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Titel der Diplomarbeit

"Selective and accurate translation by protein-depleted ribosomes formed in *E. coli* in the presence of kasugamycin *in vivo*"

Verfasserin

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1. Abstract

The aminoglycoside antibiotic kasugamycin inhibits translational initiation on canonical mRNAs, as it binds to the mRNA path in the ribosomal P-site and thus blocks the positioning of the mRNA and the initiator tRNA during translation initiation complex formation. In contrast, the antibiotic does not inhibit translation initiation on leaderless mRNAs, which contain a 5´-terminal start codon and therefore are devoid of ribosome recruitment signals present in the 5´-UTR. Recently, it has been shown that treatment of *E. coli* with kasugamycin results in the formation of ribosomes, which are deficient for several proteins from the 30S subunit. These particles -termed 61S particles, according to their sedimentation rate- lack the small ribosomal subunit proteins S1 and S2, which are essential for the translational of canonical mRNAs, and protein S12. As S12 has been shown to negatively influence the translational fidelity, and kasugamycin, unlike other aminoglycosides, does not induce frame shifting, misreading or read through, these data suggested that 61S particles might exhibit a higher translational fidelity. Therefore, the first aim of the study was to test for this assumption. Employing a leaderless GFP-construct, *in vivo* studies revealed an increased translational accuracy of the 61S particles, which might be attributed to the lack of the ribosomal protein S12.

The addition of kasugamycin blocks the translation of bulk mRNA, as indicated by pulse labeling experiments. However, prolonged kasugamycin treatment results in restored translation of specific proteins. Therefore, the second aim of the study was the identification of the respective proteins by 2D gel analysis and subsequent mass spectrometry. Primer extension analysis of the corresponding mRNAs revealed that

they were rendered leaderless upon kasugamycin treatment, either by alternative transcription or by a cleavage event closely upstream of the AUG start codon. Furthermore, the data suggested the possible implication of a known RNA interferase, MazF, which represents the toxin of a toxin-anti toxin module in *E. coli*. Overexpression of *mazF* resulted in the same cleavage in the leader region of the respective mRNAs, however, not in the coding region of these mRNAs.

Taken together, these results may indicate a survival strategy for bacteria under conditions where translation is compromised, based on the formation of leaderless mRNAs, which are selectively translated by aberrant ribosomes deficient for some essential proteins. As the proteins synthesized in the presence of the antibiotic are primarily stress response proteins, the selective translation of this conditionally leaderless mRNA regulon might contribute to the viability of the cells under adverse conditions.

2. Zusammenfassung

Kasugamycin ist ein Antibiotikum aus der Klasse der Aminoglycoside. Es inhibiert die Ausbildung des Translationsinitiationskomplexes an kanonischen mRNAs, indem es die Bindung der mRNA in der ribosomalen P-Stelle blockiert. Im Gegensatz dazu inhibiert es aber nicht die Initiation der Translation von sogenannten „leaderless mRNAs“, die direkt mit einem 5´-terminalem Startcodon beginnen und somit keine zusätzlichen Startsignale für das Ribosom aufweisen. Vor kurzem wurde gezeigt, dass in *E. coli* in Anwesenheit von Kasugamycin Ribosomen gebildet werden, denen einige Proteine der 30S Untereinheit fehlen. Zu den fehlenden Proteinen gehören unter anderem die essentiellen Proteine S1 und S2, und das Protein S12. Da bekannt ist, dass Kasugamycin –im Gegensatz zu anderen Aminoglycosiden- keinen Einfluss auf die Fehlerrate hat und nicht zu einer Verschiebung des Leserahmens während der Translation führt, könnte das Fehlen von S12, das die Translationsgenauigkeit des Ribosoms negativ beeinflusst, ein Hinweis darauf sein, dass die protein-defizienten Partiklen eine höhere Genauigkeit in der Translation aufweisen. Daher war das erste Ziel meiner Arbeit, diese Hypothese zu bestätigen. Mit Hilfe von „leaderless“ GFP-Konstrukten würde die Translationsgenauigkeit *in vivo* nach Zugabe von Kasugamycin bestimmt. Die Resultate deuten darauf hin, dass die Fehlerrate der ribosomalen Partikel im Vergleich zu vollständig assemblierten Ribosomen geringer ist.

