).

The ribosome, which serves as a model for a contemporary ribozyme mainly consisting of ribosomal RNA is structurally conserved in all three domains of life. The crystal structure of the large ribosomal subunit has evidenced that the ribosome is fundamentally a peptidyl-transferase ribozyme (Ban et al., 2000).

4. INTRODUCTION

4.1. The ribosome

One key process in life is the decoding of the messenger RNA (mRNA) into polypeptides, denominated protein synthesis. This intricate process is performed by a large and complex ribonucleoprotein particle, the ribosome.

4.1.1. The composition and structure of the ribosome

The key components of the ribosome are conserved in all three kingdoms of life, bacteria, archaea and eukarya. The bacterial ribosome has a sedimentation coefficient of 70S, a mass of approximately 2.6 – 2.8 MDa and a diameter of 200 – 250Å. The 70S ribosome is composed of two unequal subunits, the small 30S and the large 50S subunit (Tissieres et al., 1959). The mass of each subunit is divided into one-third protein and two-thirds RNA respectively. The 30S subunit is made up of a single 16S rRNA and 21 proteins, whereas the 50S subunit has two rRNAs, namely 5S and 23S rRNA, and 33 proteins.

At the beginning of the new millennium the first high-resolution structures of the bacterial ribosomal subunits were elucidated. These results, which were awarded 2009 with the Nobel Prize to Ada E. Yonath, Venkatraman Ramakrishnan and Thomas A. Steitz, indicated that the main component of the functional centers of 30S and 50S subunits as well as the subunit interface consist mainly of RNA. The ribosomal proteins are arranged primarily at the periphery of both subunits and serve as scaffold for the translational machinery (Ban et al., 2000; Carter et al., 2000; Schluenzen et al., 2000; Wimberly et al., 2000; Nissen et al., 2000). However, both

components – RNA and proteins – have to be present for various steps leading to protein synthesis.

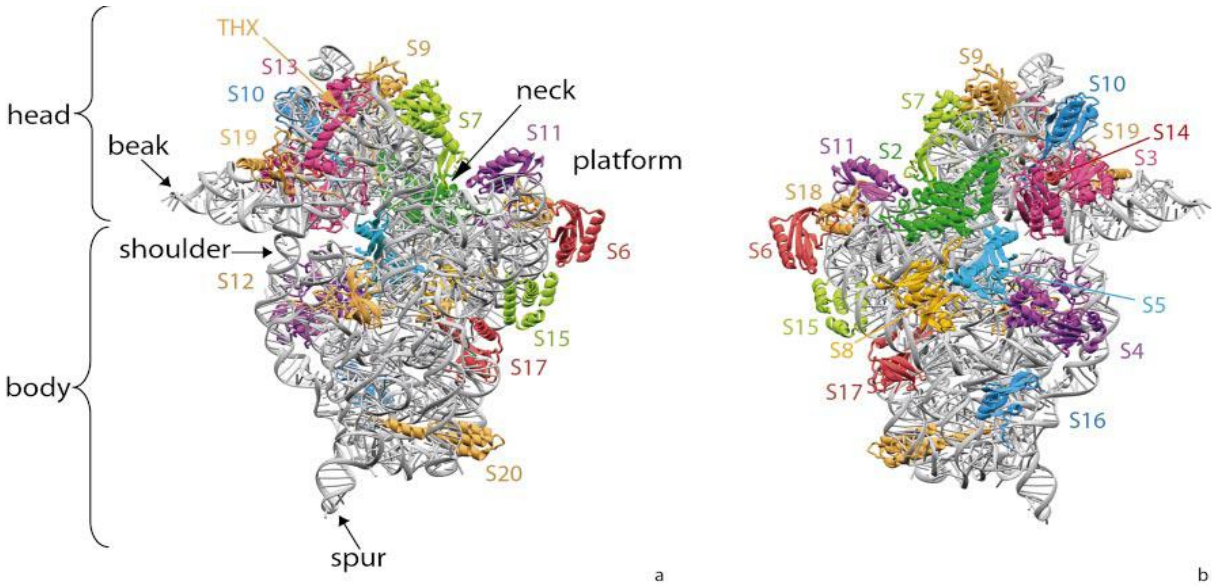

Figure 1 - The 30S ribosomal proteins

The 16S RNA is depicted as a grey ribbon-stick. The ribosomal proteins are overlayed. Morphological characteristics which were found in the early EM reconstructions are indicated. (a) The front side (or interface) of the 30S subunit, (b) the back of the 30S subunit.

(taken from Brodersen et al., 2002)

Decoding of the mRNA is performed on the ribosome by transfer RNAs (tRNAs) (Brodersen et al., 2002; Figure 1). Every tRNA carries a specific amino acid that is added to the growing polypeptide chain. The ribosome provides three binding sites for the tRNAs, namely the A-site, P-site and E-site (Frank, 2003). At the A-site decoding is taking place. Here selection of the correct aminoacyl-tRNA (aa-tRNA) is performed by codon-anticodon interaction. The P-site carries the peptidyl-tRNA, which bears the growing polypeptide chain (Frank, 2003). Upon peptide-bond formation the deacylated tRNA moves to the E(exit)-site. Therefore, the protein synthesis may be

divided into three individual phases, namely first the initiation phase, second the translation and elongation phase and third the termination and ribosome recycling phase (Frank, 2003).

4.1.1.1. Initiation of translation

One of the rate-limiting steps of protein synthesis is the translation initiation (Gualerzi and Pon, 1990). Generally, the ribosome is programmed with an initiator tRNA and mRNA, such that the start codon and tRNA are both positioned at the P-site (Nierhaus and Wilson, 2004). To assure the fidelity of translation initiation, in bacteria this process is assisted by three initiation factors (IFs), namely IF1, IF2 and IF3.

In the first step, IF1 and IF3 bind to the 30S subunit. IF3 is a two domain protein and recent crystallization analysis as well as site directed hydroxylradical probing experiments have shown that its C-terminal domain is located at the subunit interface (Ramakrishnan, 2002). Therefore one function of IF3 is to prevent the premature association of the 30S and 50S subunits. When the mRNA binds to the 30S subunit, the start codon is positioned in the P-site by the interaction between the Shine-Dalgarno (SD) sequence, located on the mRNA upstream of the start codon, and the anti-SD at the 3' terminus of the 16S rRNA (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Jacob et al., 1987). The exact role of IF1 is still unknown, however it binds to the A-site thereby preventing premature binding of elongator tRNAs (Dahlquist and Puglisi, 2000; Carter et al., 2001). Additionally, IF3 selects for the correct codon-anticodon interaction at the P-site and thereby selects for the initiating formylmethionine - tRNA (fMet-$tRNA_f^{Met}$) (Hartz et al., 1990).

Next the complex is joined by GTP-bound IF2 which facilitates the binding of fMet-$tRNA_f^{Met}$ to the 30S subunit (Carter et al., 2001). Accordingly, a codon-anticodon interaction of the mRNA start codon and the fMet-$tRNA_f^{Met}$ enables correct binding (Mayer et al., 2003). Finally, the 50S subunit assembles upon IF2 dependent GTP hydrolysis and all three IFs from the functional 70S ribosome are released.

4.1.1.2. Elongation and peptide bond formation

In bacteria, elongation is catalyzed by the elongation factors EF-Tu, EF-Ts and EF-G. First, the cognate aa-tRNA-EF-Tu-GTP complex binds to the A-site of the 70S initiation complex. Upon GTP hydrolysis the EF-Tu-GDP complex is released from 70S (Sprinzl, 1994; Rodnina et al., 1996). The regeneration of EF-Tu-GDP takes place in a process including EF-Ts and GTP.

Next, peptide bond formation occurs between the A-site bound aa-tRNA and the P-site bound peptidyl tRNA (Moazed and Noller, 1989). By this reaction a dipeptidyl-tRNA is produced in the A-site, and the now deacylated $tRNA_f^{Met}$ is still bound to the P-site.

Peptide bond formation occurs in the peptidyl transferase center (PTC) of the 50S subunit. Identification of the PTC was achieved by usage of a putative transition state analogue of the peptidyl transferase reaction, soaked into crystals of the 50S subunit (Nissen et al., 2000). Further, this analogue mimics the CCA end of the P-tRNA which is attached to puromycin in the A-site and is a strong competitive inhibitor of the A-site substrate (Welch et al., 1995). The region moulding the inhibitor binding site is tightly packed with the highly conserved bases of the 23S rRNA, which are mainly derived from the peptidyl transferase ring of the domain V. The ring structure of the peptidyl

transferase contains 41 nucleotides (Agmon et al., 2003) and is highly conserved in rRNA.

The final step of elongation phase is the translocation, which moves the ribosome one codon towards the 3' end of the mRNA (Frank and Agrawal, 2000). As a result, peptidyl-tRNA located in the A-site is shifted to the P-site. This leads to a shift of the deacylated tRNA from the P-site to the E-site, where release of the tRNA into the cytosol takes place. Thereby, the third codon of the mRNA is positioned in the A-site and the second codon in the P-site. Elongation factor EF-G is required for the movement of the ribosome along the mRNA and the energy is provided by hydrolysis of GTP (Frank and Agrawal, 2000).

4.1.1.3. Termination and ribosome recycling

If one of three termination codons, namely UAA, UAG or UGA, occupies the A-site in bacteria, three release factors, so-called RF1, RF2 and RF3, signal termination to the ribosome. The domains of the release factors are thought to mimic the tRNA structure. The release factor RF1 specifically recognises the termination codons UAA as well as UAG, and RF2 recognises UAA as well as UGA. RF1 or RF2 binds to the termination codon and induces a peptidyl transferase reaction, where a water molecule is transferred to the growing polypeptide chain rather than another amino acid. Next, RF3 releases RF1 and RF2 from the post-termination complex by means of GTP hydrolysis. The final dissociation of the 70S ribosome into its 30S and 50S subunit occurs with the ribosome recycling factor RRF, in combination with the elongation factor EF-G and IF3 (Kisselev and Buckingham, 2000). Finally, after the

dissociation of the 70S ribosome, IF3 binds to the 30S subunit and prevents a reassociation (Hirashima and Kaji, 1973; Zavialov et al., 2005; Peske et al., 2005).

4.1.2. The protein complement of the bacterial ribosome

The 30S subunit from *Escherichia coli* (*E. coli*) contains 21 ribosomal proteins, which are termed S1 – S21. The 50S subunit harbours 33 proteins termed L1 – L36. However, several numbers of the 50S subunit were eliminated. This concerns the ribosomal protein L7, which represents a modified version of L12. Furthermore, L8 was deleted because it was proved to be a complex of L7/L12 and L10 (Pettersson et al., 1976) and ribosomal protein L26 equals S20.

The 54 bacterial ribosomal proteins differ strongly in structure and size. Their molecular weights range from 6.000 to 75.000. In general, ribosomal proteins have globular domains placed on the ribosomal surface. Besides, some have snakelike protein extensions without ordered structures which protrude into the rRNA core of the ribosome, thereby anchoring the proteins and stabilising the ribosome structure (Nierhaus and Wilson, 2004).

Each ribosome holds one copy per ribosomal protein (Hardy, 1975), with the exception of L7/L12 which occurs four-times. Approximately two-thirds of all ribosomal proteins belong to the RNA-binding family and bind to naked rRNA *in vitro*.

In contrast to several copies of rRNA genes in *E. coli*, there is only one copy of each gene encoding a ribosomal protein (Wagner, 2001), most of which are arranged in the *str*-, S10-, *spc*- and *α*-operons (Zengel and Lindahl, 1994; Wagner, 2001). The residual ribosomal proteins are distributed over the *E. coli* chromosome (Wagner, 2001). The expression of ribosomal proteins is supposed to be adapted to the level of

rRNA by autogenous control (Wagner, 2001). If this balance is affected due to overproduction of a ribosomal protein, a negative feedback regulation is triggered. Briefly, free ribosomal proteins inhibit translation of their corresponding polycistronic mRNAs by binding to mRNA structures that are similar to their binding site in the ribosome (Wagner, 2001).

Already more than 40 years ago, it was shown that the protein complement of the ribosome varies in *E. coli* as a function of growth rate (Deusser and Wittmann, 1972). *In vivo* studies performed under different growth conditions, namely in rich or minimal medium, resulted in the reduction of the ribosomal proteins L12, S6 and S21 and the increase of L7 in minimal medium (Deusser and Wittmann, 1972). Moreover, purified 30S subunits of *E. coli* are, functionally and structurally, heterogenous *in vitro* (Kurland et al., 1969; Voynow and Kurland, 1971). These results are supported by the observation that single deletions of ribosomal proteins from *E. coli* do not affect cell viability (Dabbs et al., 1983). In these experiments mutants were used lacking one or two ribosomal proteins (Dabbs et al., 1983). Furthermore, comparison of the genomes of bacteria, archaea and eukarya, indicates that ribosomal proteins are conserved (Lecompte et al., 2002), next to translation factors (Pandit and Srinivasan, 2003).

4.1.2.1. Ribosomal protein S1

Protein S1 consists of 557 amino acid residues and features two N-terminal RNA-binding motifs, which are required for ribosome binding, and four C-terminal RNA-binding motifs. It represents with 61,2 kDa the largest ribosomal protein, which is present in the 30S subunit of *E. coli* (Wittmann, 1974) and is essential for translation

initiation in gram-negative bacteria (Tzareva et al., 1994). In contrast to other ribosomal proteins, S1 has a high affinity for mRNA (Draper and Hippel, 1978) and it is implicated in the selection of the translational start sites on the mRNA (Subramanian, 1984). It was suggested that it binds a pyrimidine-rich region (Sengupta et al., 2001) upstream of the SD sequence of the mRNA (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Jacob et al., 1987). Thereby it stabilises the mRNA on the ribosome and is important for the recognition of the 5' untranslated region (UTR). Moreover, S1 is associated weak and reversible to the ribosome by means of protein-protein interaction whereas most of the ribosomal proteins show strong binding. Additionally, all ribosomal proteins are basic, except for the protein L7/L12 and the acidic protein S1.

4.1.3. Antibiotics interacting with the ribosome

A large number of antibiotics target the protein synthesis machinery, which harbours numerous potential sites for the functional interference. The specific inhibition of nearly every step in protein synthesis (Nierhaus and Wilson, 2004; Yonath, 2005) enables antibiotics to be a valuable tool in the study of protein biosynthesis. Previous studies showed that the interaction sites of antibiotics overlap with ribosomal functional centers, like the tRNA binding sites and the decoding center (Tenson and Mankin, 2006). Generally, ribosome targeting antibiotics interact with the rRNA rather than with proteins (Sutcliffe, 2005), e. g. tetracycline, which inhibits protein synthesis in bacteria by blocking the ribosomal A-site and preventing the binding of aa-tRNAs (Pioletti et al., 2001), and chloramphenicol, which blocks peptidyl transfer of bacterial ribosomes (Hansen et al., 2003) and affects also mitochondrial

and chloroplast ribosomes in eukaryotes. The aminoglycoside antibiotics gentamycin, kanamycin, neomycin and paramycin bind to the 30S subunit and disrupt mRNA-decoding fidelity (Sutcliffe, 2005).

4.1.3.1. The aminoglycoside antibiotic kasugamycin

The aminoglycoside antibiotic kasugamycin (Ksg) acts as inhibitor of translation initiation of canonical mRNAs without affecting elongation (Kozak and Nathans, 1972). However, translation of leaderless mRNAs continues in the presence of the antibiotic (Chin et al., 1993; Moll and Bläsi, 2002). It has been shown that binding of the fMet-tRNA$_f^{Met}$ to the P-site of 30S is prevented by Ksg (Okuyama et al., 1971; Poldermans et al., 1979). Consequently, the pre-initiation complex formation is inhibited in the presence of Ksg.