Vorangegangene Experimente haben gezeigt, dass die Behandlung von *E. coli* mit Kasugamycin die Translation vollständig hemmt. Interessanterweise haben wir im Laufe der oben genannten Studien beobachtet, dass nach längerer Inkubation mit dem Antibiotikum die Synthese von einigen Proteinen wieder beginnt. Da in *E.*

coli keine leaderless mRNAs bekannt sind und die Synthese der Proteine resistent gegen Kasugamycin ist, war das zweite Ziel meiner Arbeit, dieses Phänomen näher zu untersuchen. Nach der Identifizierung der Proteine mittels 2D-Gel-Analyse und Massenspektrometrie wurden die 5´-Enden der korrespondierenden mRNAs bestimmt. Die Studien haben gezeigt, dass diese mRNAs in Gegenwart von Kasugamycin durch einen alternativen Transkriptionsstart nahe am AUG Startcodon oder durch RNA-Degradierung „leaderless“ geworden sind. Weiters deuten die Ergebnisse darauf hin, dass eine RNA Interferase, MazF, an diesem Prozess beteiligt ist.

Zusammengefaßt, weisen die Resultate meiner Arbeit auf eine noch nicht beschriebene Überlebensstrategie von Bakterien hin, die induziert wird, wenn die Translation gehemmt wird. Unter diesen Bedingungen könnte es zur Bildung von „leaderless“ mRNAs kommen, die selektiv von protein-defizienten Ribosomen translatiert werden können. Die Synthese dieser spezifischen Proteine könnte zum Überleben der Bakterien unter ungünstigen Bedingungen beitragen.

3. Preface

50 years have passed since Francis Crick first introduced his concepts which he believed to be the foundation of all biology (Morange 2008): the Central Dogma of Molecular Biology. A concept further supplemented by the Sequence Hypothesis (Crick, 1958), it describes the direction in which the information is passed in a cell, from DNA, to RNA and then to protein (Figure 1), and states: "…the specificity of a piece of nucleic acid is expressed solely by the sequence of its bases, which provides a simple code for the amino acid sequence of a particular protein".

The central dogma has since been questioned, and has suffered serious doubt after the discovery of the reverse transcriptase, prions and chaperons, but has only been supplemented over the years, and never reversed.

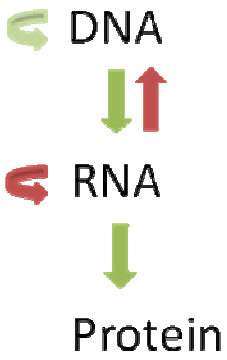


Figure 1: The central dogma of molecular biology; simplified and modernized. Green: pathway present in all organisms; Red: pathway present only under certain conditions.

Cricks theories have also been modified over time, and, among others, James Watson (Watson, 1965) tried to phrase his own version. Regrettably, the wording proved unfortunate, and his version never lived. However, in the same year, Watson came up with successful theories of his own, published in a book "Molecular Biology of the Gene", where he described a model for protein synthesis (Wilson and Nierhaus 2006). The three steps, as described by him, were:

1. Occupation of the decoding site (A site) by an aminoacyl-tRNA corresponding to the mRNA at this site. Adjacent, a second binding site (P site) is occupied with a peptidyl tRNA carrying the growing polypeptide chain.
2. Peptide bond formation. The polypeptide chain is cleaved off the second tRNA, and transferred to the tRNA still located in the A site.
3. Translocation, in which the peptidyl-tRNA is moved by the length of three nucleotides, thus entering the P site, and exposing a new anti-codons in the A site.

The model Watson proposed fitted with the theories of the time, and it remained so until a saturation experiment with ^{32}P labeled deacylated tRNAs in polysomes suggested a third site, where the deacylated tRNA could reside before parting from the ribosomes (Wettstein and Noll 1965). The site was named the “exit” site (E site). The conformation for the existence of this site came 15 years later (Rheinberger et al. 1981), and its full significance in maintaining the translational fidelity was only determined recently (Wilson and Nierhaus 2006).