Resistance to Ksg occurs in mutants lacking a functional *ksgA* gene, named after the phenotype of the resistance (Sparling, 1970; Helser et al., 1971; Helser et al., 1972). The gene *ksgA* encodes an adenine dimethylase that post-transcriptionally modifies the universally conserved bases A1518 and A1519, corresponding to the *E. coli* numbering, which are located in helix 45 close to the 3'-end of the 16S rRNA (Sparling, 1970; Helser et al., 1971; Helser et al., 1972). Furthermore, it was previously shown, that nearly every base mutation at the position A1519 confers resistance to Ksg (Vila-Sanjurjo et al., 1999). Moreover, this work determined two other universally conserved 16S rRNA bases at the positions A794 and G926, which confer Ksg resistance (Vila-Sanjurjo et al., 1999). These data verified earlier studies that revealed that these bases which are protected from chemical modification by P-

tRNA (Moazed and Noller, 1987) are likewise protected by binding of Ksg to the ribosomes, assayed by chemical probing (Woodcock et al., 1991).

Recently the binding sites of Ksg were determined on the *Thermus thermophilus* 30S ribosomal subunit as well as on the *E. coli* 70S ribosome (Schluenzen et al., 2006; Schuwirth et al., 2006). These studies indicated that the primary binding site of Ksg overlaps with the mRNA binding site located in the P-site (Schluenzen et al., 2006).

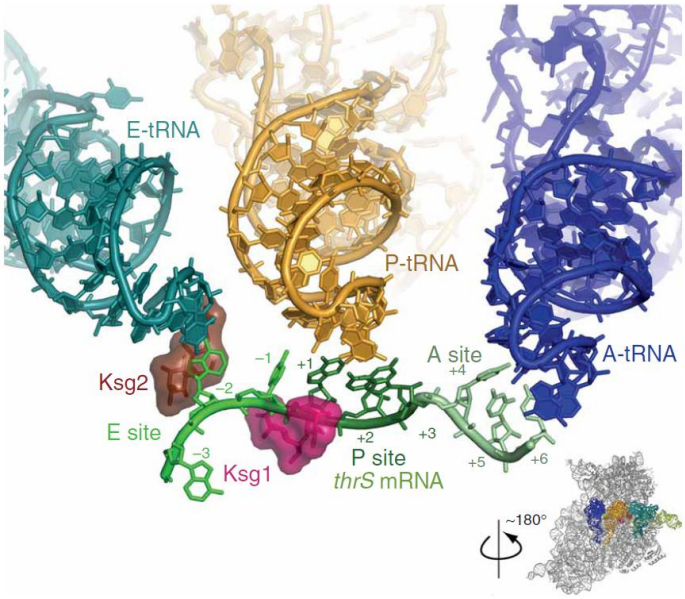

Figure 2 - The position of Ksg overlaps with P-site mRNA

Two Ksg binding sites are shown relative to A-, P- and E-tRNAs.
(taken from Schluenzen et al., 2006)

Interestingly, Ksg binds to two defined positions, namely Ksg 1 and Ksg2, at the mRNA path (Schluenzen et al., 2006; Figure 2). Ksg1 overlaps with the position of the start codon in the P-site and Ksg2 covers the mRNA located in the E-site (Figure 2).

4.2. The bacterial ribosome binding site

4.2.1. Canonical messenger RNA

In bacteria, most mRNAs contain a 5' UTR with a length of 26 to over 200 nucleotides (nt). The correct start codon is defined by the presence of a RBS, which is defined as the sequence of mRNA which is protected against RNase digestion and extends over about 15 nt up- and downstream of the start codon (Steitz and Jakes, 1975). It harbours signals for ribosome recognition, namely the start codon, the SD sequence and a region rich in pyrimidines for S1 binding as specified before (3.2.2.1.).

4.2.1.1. The start codon

Mainly, AUG is used as start codon. By codon-anticodon interaction with the fMet-$tRNA_f^{Met}$ positioned in the ribosomal P-site, the AUG start codon is recognised and determines the reading frame (Mayer et al., 2003). If AUG is replaced by an alternative start codon, the pairing with fMet-$tRNA_f^{Met}$ is weaker and results in less efficient translation initiation. Nevertheless, in *E. coli* 14% of the genes harbour GUG and 3% UUG as start codon (Blattner et al., 1997). In Gram-positive bacteria and several bacteriophages the usage of UUG as start codon is more common (Kunst et al., 1997; Lobocka et al., 2004). AUU as start codon is only used for two *E. coli* genes, one is coding for the poly(A) polymerase PcnB (Binns and Masters, 2002) and the other encodes IF3, where it is implicated in autoregulation of IF3 synthesis (Dallas and Noller, 2001).

4.2.1.2. The Shine Dalgarno sequence

The purine-rich SD sequence is located 5 to 8 nt upstream of the start codon and has in general a length of 4 to 5 nt. During initiation it base pairs with the anti-SD sequence, which is located at the 3' end of the 16S rRNA (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Jacob et al., 1987). This complementarity is important for positioning of the AUG start codon in the P-site and consequently it enhances the formation of the translation initiation complex. The optimal distance of the SD sequence to the start codon depends on the interacting bases at the 3' end of the 16S rRNA (Chen et al., 1994a).

4.2.2. Leaderless mRNAs

Leaderless mRNAs start directly with the AUG start codon and therefore lack all ribosome recruitment signals besides the AUG start codon. Therefore, mutations of the AUG start codon reduce the translational efficiency (Moll et al., 2002) and *in vivo* studies identified AUG as the only functioning start codon in the absence of a 5' leader (Van Etten and Janssen, 1998). They are present in all kingdoms of life. However, they are prevalent in Gram-positive bacteria and archaea. In *E. coli*, the known leaderless mRNAs are derived from mobile genetic elements like the *λcI* mRNA (Walz et al., 1976) and the *tetR* mRNA from transposon Tn*1721* (Baumeister et al., 1991). Additionally, a number of leaderless mRNAs were determined in *Mycoplasma pneumoniae* (Weiner et al., 2000) and the leaderless *argF* mRNA encoding ornithine carbamoyltransferase was determined in *Thermus thermophilus* (Sanchez et al., 2000).

Previous studies revealed that translation of leaderless mRNAs is carried out by 70S ribosomes (Balakin et al., 1992; Moll et al., 2004), which is supported by the observation that the initiation complex formed with 70S ribosomes show a higher stability with 70S ribosomes compared to 30S subunits (Udagawa et al., 2004; O'Donnell and Janssen, 2002). Furthermore, it was demonstrated that translation of leaderless mRNAs is possible in the absence of IFs (Udagawa et al., 2004).

It was shown that IF2 stimulates translation of leaderless mRNA, whereas IF3 has an inhibitory effect. Hence, the ratio between IF2 and IF3, is pivotal for the regulation of translation on leaderless mRNAs (Grill et al., 2001). Furthermore, it was evidenced that modulation of the translational efficiency on leaderless mRNAs also depends on protein S1 (Moll et al., 1998). S1 was shown to be dispensable for translation of leaderless mRNAs in contrast to canonical mRNAs, where it is essential for ternary complex formation (Tedin et al., 1997). Since S1 binds to a pyrimidine-rich region upstream of the SD-sequence on the mRNA, the dispensability of S1 can be attributed to the lack of the 5' UTR.

Furthermore, due to the lack of species specific ribosome recruitment signals, leaderless mRNAs can be translated in heterologous systems, which implies a conserved function of the translation machinery during evolution. Consequently, it was suggested that leaderless mRNAs could represent relics from ancestral mRNAs (Moll et al., 2002).

4.2.2.1. Selective translation of leaderless mRNAs in the presence of kasugamycin

In contrast to canonical mRNAs, the presence of the aminoglycoside antibiotic Ksg does not inhibit translation of leaderless mRNAs *in vivo* (Chin et al., 1993; Moll and Bläsi, 2002). Additionally, it was shown that 70S translation initiation complexes are relatively resistant to this antibiotic (Moll and Bläsi, 2002).

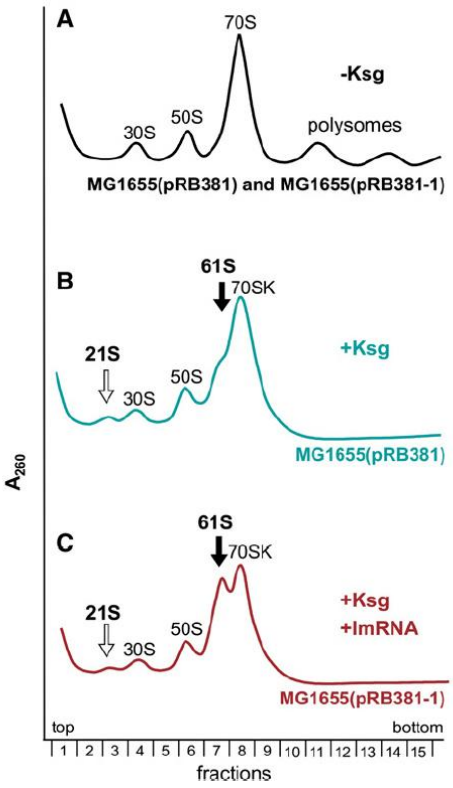

Figure 3 - The formation of 61S and 21S protein-deficient ribosomes (taken from Kaberdina et al., 2009)

Previous data revealed the formation of 61S ribosomes deficient in several small ribosomal proteins in the presence of Ksg *in vivo* (Kaberdina et al., 2009). Ribosome profile analysis revealed that in the presence of Ksg a small 21S peak was formed, which represents precursor particles inhibited in assembly (Figure 3B and Figure 3C). Moreover, in the presence of leaderless mRNA, addition of Ksg induced the formation of a clear 61S peak (Figure 3C). The protein composition of the 61S peak was identified by mass spectrometry and western blot analysis. The results showed a deficiency in the 30S ribosomal proteins S1, S2, S6, S12, S18 and S21 and displayed a significant reduction of S3, S5, S11, S16 and S17. In contrast, the 50S subunit remained unchanged (Kaberdina et al., 2009). Furthermore, this work showed that selective

translation of leaderless mRNAs can be attributed to the protein-deficient ribosomes even in the presence of Ksg (Kaberdina et al., 2009).

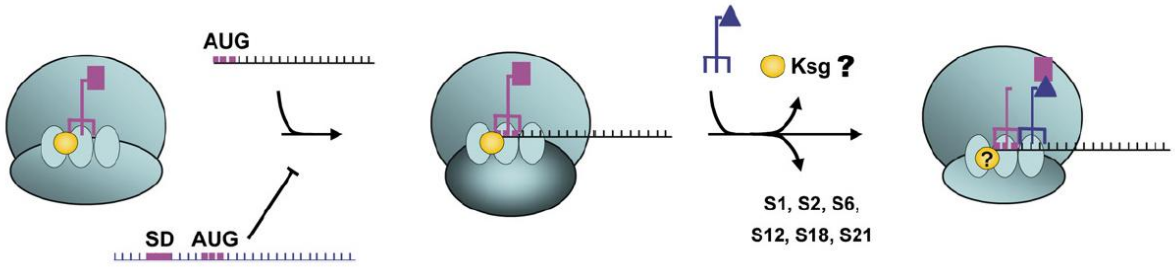

Figure 4 - Model for the 61S particle formation in the presence of the antibiotic Ksg and concomitant overexpression of a leaderless mRNA

(taken from Kaberdina et al., 2009)

Taken together, a model (Figure 4) for the formation of 61S particles was proposed, wherein the formation of an initiation complex by 70S ribosomes on a leaderless mRNA in the presence of Ksg leads to a conformational change in the 70S ribosome, which results in the release of several ribosomal proteins (Kaberdina et al., 2009).

4.3. Toxin-antitoxin modules in *E. coli*

The genomes of bacteria encode a number of genetic elements called toxin-antitoxin (TA) modules (Gerdes, 2000; Gerdes et al., 2005). Typically, the modules are composed of two translationally coupled genes. One of these genes codes for a stable toxin and the other encodes an unstable antitoxin. Generally, the toxin inhibits several important cellular functions, whereas the antitoxin is responsible for the neutralisation of the toxin by direct protein-protein interaction with the toxin.

Up to now, more than six TA systems were determined in *E. coli*. These are *relBE* (Bech et al., 1985), *chpBIK* (Masuda et al., 1993), *mazEF* (Aizenman et al., 1996), *yefM-yoeB* (Gotfredsen and Gerdes, 1998), *dinJ-yafQ* (Motiejunaite et al., 2007) and *mqsR-ygiT* (Kasari et al., 2010). However, the physiological role of TA modules is still a matter of debate. First, it was proposed that TA modules trigger programmed cell death (PCD) as response to stress, like antibiotic treatment, starvation and heat (Aizenman et al., 1996; Sat et al., 2001; Hazen et al., 2004). In contrast, Gerdes and co-workers suggested a role of TA modules in inhibition of macromolecular synthesis, which leads to reversible growth inhibition, also known as bacteriostasis (Christensen et al., 2001; Gerdes, 2000).

4.3.1. MazEF

The best studied TA module is *mazEF* (Metzger et al., 1988; Masuda et al., 1993), located downstream of the *relA* gene in the same operon (Metzger et al., 1988; Masuda et al., 1993). The module is composed of two contiguous and translationally coupled genes, namely *mazE* and *mazF* (Figure 5). The stable toxin MazF is an endoribonuclease, which specifically cleaves single stranded RNA at ACA-sites. It is

inhibited by interaction with the labile antitoxin MazE (Aizenman et al., 1996; Kamada et al., 2003; Figure 5). However, upon stress treatment MazE is degraded by the ATP-dependent ClpPA serine protease *in vivo* (Aizenman et al., 1996; Figure 5). In addition, the module is negatively autoregulated at the transcriptional level by binding of both proteins to the *mazEF* promoter P_2 (Marianovsky et al., 2001; Figure 5).

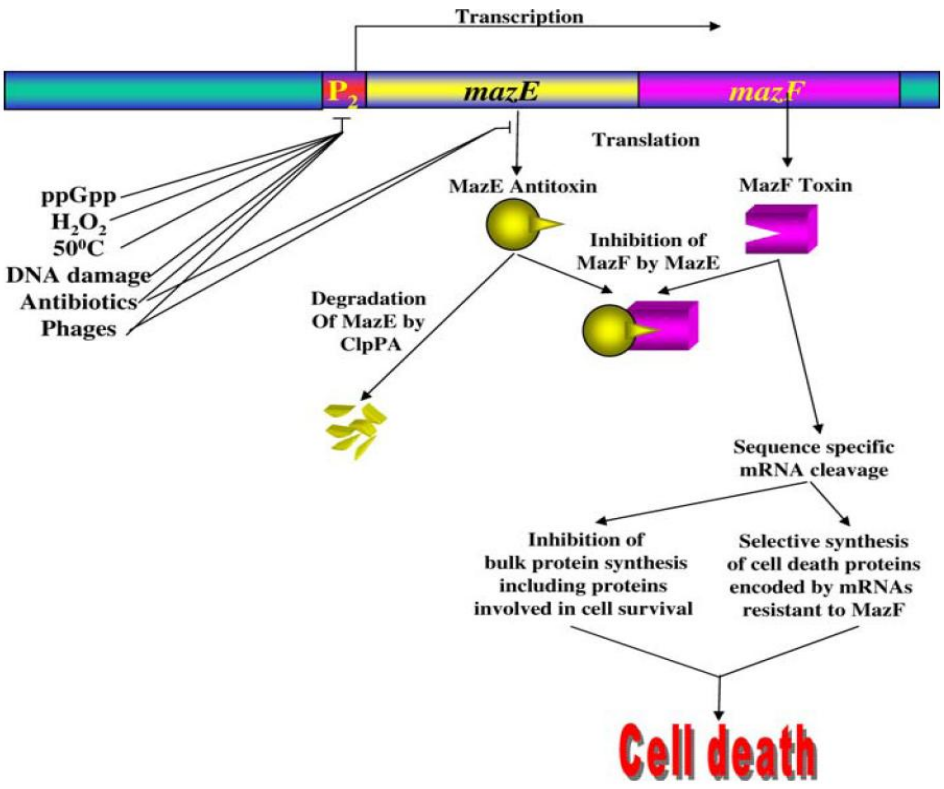

Figure 5 - Genomic organisation and function of the *mazEF*- module (taken from Engelberg-Kulka et al., 2006)

5. AIMS OF THE STUDY

When pathogenic bacteria enter their host, they encounter changes in the environmental conditions, like temperature and pH, as well as nutrient or magnesium depletion. Previous experiments in the laboratory revealed that stress conditions, like antibiotic treatment, result in the formation of protein-depleted ribosomes. Furthermore, these ribosomes were shown to selectively translate leaderless mRNAs, which start directly with the AUG start codon. In contrast, bulk mRNA translation was inhibited. Here, the aim of the study was to test diverse stress conditions for the stimulation of leaderless mRNA translation as a means to identify conditions affecting the protein complement of the ribosome.

For this purpose, I first constructed a plasmid coding for a bicistronic mRNA harbouring a leaderless *gfp* mRNA (encoding the green fluorescent protein) followed by a canonical *yfp* mRNA (encoding the yellow fluorescent protein). To validate the functionality of the construct, *E. coli* harbouring the plasmid was grown in the presence of the aminoglycoside antibiotic Ksg and the amount of active GFP and YFP was determined by fluorescence measurement upon native gel electrophoresis. Upon quantification, the results indicate a stimulated translation of leaderless mRNA compared to canonical mRNA in the presence of Ksg. Since these data are consistent with the results presented by Kaberdina et al. (2009), the construct was used for further analysis of diverse stress conditions including cold and heat stress, some of them leading to a stimulated translation of leaderless *gfp* mRNA. As this result could hint towards the presence of protein-depleted ribosomes, under these conditions the protein complement of the ribosomes will be studied.

Furthermore, using a construct encoding the leaderless *cI-lacZ* fusion gene I studied the effect of (p)ppGpp on the translation of leaderless mRNAs. Previous experiments in our laboratory revealed resumed expression of several stress response genes upon Ksg treatment. Here, I studied possible mechanisms resulting in selective translation of leaderless mRNAs upon Ksg treatment. These data support a correlation between the selective translation and a transcriptional reprogramming due to increased (p)ppGpp levels by the stringent response.

In addition, I studied the effect of the toxin MazF on translation of leaderless mRNAs. MazF represents the stable toxin component of the toxin-antitoxin module MazEF in *E. coli*, which consists of two translationally coupled genes, the first encoding the labile antitoxin MazE, the latter being MazF, a RNA interferase that cleaves mRNA site-specifically (Zhang et al., 2003; Zhang et al., 2005). Previous experiments indicated that MazF activity generates leaderless mRNA *in vivo* and *in vitro*. Therefore, the arising question I addressed was, whether overexpression of *mazF* might concomitantly affect the protein synthesis machinery resulting in selective translation of leaderless mRNAs. Employing a leaderless *cI-lacZ* construct together with the technique of pulse labeling, I was able to confirm this assumption.

6. RESULTS AND DISCUSSION

6.1. Testing the translational activity of a leaderless mRNA *in vivo* employing a *gfp*-based reporter system

As mentioned before, translation of leaderless mRNAs was stimulated in *E. coli in vivo* by treatment of the cells with the antibiotic Ksg (Kaberdina et al., 2009). This effect could be attributed to the formation of protein-depleted ribosomes in the presence of the antibiotic. In this study, one aim was to determine the relative translation of a leaderless mRNA compared to canonical transcripts under a variety of different conditions, as a means to identify conditions affecting the protein complement of the ribosome. In order to facilitate and accelerate this screen, the leaderless *cI*$_{18}$-*gfp* fusion gene (described in Material and Methods 7.1.2.1.) was used, which would allow a fast measurement of gene expression without the need of pulse labeling. Expression of the leaderless *cI*$_{18}$-*gfp* fusion gene was monitored under diverse conditions and compared to the expression of the canonical *gfp* gene (described in Material and Methods 7.1.2.), which served as a control. Briefly, wildtype *E. coli* strain MG1655 (Blattner et al., 1997), which harboured either the leaderless construct *cI*$_{18}$-*gfp* or the canonical construct can-*gfp* was grown in Luria-Bertani (LB) media (Miller, 1972) in the presence of 100 µg/ml ampicillin. Both genes were placed under control of a constitutive *lac* promoter.

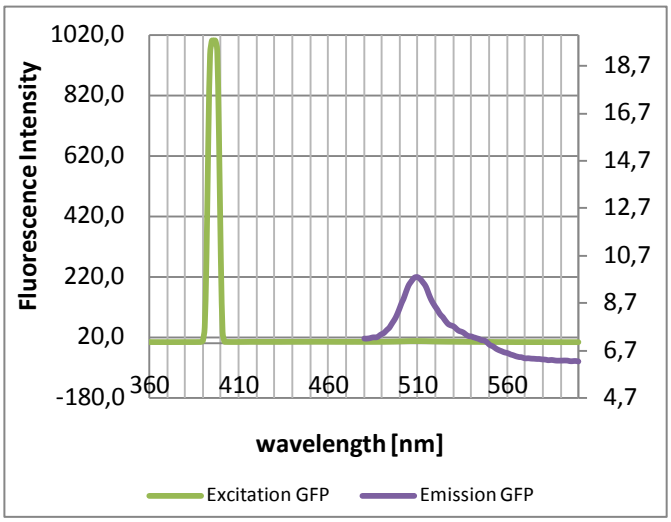

Figure 6 - Emission Spectrum of GFP

The correct excitation and emission wavelengths were determined by spectrum measurements with a spectrofluorometer (FP-6300, Jasco). The excitation wavelength of GFP is 395nm and the emission wavelength is 509nm.

Before (time point 0) as well as 15 min, 30 min, 60 min and 90 min after the stress was applied to the cells, triplicate samples were withdrawn. As control, the same experiment was performed without stress treatment. Concomitantly, growth was monitored at 600nm (OD_{600}). The fluorescence of the active GFP was measured with a spectrofluorometer (FP-6300, Jasco) with the excitation wavelength of 395nm and the emission wavelength of 509nm. The correct excitation and emission wavelengths were determined with spectrum measurements (Figure 6). Since relative translation of leaderless mRNAs is severely reduced when compared to canonical mRNAs, the GFP fluorescence encoded by the leaderless *cI*$_{18}$-*gfp* construct was measured with a higher sensitivity, to ensure better comparability with the fluorescence expressed from the canonical can-*gfp* gene. The sample preparation is described in Material and Methods (7.2.3.). Finally, the GFP fluorescence was divided by the OD_{600} to calculate the relative expression of the *gfp* genes. In order to ensure better comparability these values were normalised to the value obtained at time point 0 which was set to 1, indicated by relative ratios in the Figures. This experimental setup was employed to study diverse stress conditions.

6.1.1. Kasugamycin stimulates the translation of the leaderless *cI*$_{18}$-*gfp* fusion gene *in vivo*

First, the applicability of the GFP-based reporter system to indicate stress conditions stimulating leaderless mRNA translation was tested. Therefore the antibiotic Ksg was employed *in vivo* to reproduce the results obtained by pulse labelling experiments upon Ksg treatment, using the leaderless *cI-lacZ* fusion gene (Kaberdina et al., 2009). Briefly, *E. coli* strain MG1655 harbouring the leaderless *cI*$_{18}$-*gfp* fusion gene or the canonical *gfp* gene was grown in LB-media in the presence of 100 µg/ml ampicillin for plasmid maintenance. Each strain was grown in duplicate. At OD_{600} of 0.25, the antibiotic Ksg (final concentration 1 mg/ml) was added to one flask, whereas the other was incubated without antibiotic. Growth was monitored and triplicate aliquots were withdrawn before (time point 0) and at time points 15 min, 30 min, 60 min and 90 min after treatment with Ksg. The cells were pelleted by centrifugation as specified in Material and Methods (7.2.3.). Upon removal of LB the cells were resuspended in TE-buffer and the fluorescence was determined employing a spectrofluorometer. As shown in Figure 7, growth was severely inhibited by addition of the antibiotic.

Determination of the GFP fluorescence during growth in the absence of Ksg revealed a constant level of expression of both, the leaderless as well as the canonical constructs (Figure 8A). However, upon treatment with the antibiotic the GFP fluorescence was severely reduced using the canonical transcript. In contrast relative translation of the leaderless *cI*$_{18}$-*gfp* fusion gene remained constant for 60 min and even increased after prolonged treatment in the presence of Ksg (Figure 8B, time point 90 min). Taken together, these results revealed a stimulating effect of Ksg

treatment on translation of the leaderless *cI*$_{18}$-*gfp* fusion gene. As these data clearly mirror the observations obtained before by pulse labelling experiments with the *cI-lacZ* fusion gene (Kaberdina et al., 2009), the system could be employed to determine the effect of a variety of different growth conditions to determine conditions which might result in formation of protein depleted ribosomes.

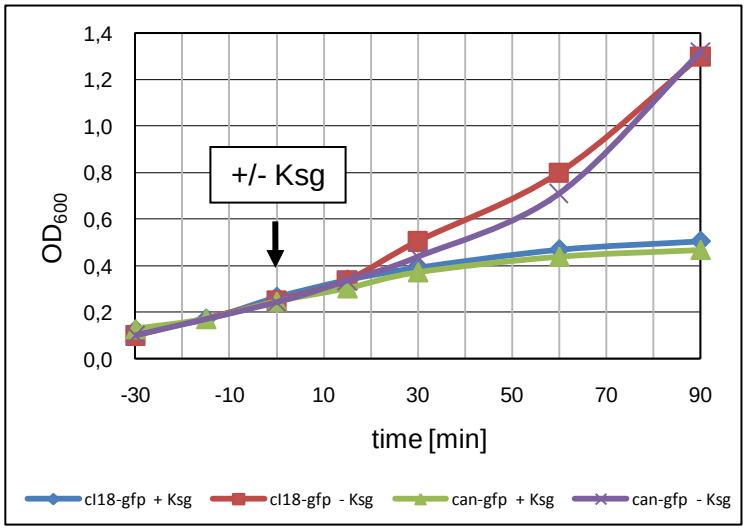

Figure 7 - Growth of the wildtype strain *E. coli* MG1655, harbouring either *cI*$_{18}$-*gfp* or can-*gfp* in the absence and presence of the antibiotic Ksg (indicated by an arrow). Growth was reduced upon addition of Ksg.

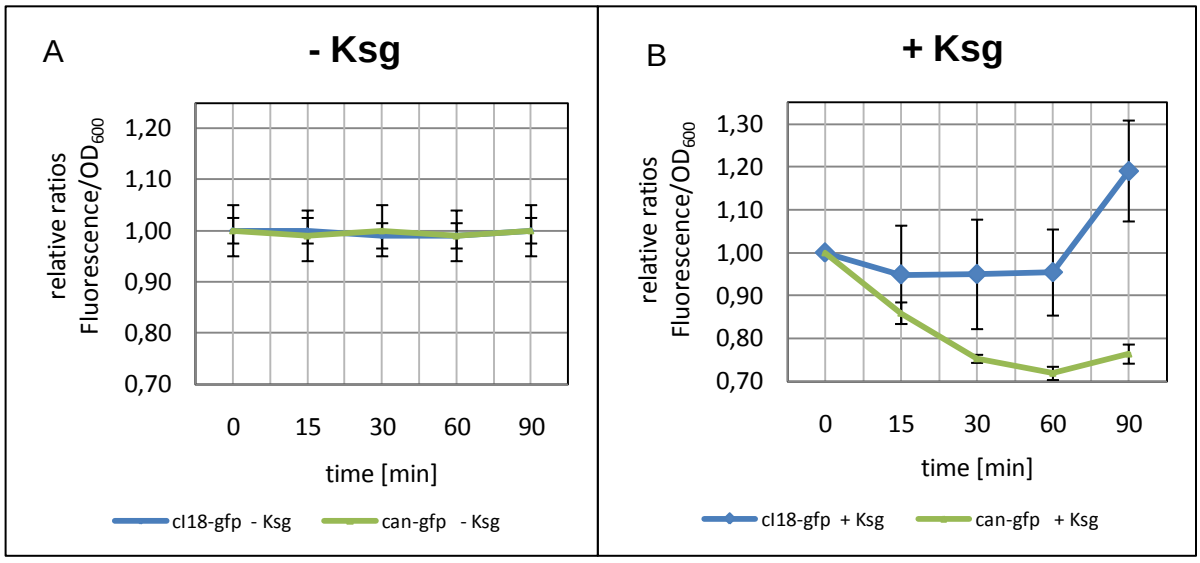

Figure 8 - Relative translation of the leaderless *cI*$_{18}$-*gfp* fusion gene and the canonical can-*gfp* gene in the absence (A) and presence (B) of Ksg

The relative translation was calculated by dividing the measured GFP fluorescence with the OD_{600} in the absence (A) and presence (B) of Ksg, respectively. These values were normalised to the value obtained at time point 0 which was set to 1. The fluorescence of the active GFP was measured with a spectrofluorometer (FP-6300, Jasco) with the excitation wavelength of 395nm and the emission wavelength of 509nm.

6.1.2. Cold stress stimulates translation of the leaderless *cI*$_{18}$-*gfp* fusion gene *in vivo*

Several lines of evidence indicate enhanced translation of leaderless mRNAs at low temperatures, e.g. 20°C (Grill et al., 2002). Since IF2 stimulates translation of leaderless transcripts (Grill et al., 2001) and moreover, IF2 levels are increased under cold shock conditions, these results were attributed to IF2 *in vivo*. In contrast, *in vitro* translation experiments performed at different temperatures in the presence of equimolar concentrations of IFs employing the leaderless *cI* mRNA and the canonical *ompA* mRNA, support the notion, that the relative translation rate of leaderless mRNAs was stimulated in the cold irrespective of the amount of IF2 (Grill et al., 2002). Taken together, these observations provide the basis for another growth condition which was used to test for the applicability of the GFP-based reporter system.

Therefore, *E. coli* strain MG1655 harbouring the leaderless *cI*$_{18}$-*gfp* fusion gene or the canonical *gfp* gene was grown in LB-media at 37°C in the presence of 100 µg/ml ampicillin. Each strain was grown in duplicate. At OD_{600} of 0.25, one flask was kept at 37°C and the other was incubated at 20°C to apply “cold stress”. Before and 15 min, 30 min, 60 min and 90 min upon stress treatment, triplicate aliquots were withdrawn and the GFP fluorescence was determined as specified in Material and Methods (7.2.3.).

As shown in Figure 9, growth was severely inhibited upon temperature shift to 20°C, indicating a “cold shock”. When *E. coli* was grown at 37°C, translation of both, the leaderless and the canonical reporter genes remained constant (Figure 10A). In contrast, translation of the canonical GFP stops upon cold shock and the GFP activity

decreased upon prolonged cold treatment (Figure 10B, time point 90 min). In accordance with the data obtained by Grill et al. (2002), the translation of the leaderless construct increased continuously, although growth ceased completely (Figure 10B).

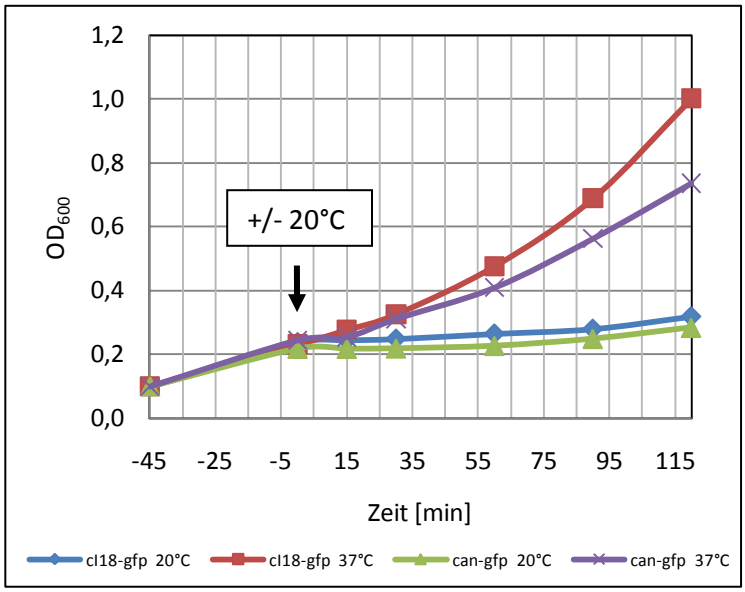

Figure 9 - Growth of the wildtype strain *E. coli* MG1655, harbouring either *cI*$_{18}$-*gfp* or can-*gfp* at 37°C and upon shift to 20°C (indicated by an arrow). Growth was inhibited upon cold stress.