4. Introduction

4.1. The Ribosome: the protein synthesis machinery

The bacterial ribosome is a 2,5 MDa ribonucleoprotein particle composed of three large, stable RNA molecules and 51 proteins, which form 2 asymmetric subunits, the large 50S and the small 30S subunit. The 50S subunit, which catalyzes peptide-bond formation at the peptidyl transferase center, consists of two different rRNAs, the 23S rRNA (~2900nt) and the 5S rRNA (~120nt), and 34 proteins (L1-L36). The smaller subunit, which sediments at 30S, is composed of the 16S rRNA (~1500 nt) and 21 proteins. It comprises the messenger decoding-site and mediates the interaction between messenger RNA (mRNA) and transfer RNA (tRNA). The association of the two subunits is mediated mainly by rRNA-rRNA contacts, although rRNA-protein and protein-protein contacts are also involved. (Frank 2003; Berk and Cate 2007).

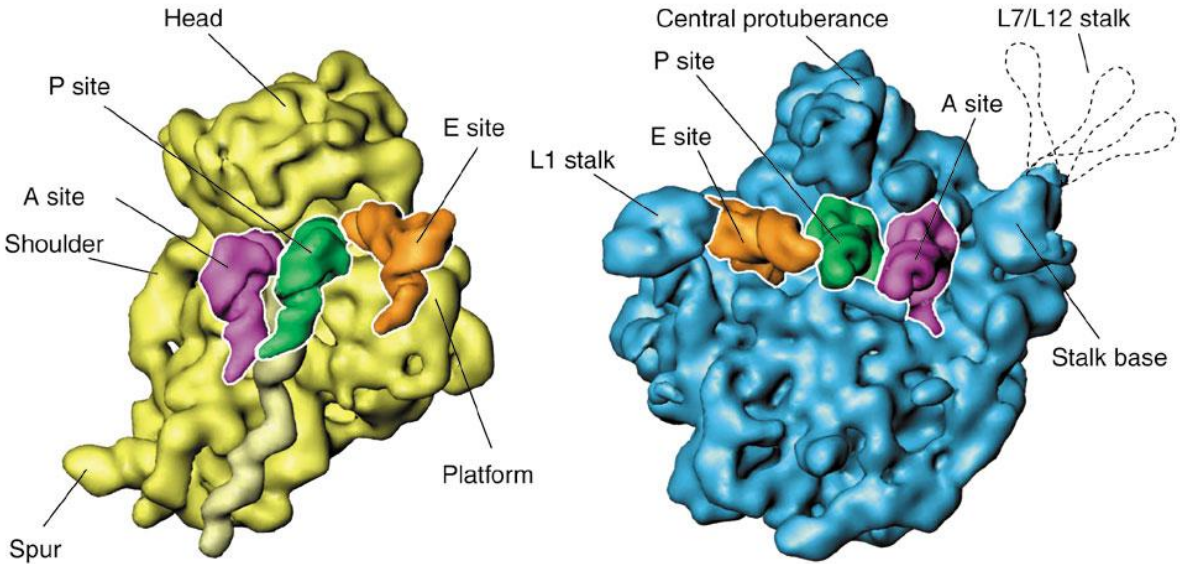


Figure 2: The 30S ribosomal subunit (in yellow), with the head, platform, shoulder and body domains and 50S ribosomal subunit (in blue), with three protrusions: the L1 stalk, the central protrusion, and the flexible L7/L12 stalk (taken from Frank, 2003).

The structure of bacterial ribosomes has been solved at atomic resolution, and recently it became clear that it acts as a protein stabilized ribozyme (Moore and Steitz, 2002). The architecture of the ribosome is determined by the shapes of the subunits, the inter-subunit cavity which serves for the passage of the tRNAs, and the mRNA path. As shown in Figure 2, the small subunit has an elongated shape and pronounced domains named the “head”, “platform”, “shoulder” and “body”. The large subunit is circular, and monolithic in shape, except for three protrusions: the L1 stalk, the central protuberance and the L7/L12 stalk (Figure 2, Frank 2003). The inter-subunit cavity is shaped for the passage of the tRNAs from the A- and P-site, where the aminoacyl- and peptidyl-tRNAs reside, to the E–site, which is specific for the deacylated tRNA before it exits the ribosome (Nierhaus, 2006). The “entrance” for the tRNA at the A-site harbors binding sites for several protein factors essential for translation, elongation factors (EF) EF-Tu, and EF-G, release factors (RF) 1 to 3 and the ribosomal recycling factor (RRF). In addition, the long, flexible ribosomal L7/L12 stalk is crucial for recruiting translation factors to the ribosome and enhancing their GTPase activity, and has therefore been termed the “GTPase activating region” or GAR (Frank 2003). As shown in Figure 3, the region is formed by the proteins L10, L11, four copies of L12/L7, and 58 nucleotides of the 23S rRNA. L12 is modified posttranslationally by an N-terminal acetylation to form L7, with a mass increase of 42Da. Increased acetylation of L12 was observed in cells under stressful conditions and results in an increased stability of the stalk complex (Gordiyenko and Deroo, 2008). The C-terminal domain of proteins L7/L12 form the binding site for the major translation factors, IF2, EF-Tu, EF-G and RF3 (Helgstrand *et al*. 2007), and the N-terminus is responsible for dimerization of L7/L12, and binding to the ribosome *via* interaction with L10 (Bocharov *et al.* 2004).