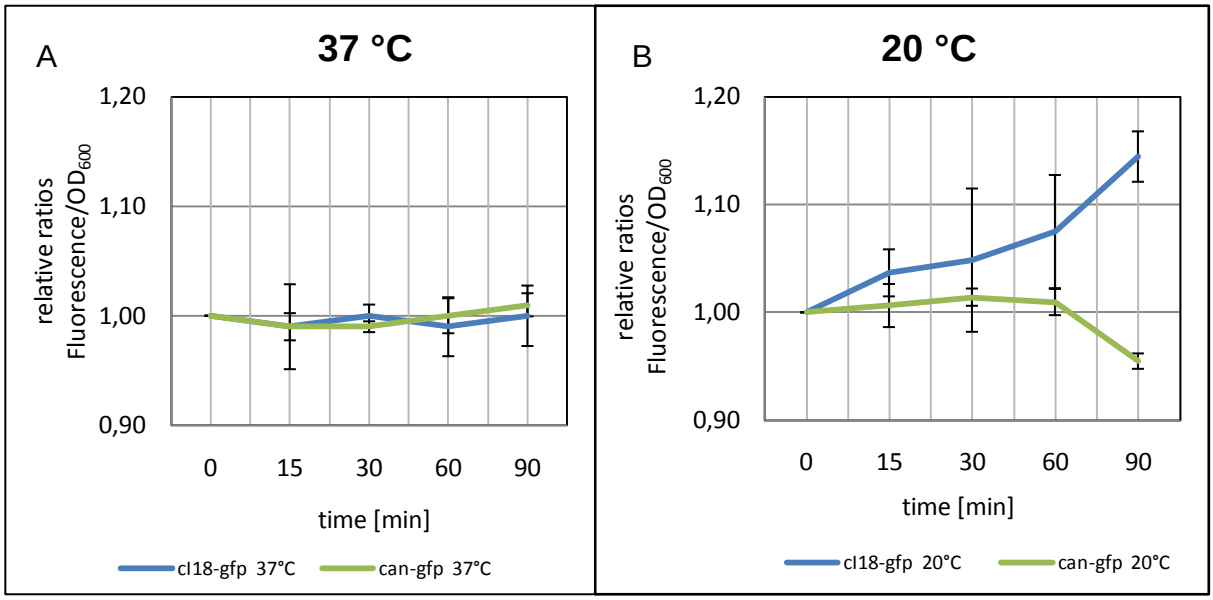

Figure 10 - Relative translation of the leaderless *cI*$_{18}$-*gfp* fusion gene and the canonical can-*gfp* gene at 37°C (A) and upon shift to 20°C (B)

The relative translation was calculated by dividing the measured GFP fluorescence with the OD_{600} at 37°C (A) and upon shift to 20°C (B), respectively. These values were normalised to the value obtained at time point 0 which was set to 1. The fluorescence of the active GFP was measured with a spectrofluorometer (FP-6300, Jasco) with the excitation wavelength of 395nm and the emission wavelength of 509nm.

6.1.3. Translation of the leaderless *cI*$_{18}$-*gfp* fusion gene remains unchanged by heat stress *in vivo*

Previous studies pointed to a reduced translation of leaderless mRNAs in comparison to canonical mRNAs at high temperatures like 42°C (Grill et al., 2002). These results were attributed to structural features within the mRNA and reduced stability of the translation initiation complex formed at a 5'-terminal start codon at elevated temperature (Grill et al., 2002). Due to this study, another growth condition was applied to the GFP-based reporter system in order to determine the relative translation of leaderless mRNAs under heat stress. *E. coli* strain MG1655 harbouring either the leaderless *cI*$_{18}$-*gfp* fusion gene or the canonical *gfp* gene was grown in duplicates at 28°C in LB-media in the presence of 100 µg/ml ampicillin. At OD_{600} of 0.25, one flask was maintained at 28°C and the other was incubated at 42°C to induce "heat shock". Triplicate samples were withdrawn before (time point 0) and 15 min, 30 min, 60 min and 90 min upon stress treatment and the GFP fluorescence was determined as described in Material and Methods (7.2.3.).

Growth was stimulated upon shift from 28°C to 42°C *in vivo* mimicking "heat stress". As shown by the growth curve in Figure 11, the shift to 42°C stimulated growth, already indicating that the temperature shift to 42°C does not represent a strong stress situation. As indicated in Figure 12A, at 28°C expression of the leaderless *cI*$_{18}$-*gfp* fusion as well as the canonical *gfp* gene resulted in constant GFP amounts (Figure 12A). Interestingly, at 28°C translation of leaderless mRNA is not stimulated like it was shown for 20°C (Grill et al., 2002; this work 6.1.2.). Moreover, the obtained GFP fluorescence upon shift to 42°C, showed constant expression for both, the leaderless as well as the canonical construct, and hence mirrors the results

shown before at 37°C (6.1.1. and Figure 8A; 6.1.2. and Figure 10A). Taken together, these results indicate that a temperature shift to 42°C might not represent a strong stress condition and moreover, the data do not point towards the formation of protein depleted ribosomes.

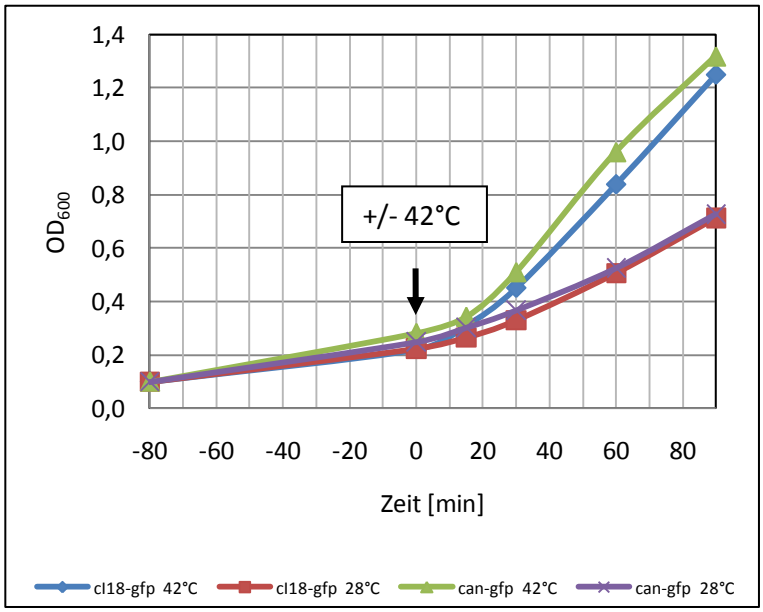

Figure 11 - Growth of the wildtype strain *E. coli* MG1655, harbouring either *cI*$_{18}$-*gfp* or can-*gfp* at 28°C and upon shift to 42°C (indicated by an arrow). Growth was stimulated upon heat stress.

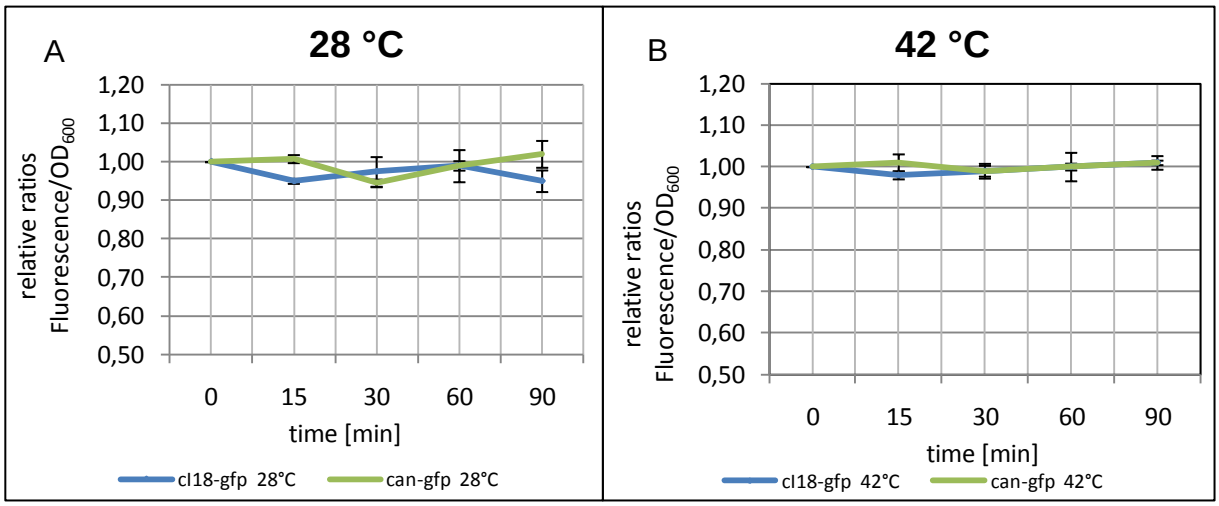

Figure 12 - Relative translation of the leaderless *cI*$_{18}$-*gfp* fusion gene and the canonical can-*gfp* gene at 28°C (A) and upon shift to 42°C (B)

The relative translation was calculated by dividing the measured GFP fluorescence with the OD_{600} at 28°C (A) and upon shift to 42°C (B), respectively. These values were normalised to the value obtained at time point 0 which was set to 1. The fluorescence of the active GFP was measured with a spectrofluorometer (FP-6300, Jasco) with the excitation wavelength of 395nm and the emission wavelength of 509nm.

6.1.4. Applicability of the used *gfp*-based reporter system *in vivo*

In this study, I determined translation of leaderless mRNAs under different stresses employing a *gfp*-based reporter system. As shown before, upon addition of Ksg as well as upon cold stress at 20°C, translation of leaderless mRNA was stimulated (6.1.1. and Figure 8B; 6.1.2. and Figure 10B). Interestingly, at 28°C the translation of leaderless mRNA is not increased in comparison to 20°C (6.1.3. and Figure 12A). Moreover, “heat stress” at 42°C resulted in constant leaderless mRNA translation (6.1.3. and Figure 12B), when compared to canonical mRNA translation.

The experiments were performed with the wildtype *E. coli* strain MG1655 (Blattner et al., 1997), which harboured either the leaderless construct *cI*$_{18}$-*gfp* or the canonical construct can-*gfp*. Both genes were placed under control of a constitutive *lac* promoter (Backman et al., 1976), which results in a high amount of active GFP before stress induction (time point 0). Furthermore, mature and active GFP is highly stable (Tombolini et al., 1997). Taken together, these features lead to a high background activity, which negatively affects the fluorescence measurements. Moreover, to study translational activity the fluorescence values have to be normalised to the mRNA levels present at the respective time point.

In order to facilitate the screen, I therefore constructed a bicistronic mRNA, consisting of a 5’-terminal leaderless *cI*$_{18}$-*gfp* gene followed by a structured intergenic region and a canonical *yfp* gene. This construct would allow the concomitant measurement of both, a leaderless and a canonical mRNA translation present on one mRNA. Furthermore, this construct was placed under control of an inducible promoter to reduce the amount of background fluorescence protein before applying the various stress conditions.

6.2. Comparing translation of a leaderless and canonical mRNA *in vivo* employing a bicistronic *gfp*/*yfp*-based reporter system

In order to allow concomitant measurement of translation of a leaderless and a canonical mRNA, I constructed a bicistronic transcript harbouring a 5'-terminal *cI*$_{18}$-*gfp* fusion mRNA starting directly with the AUG start codon, followed by a canonical *yfp* gene as specified in Material and Methods 7.1.2.3. The presence of a structured intergenic region provides a means to prevent translational coupling, since the 70S ribosomes translating the leaderless *cI*$_{18}$-*gfp* mRNA have to terminate and dissociate after translation of the leaderless *gfp* mRNA. Since placing a leaderless mRNA under control of a *lac* promoter, as used for the monocistronic *cI*$_{18}$-*gfp* reporter fusion gene, results in the loss of the main operator sequence and thus leads to constitutive expression, the bicistronic mRNA was placed under control of a *T7* promoter, which allows controlled transcription of a leaderless construct.

To analyse translation, *E. coli* strain Rosetta (DE3) pLysE (Novagen, 2004) was used, which harbours the gene *1* of phage T7, encoding the T7 RNA polymerase under control of an inducible *lac* promoter as well as the *lacI*^{q} gene on the λ prophage DE3. The pLysE plasmid ensures tight control of the expression of the T7 RNA polymerase by expression of the T7 lysozyme, which results in inhibition of basal levels of T7 RNA polymerase through direct protein-protein interaction (Moffatt and Studier, 1987). This strain harbouring the plasmid derivative pK-*cI*$_{18}$*gfp*-can-*yfp* encoding the bicistronic reporter mRNA was grown in LB media (Miller, 1972) in the presence of 50 µg/ml kanamycin and 10 µg/ml chloramphenicol. IPTG (final concentration 0.5 mM) was added to the culture, to induce the T7 RNA polymerase.

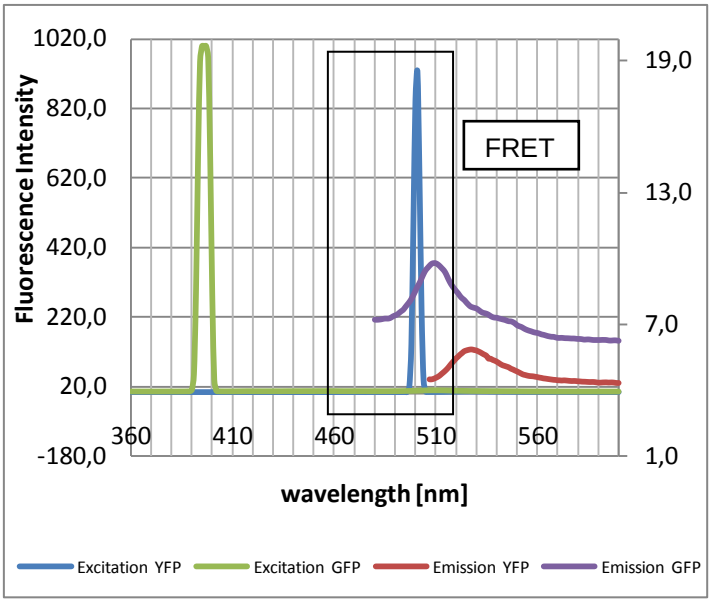

Figure 13 - Emission spectrum of GFP and YFP

The correct excitation and emission wavelengths were determined by spectrum measurements with a spectrofluorometer (FP-6300, Jasco). The excitation wavelength of GFP is 395nm and the emission wavelength is 509nm. The excitation wavelength of YFP is 500nm and the emission wavelength is 530nm. Due to the excitation wavelength of YFP at 500nm and the emission wavelength of GFP at 509nm, fluorescence resonance energy transfer (FRET) takes place (indicated by the box).

To optimise the induction of the bicistronic reporter mRNA before stress treatment, different time points for induction of the T7 RNA polymerase were tested. Briefly, duplicate samples were withdrawn before the addition of IPTG (time point -15/-25/-35 min), before the stress treatment (time point 0) as well as 15 min, 30 min, 60 min and 90 min after stress treatment. As control, the same experiment was performed without stress. Growth was measured at OD_{600}. The fluorescence of the active GFP and YFP were measured with a spectrofluorometer (FP-6300, Jasco). The correct excitation and emission wavelengths were determined with spectrum measurements. As shown in Figure 13, the excitation wavelength of GFP is 395nm and the emission wavelength is 509nm, and the excitation wavelength of YFP is 500nm and the emission wavelength is 530nm. Since the emission wavelength of GFP at 509nm overlaps with the excitation wavelength of YFP at 500nm, we observed fluorescence resonance energy transfer (FRET). By definition, FRET is an energy transfer, which is non-radioactive, from an excited donor fluorophore, in this case GFP, to an acceptor, here YFP. In order to prevent measurement errors due to FRET, for further experiments the samples were loaded on native gels in case of Ksg

treatment (6.2.1.) and cold stress induction (6.2.2.) and the fluorescence of active GFP and YFP was scanned with Typhoon^{TM} Trio Variable Mode Imager (GE Healthcare).