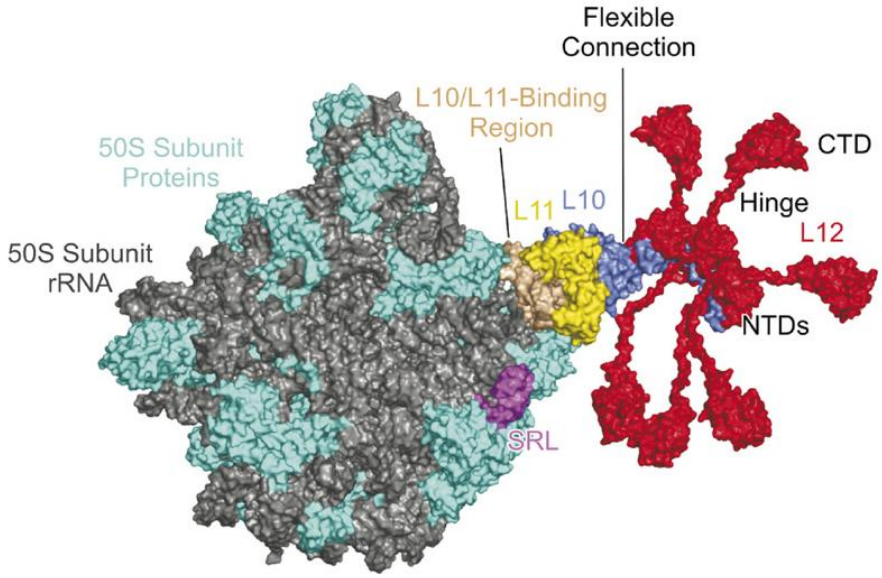


Figure 3: The 50S ribosomal subunit, with the L7/L12 ribosomal stalk, compromised of the proteins L10 (blue), L11 (yellow), and four L7/L12 copies (red). The stalk is highly flexible and dynamic, due to the flexible hinge region (Berk and Cate 2007)

The structural complexity of the ribosome is mirrored by the functional complexity of protein synthesis, which can be divided into four phases: initiation, elongation, termination, and ribosome recycling (Cate *et al.* 1999; Frank, 2003):

Initiation: In the first step of translation initiation, the 30S subunit, the initiator tRNA charged with formyl-methionine, and the mRNA form a ternary complex, aided by the initiation factors (IF) 1, 2 and 3. Initially, the binding of the small ribosomal subunit to the mRNA is mediated by the largest ribosomal protein S1, which binds to the mRNA in a region rich in pyrimidines upstream of the Shine-Dalgarno (SD) sequence (Boni *et al*., 1991, Sengupta *et al*., 2001), thereby increasing the concentration of the translational start site in the vicinity of the decoding site on the ribosome. The SD sequence is located 4-7 nucleotides upstream of the start codon. It is complementary to the sequence at the 3´-terminus of the 16S rRNA,

the so-called anti-SD sequence. The subsequent SD-anti-SD interaction contributes to the correct positioning of the start codon in the P-site of the 30S subunit (Boni *et al*, 1991, Shine and Dalgarno, 1974). The initiation factor IF2 facilitates the binding of the initiator fMet-$tRNA_f^{met}$ to the P-site (Carter *et al*, 2001), which interacts *via* codon-anticodon base-pairing with the start codon on the mRNA. IF3 is responsible for the discrimination against non-AUG start codons (Hartz *et al*., 1990) and IF1 participates in initiation complex formation by specifically binding to the A-site of the 30S subunit. It is suggested to block the A-site and thereby it positions the initiator tRNA towards the P-site (Carter *et al*., 2001; Dahlquist and Puglisi, 2000). After formation of this primary initiation complex, the 50S subunit joins and concomitantly the IFs are released, resulting in the formation of the translation initiation complex.