6.2.1. The presence of kasugamycin favours translation of leaderless mRNA *in vivo*

To validate the *gfp*/*yfp*-based reporter system, the cells were again stressed by the addition of Ksg as specified before (6.1.1.) to check for the reproducibility of the results obtained by pulse labeling using the leaderless *cI-lacZ* fusion gene (Kaberdina et al., 2009).

Briefly, the *E. coli* strain Rosetta (DE3) pLysE (Novagen, 2004), which harboured the bicistronic construct (described in Material and Methods 7.1.2.3.) on plasmid pK-*cI*$_{18}$*gfp*-can-*yfp*, was grown in LB media (Miller, 1972) in the presence of 50 µg/ml kanamycin and 10 µg/ml chloramphenicol. The strain was grown in duplicate. IPTG (final concentration 0.5 mM) was added to the culture, in order to induce the T7 RNA polymerase at OD_{600} of 0.2. 15 min thereafter the antibiotic Ksg (final concentration 1 mg/ml) was added into one flask and the other flask was incubated in the absence of Ksg. Growth was monitored and duplicate samples were withdrawn before IPTG addition (time point -15 min), before stress induction (time point 0), as well as 15 min, 30 min, 60 min and 90 min after stress induction. As indicated in Figure 14, growth was inhibited growth upon addition of the antibiotic Ksg.

In order to measure GFP and YFP separately without the occurrence of FRET, the samples were prepared as specified in Material and Methods (7.2.4.) and loaded

on native gels (Figure 15). The fluorescence of active GFP and YFP was scanned with a Typhoon™ Trio Variable Mode Imager (GE Healthcare). Furthermore, two controls which were obtained *in vivo* were loaded next to the samples on each native gel. First, a positive-control for the leaderless GFP was used, displayed by the *cI*$_{18}$-*gfp* fusion gene under control of a *T7* promoter on the plasmid *cI*$_{18}$-*gfp*(P*T7-XbaI*)-pUC18 (described in Material and Methods 7.1.2.3.; Figure 15 lane 7). Further, a positive-control for the canonical YFP was loaded, encoded by a *yfp* gene harbouring the 5'-UTR of *ompA*, placed under control of a *T7* promoter on the plasmid *ompA-yfp*-pETDuet-1 (described in Material and Methods 7.1.2.3.; Figure 15 lane 8). These positive-controls, namely the leaderless *cI*$_{18}$-*gfp* and the canonical *yfp* genes, were expressed separately in the *E. coli* strain Rosetta (DE3) pLysE (Novagen, 2004) and IPTG (final concentration of 0.5 mM) was added for induction of the T7 RNA polymerase.

The results are shown in Figure 15. The absence of the fluorescence GFP and YFP proteins at time point -15 min (Figure 15, lane 1) and time point 0 (Figure 15, lane 2) indicate that translation of the leaderless *cI*$_{18}$-*gfp* and can *yfp* mRNA was determined solely in the presence of the antibiotic and no background expression causes a measurement error.

Determination of the GFP/YFP ratio revealed an increase of the GFP fluorescence in the presence of the antibiotic (Figure 16), indicating a stimulation of leaderless mRNA translation. In striking contrast, the GFP/YFP ratio decreased in the absence of Ksg (Figure 16). These data verify previous results (Kaberdina et al., 2009; 6.1.1. and Figure 8B) that Ksg treatment of *E. coli* results in selective translation of leaderless mRNA *in vivo*. Therefore the system could be employed to

determine the effect of different growth conditions to detect conditions which stimulate leaderless mRNA translation.

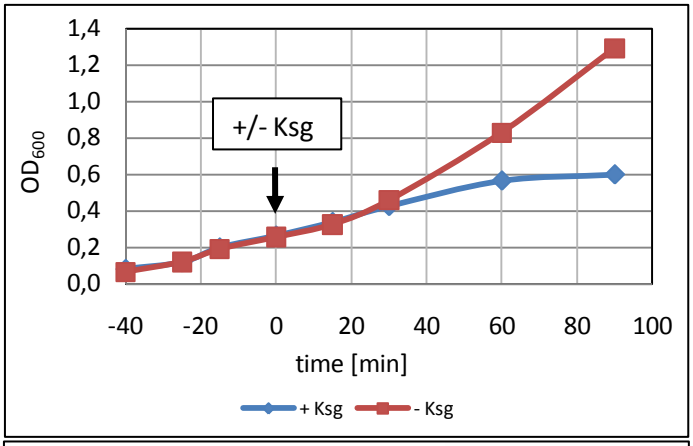

Figure 14 - Growth of *E. coli* strain Rosetta (DE3) pLysE, harbouring the bicistronic *cI*$_{18}$-*gfp*-can-*yfp* construct in the absence and upon addition of the antibiotic Ksg (indicated by an arrow). Growth was reduced upon addition of Ksg.

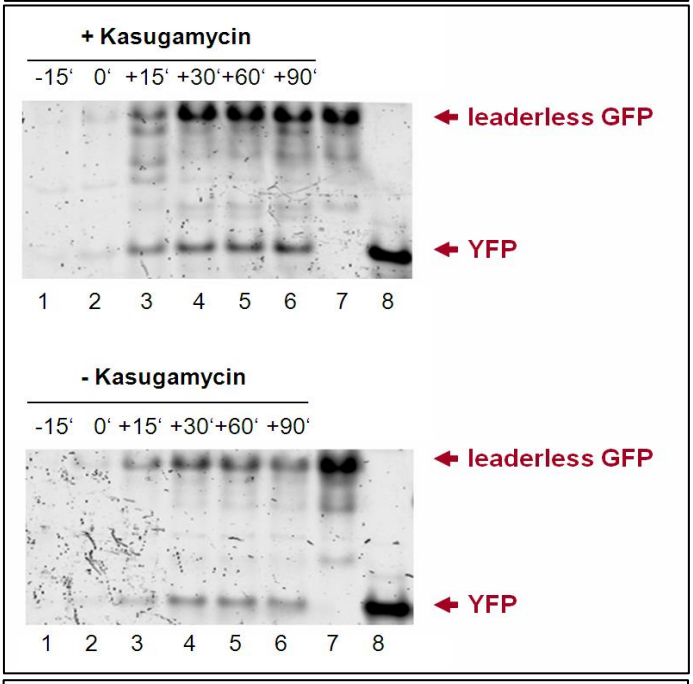

Figure 15 - Expression of *cI*$_{18}$-*gfp* and *yfp* in the absence and presence of the antibiotic Ksg

The samples were withdrawn at indicated time points and separated on native PAA gels. The fluorescence of active GFP and YFP was scanned with Typhoon™ Trio Variable Mode Imager (GE Healthcare). The sample preparation is described in Material and Methods (7.2.4.). Furthermore, CI$_{18}$-GFP and YFP proteins were loaded next to the samples on each native gel as controls (for further details see text).

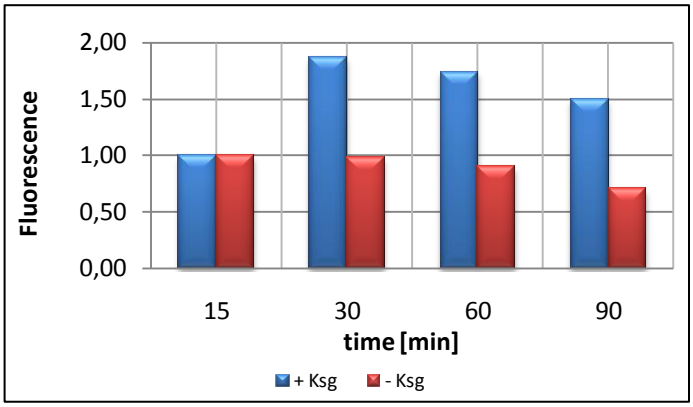

Figure 16 - The ratio of GFP/YFP fluorescence in the absence and presence of the antibiotic Ksg

Graphical representation of the relative GFP/YFP fluorescence ratios determined for each time point without or with Ksg treatment. The value obtained at time point 15 min was set to 1.

6.2.2. Cold stress stimulates the translation of leaderless mRNA *in vivo*

As mentioned before (6.1.2.), previous studies revealed enhanced leaderless mRNA translation at low temperatures of 20°C (Grill et al., 2002). These results were obtained *in vivo* due to the stimulative effect of IF2 on leaderless transcripts (Grill et al., 2001). Moreover, an increase of IF2 levels was revealed under cold shock conditions (Goldenberg et al., 1997). Therefore, cold stress was applied to test this *gfp*/*yfp*-based reporter system.

Growth was performed with *E. coli* strain Rosetta (DE3) pLysE (Novagen, 2004), which harboured the bicistronic construct (described in Material and Methods 7.1.2.3.) on the plasmid pK-*cI*$_{18}$*gfp*-can-*yfp*. This strain was grown in LB media (Miller, 1972) in the presence of 50 µg/ml kanamycin and 10 µg/ml chloramphenicol. The strain was grown in duplicate. IPTG (final concentration 0.5 mM) was added to the culture, for induction of the T7 RNA polymerase of the *E. coli* strain at OD_{600} of 0.2. 25 min afterwards one flask was kept at 37°C and the other was incubated at 20°C for "cold stress" induction. Before IPTG addition (time point -25 min), before stress induction (time point 0) and 15 min, 30 min, 60 min and 90 min after stress induction, growth was monitored and duplicate samples were withdrawn. As shown in Figure 17, growth inhibition occurs upon the induction of cold stress at 20°C.

Separate measurement of GFP and YFP without the occurrence of FRET was performed by preparing the samples as indicated in Material and Methods (7.2.4.) and loading on native gels (Figure 18). Scanning of the fluorescence of active GFP and YFP was performed with a Typhoon^{TM} Trio Variable Mode Imager (GE Healthcare). The same controls as specified in 6.2.1. were used, namely the CI_{18}-GFP and YFP protein (Figure 18 lanes 7 and 8, respectively).

As shown in Figure 18 before addition of IPTG (time point -25 min; Figure 18, lane 1) no fluorescence of GFP and YFP can be detected, which indicates that the leaderless *cI*$_{18}$-*gfp* and canonical *yfp* mRNA was translated exclusively under cold shock conditions, and hence, no background expression could cause a measurement error.

The active amount of the GFP/YFP ratio revealed stimulated translation of the leaderless mRNA 15 and 30 min upon cold stress (Figure 19, time points 15 and 30 min). In contrast, the GFP/YFP ratio declines without cold stress (Figure 19). These results verify previous data (Grill et al., 2002; 6.1.2. and Figure 10B), which revealed stimulated translation of the leaderless construct (Figure 19). However, 60 min upon cold shock, the expression of the leaderless *cI*$_{18}$-*gfp* mRNA was reduced again, which indicates adaptation to the condition and a relieve of translational stress (Figure 19, time points 60 and 90 min).

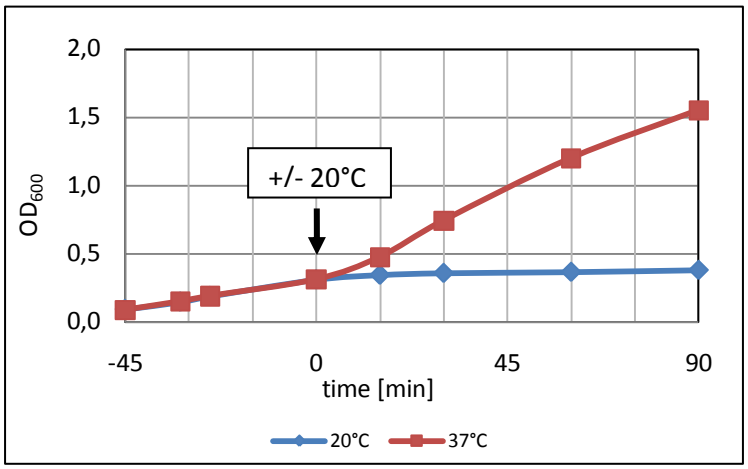

Figure 17 - Growth of *E. coli* strain Rosetta (DE3) pLysE, harbouring the bicistronic *cI*$_{18}$-*gfp*-can-*yfp* construct at 37°C and upon shift to 20°C (indicated by an arrow). Growth was inhibited upon coldstress.

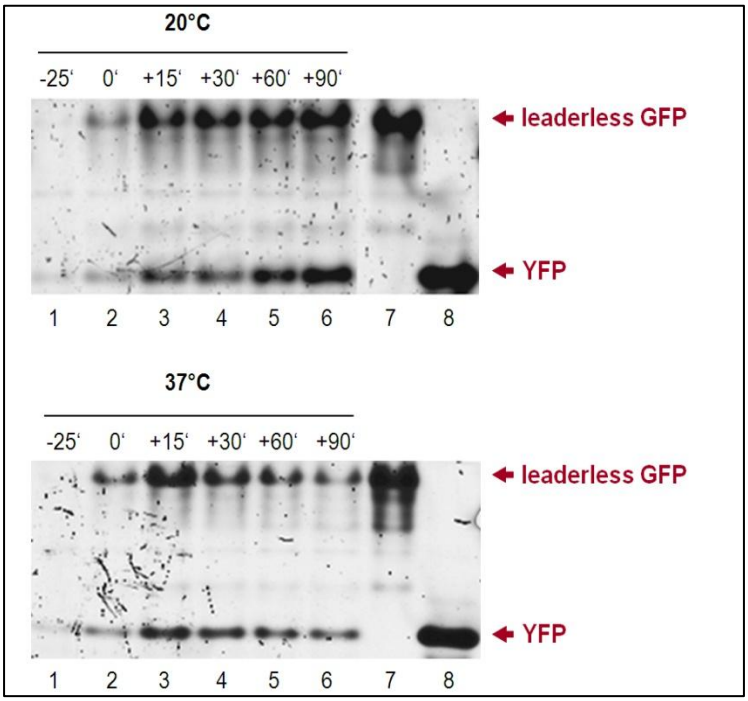

Figure 18 - Expression of *cI*$_{18}$-*gfp* and *yfp* at 37°C and upon shift to 20°C

The samples were withdrawn at indicated time points and separated on native PAA gels. The fluorescence of active GFP and YFP was scanned with Typhoon™ Trio Variable Mode Imager (GE Healthcare). The sample preparation is described in Material and Methods (7.2.4.). Furthermore, CI$_{18}$-GFP and YFP proteins were loaded next to the samples on each native gel as controls (for further details see text).

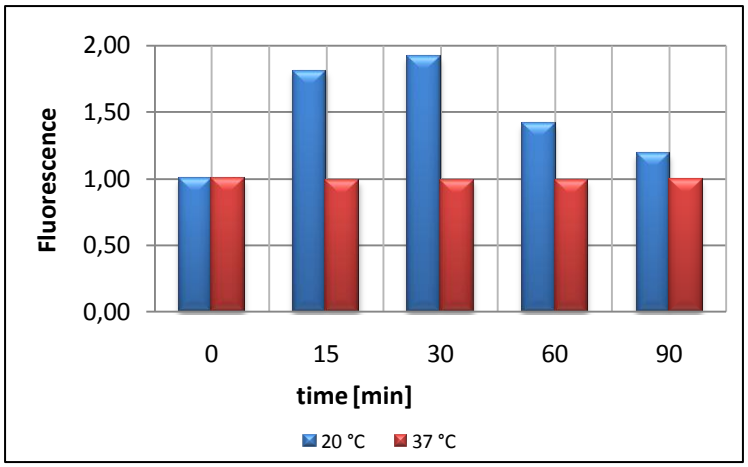

Figure 19 - The ratio of GFP/YFP fluorescence at 37°C and upon shift to 20°C

Graphical representation of the relative GFP/YFP fluorescence ratios determined for each time point without or with cold stress at 20°C. The value obtained at time point 0 was set to 1.

6.2.3. Heat stress results in constant translation of leaderless mRNA *in vivo*

As specified before (6.1.3.), at high temperatures translation of leaderless mRNAs was reduced when compared to canonical mRNAs (Grill et al., 2002). Thus, the *gfp*/*yfp*-based reporter system was tested under heat stress conditions.

The *E. coli* strain Rosetta (DE3) pLysE (Novagen, 2004), which harboured the bicistronic construct (described in Material and Methods 7.1.2.3.) on the plasmid pK-cI_{18}*gfp*-can-*yfp* was grown in duplicates at 28°C in LB media (Miller, 1972) in the presence of 50 µg/ml kanamycin and 10 µg/ml chloramphenicol. At OD_{600} of 0.25, one flask was kept at 28°C and the other was incubated at 42°C to induce “heat stress”. Triplicate samples were withdrawn before IPTG addition (time point -35 min), before stress (time point 0) as well as 30 min, 60 min and 90 min upon stress treatment and the fluorescence of the active GFP and YFP was determined as specified in Material and Methods (7.2.3.) and measured with a spectrofluorometer (FP-6300, Jasco).

As shown in Figure 20, growth increased upon shift from 28°C to 42°C. However, the ratio of GFP/YFP revealed unchanged fluorescence upon heat stress at 42°C (Figure 21), indicating consistent leaderless mRNA translation. In contrast, the obtained GFP/YFP ratio upon shift to 42°C, decreased slightly (Figure 21). In conclusion, the temperature shift to 42°C indicates no strong stress condition and hence verifies the data shown before (6.1.3. and Figure 12B), which does not indicate formation of ribosomes selective for leaderless mRNA translation.

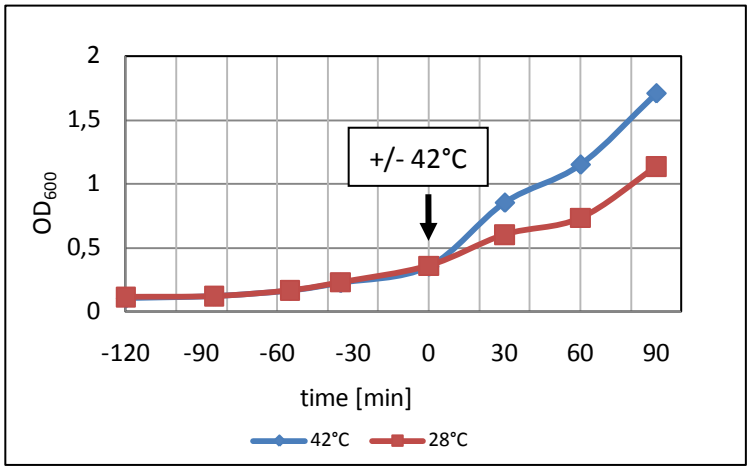

Figure 20 - Growth of *E. coli* strain Rosetta (DE3) pLysE, harbouring the bicistronic cI_{18}-*gfp*-can-*yfp* construct at 28°C and upon shift to 42°C (indicated by an arrow). Growth was stimulated upon heat stress.

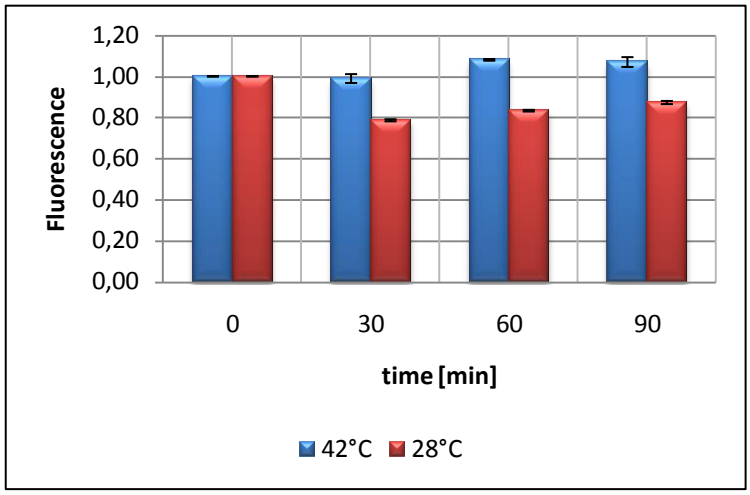

Figure 21 - The ratio of GFP/YFP fluorescence at 28°C and upon shift to 42°C

Graphical representation of the relative GFP/YFP fluorescence ratios determined for each time point without or with heat stress. The value obtained at time point 0 was set to 1.

6.2.4. Discussion

As mentioned before, I performed the experiments with a *gfp*-based reporter system using a monocistronic leaderless *cI*$_{18}$-*gfp* and canonical *gfp* construct (6.1.) as well as with a *gfp*/*yfp*-based reporter system using a bicistronic construct, harbouring a 5'-terminal leaderless *cI*$_{18}$-*gfp* gene followed by a canonical *yfp* gene (6.2.). The aim of these experiments was to simplify the screen to identify conditions which would lead to protein-deficient ribosomes, by measuring the fluorescence of the reporter proteins.

The experiments to validate the respective reporter constructs revealed that, treatment with Ksg (6.1.1. and Figure 8B; 6.2.1. and Figure 15, 16) as well as cold shock at 20°C (6.1.2. and Figure 10B; 6.2.2. and Figure 18, 19) stimulate leaderless mRNA translation. Furthermore, at 28°C the translation of leaderless mRNA was unaffected (6.1.3. and Figure 12A; 6.2.3. and Figure 21) in comparison to 20°C. Application of "heat stress" at 42°C showed consistent leaderless mRNA translation (6.1.3. and Figure 12B; 6.2.3. and Figure 21).

The experiments with the bicistronic construct, which was placed under control of an inducible *T7* promoter (6.2.) were carried out with the *E. coli* strain Rosetta (DE3) pLysE (Novagen, 2004), which allows controlled transcription of active GFP and YFP. In comparison to the preliminarily used constructs *cI*$_{18}$-*gfp* and can-*gfp*, which were under control of the constitutive *lac* promoter (6.1.), this controlled induction resulted in reduced background fluorescence.

However, since the ongoing translation of leaderless mRNA on the bicistronic construct influences the stability of the bicistronic mRNA, the translation of the consecutive canonical mRNA is affected. In addition, in the case of Ksg treatment

(6.2.1. and Figure 15) and cold stress induction (6.2.2. and Figure 18), the samples were loaded on native gels and scanned with a Typhoon™ Trio Variable Mode Imager (GE Healthcare) using a 520 nm-bandpass filter which is specific for GFP. Since YFP is a genetic mutant of GFP, this filter detects both fluorescent proteins but with higher sensitivity for GFP. Both issues, the stability of bicistronic mRNA as well as the used filter, might explain the discrepancies in signal intensity of fluorescence, when comparing treatment with and without Ksg (6.2.1. and Figure 15) as well as upon and without cold stress at 20°C (6.2.2. and Figure 18). Taken these considerations into account, this study reveals that the usage of the bicistronic *gfp*/*yfp* mRNA construct is not advantageous for the determination of the translational activity of a leaderless construct compared to a canonical mRNA under diverse stress conditions.

6.3. Molecular mechanisms resulting in stimulated translation of leaderless mRNAs under adverse conditions

Upon entering a host, pathogenic bacteria are generally subjected to a rapidly changing environment and have therefore acquired different regulatory systems for modulation of gene expression (Giuliodori et al., 2007; Marles-Wright and Lewis, 2007; Hengge, 2008). The alteration of the transcriptional program displays a common mechanism for stress reaction. The key molecules for the stimulation of alternative transcription are the guanosine tetra- and pentaphosphates (p)ppGpp, which accumulate fast upon nutritional stress by the activity of two enzymes denominated RelA and SpoT, in a mechanism termed “stringent response” (Srivatsan and Wang, 2008).

Previous data in our laboratory, revealed the formation of protein-depleted ribosomes upon Ksg treatment *in vivo*, which selectively translate leaderless mRNAs but fail to translate canonical mRNAs (Kaberdina et al., 2009). Surprisingly, upon prolonged Ksg treatment the expression of specific stress response proteins resumes. Further studies revealed that the respective mRNAs become leaderless upon Ksg treatment resulting in their selective translation by the protein depleted 61S particles. Primer extension analyses indicated that the formation of some leaderless mRNAs can be attributed to transcriptional reprogramming upon stress induction. However, the translation of leaderless mRNA is low when compared to translation of canonical mRNAs. We therefore hypothesised that concomitantly with the formation of leaderless mRNAs under stress conditions, a selective protein synthesis machinery might be formed to allow efficient expression of the respective mRNAs. Therefore one

aim of this study was to shed light on the underlying molecular mechanism how selective protein synthesis is achieved.

The observation that the formation of some leaderless mRNAs is dependent on transcriptional reprogramming based on the accumulation of (p)ppGpp, tempted us to determine, whether (p)ppGpp might as well be involved in formation of “specialised” ribosomes selective for translation of leaderless transcripts.

6.3.1. (p)ppGpp dependent stimulation of leaderless mRNA upon antibiotic treatment *in vivo*

In order to study this hypothesis, *E. coli* strain CF1678 (Xiao et al., 1991), which is a *relA spoT* deficient derivative of MG1655, was grown harbouring plasmid pKTP*lac*cI coding for the leaderless *cI-lacZ* fusion gene (Moll et al., 2001) that is transcriptionally controlled by a *lac* promoter. The expression of the leaderless *cI-lacZ* fusion gene was tested by pulse labeling experiments in the presence and absence of the antibiotic Ksg. Precisely, the cells which harboured the plasmid were grown over night in LB-media in the presence of 20 µg/ml kanamycin, 5 µg/ml chloramphenicol and 15 µg/ml tetracycline. This culture was diluted to an OD_{600} of 0.1 in M9-minimal media, in the presence of 20 µg/ml kanamycin and 5 µg/ml chloramphenicol. The strain was grown in duplicate. At OD_{600} of 0.21, the antibiotic Ksg (final concentration of 2 mg/ml) was added to one flask, whereas the other was incubated without antibiotic. Growth was monitored and samples were withdrawn for the pulse labeling experiment as specified in Material and Methods (7.2.5.) before (time point 0) and at

time points 30 min, 60 min, 90 min, 120 min and 150 min after Ksg treatment. As shown in Figure 22, growth was severely inhibited upon addition of Ksg.

The labelled proteins were separated on a 12% SDS-PAGE and upon drying the gels were exposed and scanned using a Typhoon^{TM} Trio Variable Mode Imager (GE Healthcare). Surprisingly, the results shown in Figure 23, revealed no selective expression of the leaderless *cI-lacZ* fusion gene in the presence of the antibiotic Ksg (Figure 23; lanes 2, 3, 5, 6, 7). Moreover, even in the absence of the antibiotic, expression of the leaderless mRNA was strongly reduced when compared to the expression in the wild type strain MG1655 (Figure 1A in Kaberdina et al., 2009). Taken together, these data indicate that the synthesis of (p)ppGpp upon stress treatment, might affect the ribosome, rendering it selective for leaderless mRNA translation.

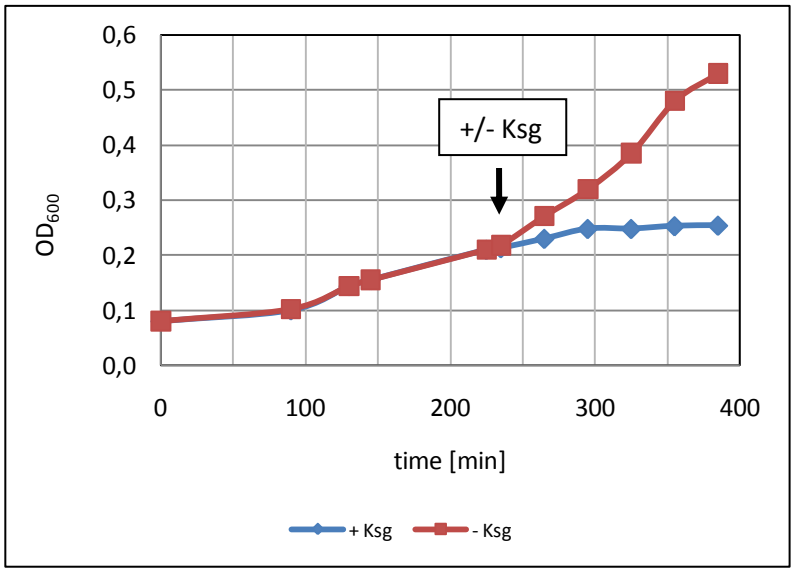

Figure 22 - Growth of *E. coli* strain CF1678, harbouring the plasmid pKTP*lacc*I, coding for the leaderless *cI-lacZ* fusion gene in the absence and presence of the antibiotic Ksg (indicated by an arrow). Growth was reduced upon addition of Ksg.

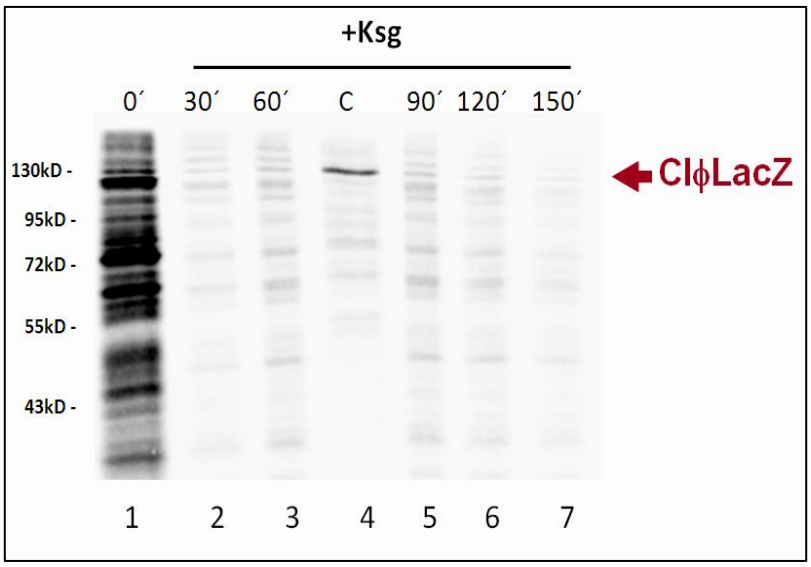

Figure 23 - Pulse labeling experiments with the *E. coli* strain CF1678, harbouring the plasmid pKTP*lac*cI, coding for the leaderless *cI-lacZ* fusion gene in the absence and presence of the antibiotic Ksg.

Lane 1, pulse labeling of strain CF1678 (pKTP*lac*cI) in the absence of Ksg. Lane 4 control, the *lacZ* gene under control of the *T7* promoter (plasmid pKT42 (Tedin et al., 1995)) was used for selective labeling of the LacZ protein *in vivo* by [^{35}S]-methionine upon induction of the T7 RNA polymerase gene in the Rosetta (DE3) strain and addition of rifampicin. Lane 2-3 and 5-7, pulse labeling of strain CF1678 (pKTP*lac*cI) in the presence of Ksg. The position of the ClϕLacZ fusion protein is indicated on the right.

6.3.2. Overexpression of *mazF* results in selective translation of leaderless mRNAs *in vivo*

Furthermore, studies performed in the laboratory indicate the formation of leaderless mRNAs by the activity of the endoribonuclease MazF under stress conditions, like antibiotic treatment. MazF is part of the toxin-antitoxin system *mazEF*, which can be triggered by elevated (p)ppGpp levels (Engelberg-Kulka et al., 2006). Therefore, the emerging question I was addressing in this diploma thesis was, whether MazF could affect the selectivity of the ribosome under stress conditions.

In order to test for this hypothesis I used *E. coli* strain MC4100 relA$^+$ (Engelberg-Kulka et al., 1998) harbouring plasmids pSA1, encoding the *mazF* gene under control of a *T5* promoter (Amitai et al., 2009), and pKTP*lac*cI, coding for the leaderless *cI-lacZ* fusion gene (Moll et al., 2001), which is transcriptionally controlled by a *lac* promoter, was used. The stimulated expression of the leaderless *cI-lacZ* fusion gene was tested by pulse labeling experiments before and upon induction of MazF expression by addition of IPTG. The cells were grown over night in LB-media in the presence of 100 µg/ml ampicillin, 15 µg/ml tetracycline and 0.5% glucose. This culture was diluted to an OD_{600} of 0.1 in M9-minimal media, in the presence of 100 µg/ml ampicillin. IPTG (final concentration 0.5 mM) was added to the culture, in order to induce the transcription of *mazF*. As control, the same experiment was performed without IPTG addition. Further, the same strain harbouring plasmids pKTP*lac*cI and plasmid pQE30 (Qiagen), the plasmid-backbone of pSA1, was grown with and without the addition of IPTG (final concentration 0.5mM), which served as a control (Figure 25A).

At the time points before *mazF* induction (time point 0) as well as 15 min, 30 min and 60 min after addition, samples were withdrawn for pulse labeling as specified in Material and Methods (7.2.5.). Concomitantly, growth was monitored at OD_{600} (Figure 24).

The autoradiography of the gels shown in Figure 25, indicate that induction of *mazF* expression results in increased and selective translation of the leaderless reporter gene (Figure 25B; lanes 4-5, indicated by an arrow). In contrast, bulk mRNA translation was severely reduced. This selective translation strongly supports the notion, that upon stress condition the TA-module might be induced by increased levels of (p)ppGpp and consequently the active riboendonuclease MazF affects the ribosome thereby making it selective for leaderless mRNA translation. This observation is supported by a recent result in the laboratory, indicating a direct effect of MazF on the 16S rRNA of the ribosome (Vesper et al., unpublished data).

Taken together, the formation of the leaderless mRNAs which are selectively translated by modified ribosomes upon activation of MazF under stress could lead to the expression of genes which are substantial for survival of bacteria under adverse conditions. Therefore, these observations point towards a novel post-transcriptional stress adaptation mechanism in bacteria to modulate gene expression.

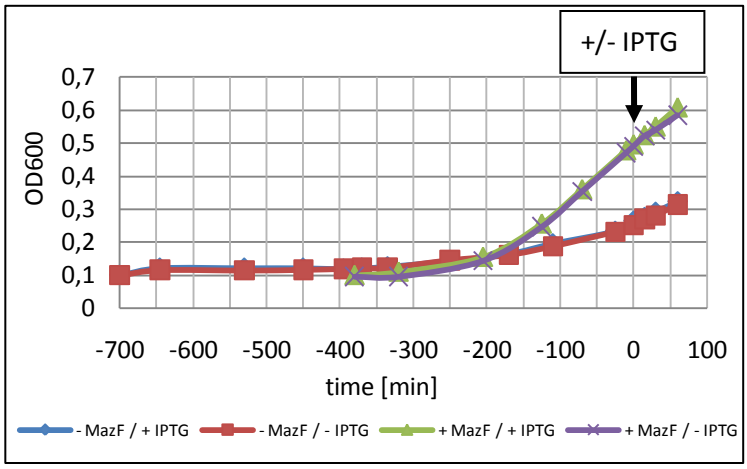

Figure 24 - Growth of *E. coli* strain MC4100 relA$^+$, harbouring the plasmid pKTP*lac*cI, coding for the leaderless cI-*lacZ* fusion gene with and without *mazF*. Experiments were performed with and without the addition of IPTG (indicated by an arrow).

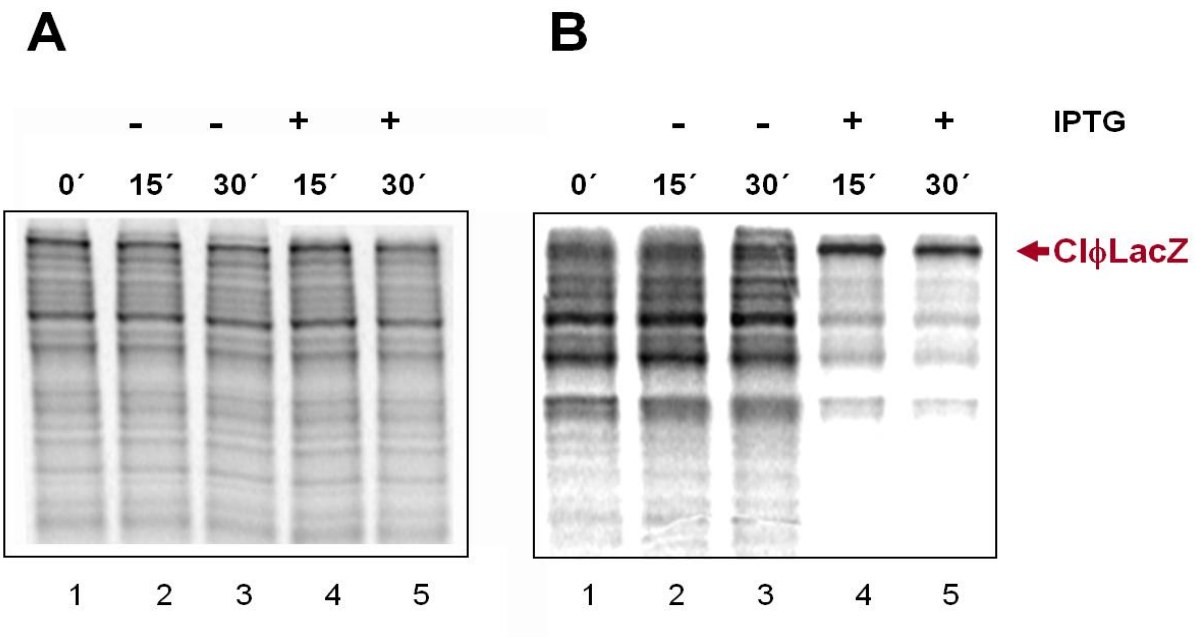

Figure 25 - Pulse labeling experiments showing translation of the *E. coli* strain MC4100 relA$^+$, harbouring the plasmids pKTP*lac*cI, coding for the leaderless *cI-lacZ* fusion gene, and pQE30 without *mazF* (A) or pSA1 coding for *mazF* (B). Experiments were performed with and without the addition of IPTG.

7. MATERIAL AND METHODS

7.1. Material

7.1.1. Bacterial strains used in this study

Strain	Genotype	Reference
BL21	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*	Novagen
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
CF1678	[MG1655] Δ*relA251::kan* Δ*spoT209::cat*	Xiao et al., 1991
MC4100 relA$^+$	araD139 Δ(*argF-lac*)205 *flb5301 pstF25 rpsL150 deoC1*	Engelberg-Kulka et al., 1998
MG1655	F$^-$ λ$^-$ ilvG- rfb-50 rph-1	Blattner et al., 1997
MG1655 F'	F$^-$ λ$^-$ ilvG- rfb-50 rph-1 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15]	this work
Rosetta (DE3) pLysE	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysE (Cam^R)	Novagen
Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$	Invitrogen
Tuner (DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1*(DE3)	Novagen
UAII	MG1655 placUV5-T7 gene 1-kan::'lacZ (F'lacI^Q lacZΔM15 proAB$^+$ tet)	Tedin et al., 1995
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Bullock et al., 1987

Table 1 - Bacterial strains

7.1.1.1. MG1655 F’

The F’ episome, carrying the *lacI*^{q} gene was transferred to *E. coli* K-12 strain MG1655 (Blattner et al., 1997), which was used as the bacterial host by conjugation. In order to distinguish donor and recipient strain, the tetracycline resistance of the F’ episome was used. The donor *E. coli* strain XL1-Blue (Bullock et al., 1987) carried the F’ episome.

The donor and recipient strains were grown separately over night at 37°C in LB media with appropriate antibiotics, namely 30 µg/ml tetracycline for the donor. Then 100µl of donor cells and 500µl of recipient cells were mixed in 10ml LB media. For the appropriate cell mixture, the OD_{600} of the overnight cultures were measured. The culture was incubated for one hour at 30rpm and 37°C. Appropriate dilutions were plated on LB selection plates with 30 µg/ml tetracycline as well as 30 µg/ml tetracycline and 40 µg/ml X-Gal. After overnight incubation at 37°C, the blue colonies were selected as MG1655 F’ are lacZ^{+} in contrast to XL1-Blue cells, which remain white on X-Gal plates.

7.1.2. Plasmids used in this study

Plasmid	Comments	Reference
pRB381*cI*	P*lac, cI-lacZ*, Amp^R	Moll et al., 2001
pKTP*laccI*	P*lac, cI-lacZ*, Tet^R	Moll et al., 2001
pRB381	*lacZ*, Amp^R	Brückner, 1992
pIVEX 2.2 GFP	P*T7*, can-*gfp*, Amp^R	Iskakova et al., 2006
pDEDH-YFP	*yfp*, 35S CaMVp	Lorkovic et al., 2004
pKS0325	P*T7*, *ompA*, Amp^R	Ried et al., 1994
pETDuet-1	P*T7*, Amp^R	Novagen
pK194	*lacZ*, Kan^R	Jobling and Holmes, 1990
pAR1219 (P266L/I810S)	P*lacUV5*, T7RNAP, Amp^R	Guillerez et al., 2005
pUC18	*lacZ*, Amp^R	Yanisch-Perron et al., 1985
pUC19	*lacZ*, Amp^R	Yanisch-Perron et al., 1985
pBAD-MazF	P*araBAD*, *mazF*, Cam^R	Amitai et al., 2004
pSA1	P*T5*, *mazF*, $lacI^q$, Amp^R derivative of pQE30	Amitai et al., 2009
pQE30	P*T5*, $lacI^q$, Amp^R	Qiagen
pRB-canGFP	P*lac*, can-*gfp*, Amp^R	Sokol, 2009
pRBcI_{18}*gfp*	P*lac*, cI_{18}-*gfp*, Amp^R	this work
pET-*yfp*	P*T7*, can-*yfp*, Amp^R	this work
pK-cI_{18}*gfp*-can-*yfp*	P*T7*, cI_{18}-*gfp*-can-*yfp*, Kan^R	this work
pUC-cI_{18}*gfp*-can-*yfp*	P*T7*, cI_{18}-*gfp*-can-*yfp*, Amp^R	this work

Table 2 - Plasmids

7.1.2.1. pRB*cI*$_{18}$*gfp*

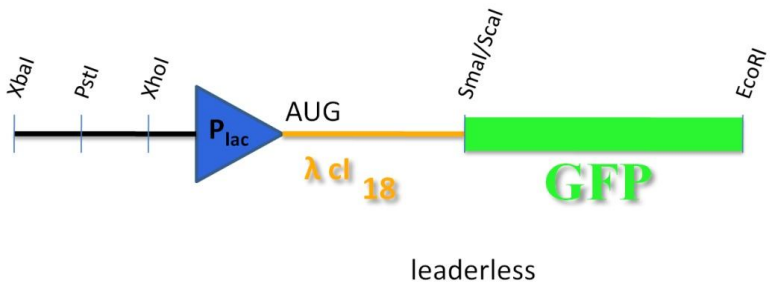

Figure 26 - *cI*$_{18}$-*gfp* construct

To construct plasmid pRB*cI*$_{18}$*gfp*, the plasmid pKTP*lac*cI (Moll et al., 2001), which encodes a leaderless *cI-lacZ* fusion gene, was used as template for a PCR employing the primers C10 (7.1.3. Table 3) and Q4 (7.1.3. Table 3). The resulting 154 nt PCR fragment encoded a P*lac*-*cI*$_{18}$ fusion gene, consisting of the *lac* promoter and the first 18 codons of the *cI* gene of phage lambda. This PCR fragment P*lac*-*cI*$_{18}$ was cleaved with the restriction enzyme *SmaI*.

A second PCR was performed with plasmid pIVEX 2.2 GFP (Iskakova et al., 2006) as template, using the primers M41 (7.1.3. Table 3) and E39 (7.1.3. Table 3). The obtained 972nt PCR fragment, harbouring the *gfp* gene starting with the third codon, was cleaved with the restriction enzyme *ScaI*.

Both PCR fragments, P*lac*-*cI*$_{18}$ and *gfp*, were ligated and the resulting fragment was amplified by PCR with the primers C10P (7.1.3. Table 3) and E39 (7.1.3. Table 3). The PCR fragment P*lac*-cI$_{18}$-*gfp* was cleaved with the restriction enzymes *EcoRI* and *XbaI* and ligated into the plasmid pRB381 (Brückner, 1992), which was cleaved with the restriction enzymes *EcoRI* and *XbaI*. The DNA manipulations of this construct were verified by sequencing.

7.1.2.2. pET-*yfp*

Primarily, the plasmid pDEDH-YFP (Lorkovic et al., 2004) was cleaved with the restriction enzymes *PstI* and *NcoI*. The DNA fragment containing the *yfp* gene was inserted in plasmid pETDuet-1, which was cleaved with the same restriction enzymes.

In order to test whether *yfp*, which was used for plants (Lorkovic et al., 2004), is also active in *E. coli*, the resulting construct of *yfp* in pETDuet-1 was transformed into the *E. coli* strain Rosetta (DE3) pLysE competent cells. These *E. coli* cells harbouring the construct were grown in LB medium until an OD_{600} of 0.2 was reached. Next, IPTG (final concentration 1 mM) was added in order to induce the T7 RNA polymerase in the strain Rosetta (DE3) pLysE. Consequently, the mRNA of *yfp* was translated by the T7 RNA polymerase. The fluorescence of active YFP was measured with a spectrofluorometer (FP-6300, Jasco) with the excitation wavelength of 500nm and the emission wavelength of 530nm.

7.1.2.3. pK-*cI*$_{18}$-*gfp*-can-*yfp* and pUC-*cI*$_{18}$-*gfp*-can-*yfp*

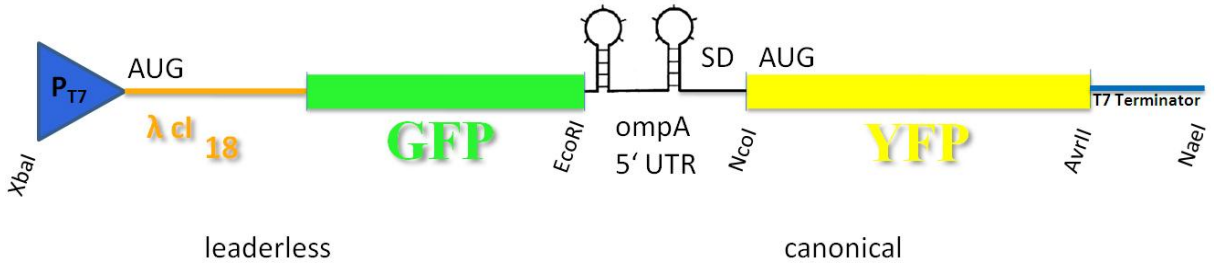

Figure 27 - Bicistronic construct *cI*$_{18}$-*gfp*-can-*yfp*

To construct plasmid pK-*cI*$_{18}$-gfp-can-*yfp*, the plasmid pKS0325 (Ried et al., 1994), which contains the outer membrane protein A (ompA), was used as template for a PCR with the forward primer IM-S2 (7.1.3. Table 3) and the reverse primer IM-T2 (7.1.3. Table 3). The obtained PCR fragment encoded the first 135nt of *ompA*, which contain the 5' UTR. It was cleaved with the restriction enzyme *NcoI* and was ligated into plasmid pDEDH-YFP (Lorkovic et al., 2004), which was likewise cleaved with the restriction enzyme *NcoI*. The ligated *ompA-yfp*-pDEDH fragment was amplified by PCR with the forward primer IM-S2 (7.1.3. Table 3) and the reverse primer IM-U2 (7.1.3. Table 3). The received PCR fragment *ompA-yfp* and the plasmid pETDuet-1 were cleaved with the restriction enzyme *AvrII* and ligated. The next PCR was performed with the forward primer IM-S2 (7.1.3. Table 3) and the reverse primer IM-V2 (7.1.3. Table 3). The received PCR fragment *ompA-yfp*-T7Terminator was cleaved with the restriction enzyme *EcoRI*.

Next the construct pRB*cI*$_{18}$*gfp* (7.1.2.1.) was used as PCR template with the forward primer R4 (7.1.3. Table 3) and the reverse primer IM-R2 (7.1.3. Table 3). Due to the forward primer R4, a *T7* promoter was placed in front of the *cI*$_{18}$-*gfp* construct. The obtained PCR fragment *cI*$_{18}$-*gfp* (P*T7-XhoI*) was used as PCR template with the

forward primer X15 (7.1.3. Table 3) and the reverse primer IM-R2 (7.1.3. Table 3). The received PCR fragment *cI*$_{18}$-*gfp* (P*T7-XbaI*) was cleaved with the restriction enzyme *EcoRI*.

Next the PCR fragment *cI*$_{18}$-*gfp* (P*T7-XbaI*) and the plasmid pUC18 were cleaved with the restriction enzyme *XbaI* as well as *EcoRI* and were ligated. The PCR fragment *ompA-yfp* and the plasmid pETDuet-1 were cleaved with the restriction enzymes *AvrII* as well as *EcoRI* and ligated together. Subsequently, the ligation products *cI*$_{18}$-*gfp* (P*T7-XbaI*)-pUC18 and *ompA-yfp*-pETDuet-1 were cut with the restriction enzymes *XbaI* as well as *EcoRI*. The cut *cI*$_{18}$-*gfp* (P*T7-XbaI*) and *ompA-yfp*-pETDuet-1 were ligated and transformed. The positive transformants were verified by colony-PCR.

The received construct *cI*$_{18}$-*gfp-ompA-yfp*-pETDuet-1 was cleaved with the restriction enzymes *XbaI* and *NaeI*, which cleaves downstream of the T7 Terminator on pETDuet-1. Additionally the plasmid pK194 was cleaved with the restriction enzymes *NheI* and *SmaI*. Finally, the cut construct *cI*$_{18}$-*gfp-ompA-yfp*-T7 Terminator and the cut plasmid pK194 were ligated. This bicistronic construct was coding for two consecutive genes, namely a leaderless *gfp* and a canonical *yfp* gene. Therefore it was denominated pK-*cI*$_{18}$-*gfp*-can-*yfp*. The DNA manipulations were verified by sequencing.

Additionally, the plasmid pUC18 was cleaved with the restriction enzymes *XbaI* and *SmaI*. The cut plasmid pUC18 and the cut construct *cI*$_{18}$-*gfp-ompA-yfp*-T7 Terminator were ligated. This bicistronic construct was coding for two consecutive genes, namely a leaderless *gfp* and a canonical *yfp* gene. Hence, it was denominated pUC-*cI*$_{18}$-*gfp*-can-*yfp*. The DNA manipulations were verified by sequencing.

7.1.3. Primer used in this study

Primer	Orientation	Sequence
R4	forward	5'-AAT AAC TCG AGT AAT ACG ACT CAC TAT AGA TGA GCA CAA AAA AGA AAC CAT TAA CAC AAG AGC-3'
Q4	reverse	5'-CAT AAA TTG CTT TCC CGG GAC GTG CGT C-3'
C10	forward	5'-AAA TCT AGA ATT CCC TTT CGT CTT CAC CTC GAG-3'
C10P	forward	5'-AAA TCT AGA CTG CAG CCC TTT CGT CTT CAC CTC GAG-3'
E39	reverse	5'-AGG AAT TCA TGG CGA CCA CAC CCG TCC TG-3'
L41	forward	5'-CCG GAA TTC TAA TAC GAC TCA GTA TAG GGA GAC CAC-3'
M41	forward	5'-ATT ATA AGT ACT AGC TGG AGC CAC CCG C-3'
X15	forward	5'-GGG CTC TAG AGT AAT ACG ACT CAC TAT ATG-3'
IM-R2	reverse	5'-AGG AAT TCG ATC TTA CCG GAT CCC-3'
IM-S2	forward	5'-TAG AAT TCG GGT GCT CGG CAT AAG CC-3'
IM-T2	reverse	5'-CTT CCA TGG TTT GCG CCT CGT TAT-3'
IM-U2	reverse	5'-TAG TCC TAG GTT ACT TGT ACA GCT CGT CC-3'
IM-V2	reverse	5'-TAC TGC AGC GCC AAT CCG GAT ATA GTT C-3'

Table 3 - Description of the used primers

7.1.4. Buffers and Solutions

7.1.4.1. Media

LB medium

1% peptone
0,5% yeast extract
1% sodium chloride (NaCl)
in H_2O_{dest}

LB plates

LB medium
1,5% agar-agar

M9-minimal medium

1x M9-salts
0,4% glycerol
5mg amino acids
0,001% thiamin (B1)
2mM magnesium sulfate ($MgSO_4$)
0,1mM calcium dichloride ($CaCl_2$)
in 500ml H_2O_{dest}

5x M9-salts

3,3% di-sodium hydrogen phosphate (Na_2HPO_4)
1,5% potassium dihydrogen phosphate (KH_2PO_4)
0,25% sodium chloride (NaCl)
0,5% ammoniumchloride (NH_4Cl)
in H_2O_{dest}

7.1.4.2. Antibiotics solutions

		Final concentration
Amp	Ampicillin	100µg/ml
Cam	Chloramphenicol	10µg/ml
Kan	Kanamycin	50µg/ml
Ksg	Kasugamycin	1mg/ml
Tet	Tetracycline	30µg/ml
IPTG	Isopropyl-β-D-1-thiogalactopyranoside	0,5mM
X-Gal	5-bromo-4-chloro-indolyl-ß-D-galactopyranoside	40µg/ml

7.1.4.3. Buffer for fluorescence measurement with a spectrofluorometer

TE buffer

10mM Tris-HCl, pH 8
1mM EDTA pH8
in H_2O_{dest}

7.1.4.4. DNA Gel

10x TBE

108g Tris-Base
55g Boric Acid
100ml 0,5M EDTA, pH8
in 1liter H_2O_{dest}

Agarose gel

0,8 to 2% agarose
in 1x TBE

Ethidium bromide bath

600µl ethidium bromide stock (10mg/ml)
in 600ml H_2O_{dest}

6x DNA loading dye

0,25% bromphenol blue
0,25% xylene cyanol FF
30% glycerol
10mM Tris, pH7.6
60mM EDTA
in H_2O_{dest}

7.1.4.5. Protein Gel

10x SDS-PAGE running buffer

3% Tris
14,4% glycine
0,1% sodium dodecyl sulphate (SDS)
in H_2O_{dest}

10x PAGE running buffer (native)

3% Tris
14,4% glycine
in H_2O_{dest}

12% SDS-PAGE

Separation Gel (12%)

3,924ml	dH_2O
2,5ml	1M Tris-HCl, pH 8.8
0,1ml	10% SDS
2,976ml	40% Acrylamide
50µl	10% APS
20µl	TEMED

Stacking Gel (4,5%)

7,1ml	dH_2O
1,25ml	1M Tris-HCl, pH 6.8
0,1ml	10% SDS
1,1ml	40% Acrylamide
40µl	10% APS
17µl	TEMED

12% PAGE (native gel)

Separation Gel (12%)

4,024ml	dH_2O
2,5ml	1M Tris-HCl, pH 8.8
2,976ml	40% Acrylamide
50µl	10% APS
20µl	TEMED

Stacking Gel (4,5%)

7,2ml	dH_2O
1,25ml	1M Tris-HCl, pH 6.8
1,1ml	40% Acrylamide
40µl	10% APS
17µl	TEMED

Coomassie staining solution

45% Methanol
10% Acetic Acid
0,25% Coomassie Blue
in H_2O_{dest}

Destain solution

45% Methanol
10% Acetic Acid
in H_2O_{dest}

2x Laemmli buffer

175mM Tris-HCl, pH 6.8
4% sodium dodecyl sulphate (SDS)
2,5% β-Mercaptoethanol
20% Glycerol
0,2% Bromphenol Blue
in H_2O_{dest}

2x native sample buffer

175mM Tris-HCl, pH 6.8
2,5% β-Mercaptoethanol
20% Glycerol
0,2% Bromphenol Blue
in H_2O_{dest}

7.1.4.6. Western Blot

10x TBS

80g NaCl
2g KCl
30g Tris-base
in 1L H_2O_{dest}, adjust to pH7.5

1x Transferbuffer

2,9g Glycin
3,7ml 10% SDS
5,8g Tris-base
200ml Methanol
in 1L H_2O_{dest}, adjust to pH8.3

AP-buffer

66µl 5% NBT (Nitroblue-Tetrazoliumchlorid)
33µl 5% BCIP (5-Brom-4-chlor-3-indolylphosphat)

7.1.5. Computer programs

Adobe Photoshop CS

Gene Runner

ImageQuant 5.1

Spectra Manager™ (Jasco)

Typhoon™ Scanner Control (GE Healthcare)

7.2. Methods

7.2.1. Competent cells

The *E. coli* cells were grown in LB media with the appropriate antibiotics overnight at 37°C under continuous shaking. The next day, the OD_{600} of the overnight culture was measured and diluted to an OD_{600} of 0.1 in 250ml LB media with the appropriate antibiotics. The cells were grown at 37°C under continuously shaking until the OD_{600} of 0.6 was reached.

Then the cells were transferred into a centrifuge tube and kept on ice for 20 to 30min. Next a centrifugation was performed for 10 min at 4.000 rpm and 4°C. The cell pellet was resuspended in 15 ml cold 0.1M $CaCl_2$ and incubated on ice for 10min. To wash the cells, centrifugation was performed for 10 min at 4.000 rpm and 4°C. The cell pellet was resuspended in 15 ml cold 0.1M $CaCl_2$ + 15% Glycerol and kept on ice for 10 min. This treatment was repeated and finally the cell pellet was resuspended in 4 ml cold 0.1M $CaCl_2$ + 15% Glycerol and aliquoted to 50 - 100µl in eppendorf tubes. These aliquots were frozen in liquid nitrogen and stored at -80°C. All these steps were carried out on ice respectively.

7.2.2. Transformation

The competent cells were thawed on ice and 1ng of the plasmid was added. The reaction was kept on ice for 30 min. Then the cells were incubated for 45 seconds at 42°C and placed on ice for 2 min. Thereafter, 1 ml of LB media was added at room temperature and the cells were incubated for 1 hour at 37°C under continuous shaking. Finally for regeneration and expression of the resistance gene, 100µl of the

cells were spread on LB plate with the appropriate antibiotics and the rest was centrifuged for 4 min at 3.000 rpm and room temperature. Only 100µl of the supernatant was left in the eppendorf tube and the cell pellet was resuspended and spread on LB plate with the appropriate antibiotics. Finally the LB plates were incubated over night at 37°C.

7.2.3. Sample preparation for fluorescence measurement with a spectrofluorometer

At each time point 3 samples of 1 ml each were withdrawn from the culture and centrifuged for 2 min at 13.200 rpm at room temperature. The supernatant was discarded and the cell pellet was frozen in liquid nitrogen and kept on ice in the dark. Then shortly before the sample measurement, 3 ml TE-buffer were added and the samples were resuspended and measured with a spectrofluorometer (FP-6300, Jasco).

Parameter of the spectrofluorometer (FP-6300, Jasco)	
Band width (Excitation)	5nm
Band width (Emission)	5nm
Response	0,5 sec

7.2.4. Sample preparation for the native gel electrophoresis and fluorescence measurement with a Typhoon Imager

At each time point 2 samples of 2 ml each were withdrawn from the culture and centrifuged for 2 min at 13.200 rpm at room temperature. The supernatant was discarded and the cell pellet was frozen in liquid nitrogen and kept on ice in the dark. According to the OD_{600} values the cell pellets were resuspended in TE-buffer containing 0,4 mg/ml lysozyme (OD_{600}=0,4 addition of 40µl). The samples were incubated on ice for 10 min. To break the cells, the samples were frozen in liquid nitrogen and thawed at room temperature three times. Upon addition of 5µl DNAseI (RNAse free), the samples were incubated on ice for 10 min and centrifuged for 2 min at 13.200 rpm at room temperature. 10µl of the supernatant were mixed with 10µl of 2x native sample buffer and 5µl were loaded on a 12% native gel.

The fluorescence of active GFP and YFP was scanned with Typhoon™ Trio Variable Mode Imager (GE Healthcare), 520 nm-bandpass filter.

7.2.5. Pulse labeling

The cells were grown in LB or M9-minimal media with the appropriate antibiotics overnight at 37°C under continuous shaking. The next day the OD_{600} of the overnight culture was measured and diluted to an OD_{600} of 0.1 in M9- minimal media and in the presence of the appropriate antibiotics. The cells were grown at 37°C under continuous shaking and the OD_{600} was monitored.

At each time point a sample of 200µl was withdrawn and 2µl of a 1:10 diluted [^{35}S]methionine were added. Upon incubation for exactly 5 min at 37°C under continuous shaking at 500 rpm, 100µl cold methionine [2mM] were added and the

sample was incubated for 1 min at 37°C under continuous shaking at 500 rpm. To precipitate the proteins, 500µl of TCA [20%] were added, mixed and incubated on ice.

Next the samples were pelleted for 60 min at 16.400 rpm at 4°C. According to the OD_{600} 1xLaemmli buffer was added to the cell pellet (OD_{600}=0,4 addition of 40µl). Then the pH was adjusted with Tris-base. After 10 min at 95°C the samples were centrifuged for 5 min at 13.200 rpm at room temperature. Finally, 5µl were loaded on a 12% SDS-PAGE and 25mA per gel were applied. The gel was dried for 30min and exposed in a phosphoimager cassette overnight. The image was scanned with Typhoon™ Trio Variable Mode Imager (GE Healthcare) in the storage phosphor mode.

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CURRICULUM VITAE

PERSONAL INFORMATION

- Name — Sanda Pasc
- Nationality — Austria
- Date of birth — February 27, 1984
- Place of birth — Oradea (Romania)

EDUCATION AND QUALIFICATIONS

- since 10/2005 — Diploma study of **Molecular Biology** at the University of Vienna, Austria
  Specialisation in Microbiology, Genetics and Neuroscience for Master's Degree

- 09/1999 - 06/2004 — 5-year secondary College of Chemistry
  Specialisation in **Biochemistry, Biotechnology and Genetic Engineering**
  Rosensteingasse 79, 1170 Vienna, Austria
  Graduation with distinction

PRACTICAL EXPERIENCE

- 01/2009 - 03/2010 — Diploma thesis at the **Max F. Perutz Laboratories**, Vienna, Austria
  Department — Microbiology and Immunobiology, Dr. Isabella Moll
  Title — Selective translation of leaderless mRNAs under different stress conditions
- 10/2008 - 06/2010 — **Tutor** at the University of Vienna, Austria
  Courses — Laboratory courses in Microbiology and Molecular Biology
- 11/2008 — Traineeship at the **Max F. Perutz Laboratories**, Vienna, Austria
  Department — Microbiology and Immunobiology, Dr. Isabella Moll
- 03/2006 - 06/2008 — Laboratory assistant at **ViennaLab Labordiagnostika GmbH**, Vienna
  Department — Research and Development
- 02/2008 — Traineeship at the **Max F. Perutz Laboratories**, Vienna
  Department — Medical Biochemistry, Univ.-Prof. Dr. Andrea Barta
- 07/2007 - 08/2007 — Traineeship at **Baxter AG**, Orth/Donau, Austria
  Department — Recombinant Cell lines, Dr. Christian Konetschny
- 03/2005 - 09/2005 — Chemical laboratory assistant at **Baxter AG**, Orth/Donau, Austria
  Department — CE Microbiology & Sterility
- 08/2004 - 03/2005 — Chemical laboratory assistant at the **Austrian Research Centers Seibersdorf GmbH**, Seibersdorf, Austria
  Department — Toxicology

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