Elongation: Upon GTP-hydrolysis by and release of IF2 the complex can enter the first step in elongation: A ternary complex, consisting of EF-Tu, GTP, and an aminoacylated tRNA, binds to the A-site on ribosome, where the codon-anticodon interaction with the second codon present on the mRNA can occur. A correct interaction results in a conformational change, which leads to GTP hydrolysis and the release of EF-Tu (Rodnina *et al*. 1996). Another proofreading step precedes tRNA accommodation, as the correct tRNA is accepted and the aminoacyl group is transferred to the peptidyl transferase center of the 50S subunit. The growing peptide bound to the P-site tRNA is then transferred to the aminoacyl-group on the A-site tRNA (Moazed and Noller, 1989). After peptidyl transfer, the GTP-hydrolysis by EF-G induces another conformational change in the ribosome, which results in translocation, where the mRNA is shifted by one triplet (Frank and Agrawal, 2000).

Termination and ribosome recycling: When a stop codon (UAA, UAG, or UGA) is located in the A-site, it is recognized by release factors 1 or 2 (RF1 and RF2). The factors catalyze the hydrolytic release of the polypeptide from the peptidyl-tRNA located in the P-site. RF1 and RF2 are released from the post-termination complex by the activity of RF3 in a GTP-dependent manner. Then the ribosome recycling factor RRF, in association with EF-G and IF3, promotes the dissociation of the ribosome into its subunits (Kisselev and Buckingham, 2000), whereby binding of EF-G to the RRF bound ribosome triggers an interdomain rotation of RRF. This conformational change destabilizes two strong intersubunit bridges of the ribosome (B2a and B3), resulting in the separation of the two subunits (Gao *et al*., 2007). Upon dissociation of the ribosomal subunits, IF3 binds to the 30S subunit, and prevents reassociation (Hirashima and Kaji, 1973; Peske *et al*., 2005; Zavialov *et al*., 2005).

4.1.1 The ribosome is a major target for antibiotics

The ribosomes function is to provide a platform where the mRNA-based information can successfully be decoded by tRNAs, in order to synthesize a protein. The translational process involves a highly dynamic interplay of the two ribosomal subunits and numerous accessory factors. This complexity makes the ribosome a perfect target for antibiotics, which are low molecular weight compounds produced by microorganisms that kill or inhibit growth of other microorganisms. Each step in protein synthesis -initiation, elongation, termination and recycling- provides many opportunities for functional interference (Yonath, 2005; Nierhaus and Wilson 2004). In general, ribosome targeting antibiotics interact with the ribosomal RNA rather than with proteins (Sutclife, 2005), and several studies revealed that most of the sites of

antibiotic action coincide with the functional centers of the ribosome, like the decoding center and the tRNA binding sites (Tenson and Mankin, 2006). The ribosomal 30S subunit is primarily targeted by pactamycin, tetracyclines, as well as by aminoglycoside antibiotics.

Pactamycin is a cyclopetane derivative and inhibits translation in all three kingdoms. Therefore it is expected to interact with the highly conserved regions in the 16S rRNA (Mankin, 1997). Binding prevents release of initiation factors from the 30S ribosomal subunit and thus inhibits the formation of 70S ribosomes. (Cohen *et al.*, 1969; Kappen and Goldberg 1976). Furthermore, the antibiotic interferes with the factor- and GTP-dependent binding of tRNA to the P site during initiation (Cohen *et at.*, 1969), and it has been suggested to promote structural changes in the 30S subunit that prevent the tRNA from binding (Mankin, 1997). More recently, a role of pactamycin as a translocation inhibitor was suggested (Dinos *et a*l., 2004). The authors stated that the E site is now emerging as the binding site of pactamycin, and furthermore that it binds in the path of the mRNA in such a way that the two ring moieties of pactamycin mimic the last two nucleotides of the E-site codon.

Tetracycline is an octahydronaphtacene derivative and has 6 binding sites on the 30S subunit. However, the site with the highest occupancy is responsible for its bacteriostatic effect (Pioletti *et al*., 2001), where it blocks the binding of the aminoacylated tRNA to the A site (Maxwell, 1967: Geigenmuller and Nierhaus, 1986). The initial binding of the ternary complex of EF-Tu with tRNA to the A site and decoding are not affected (Gordon *et al*., 1969). There is also no effect on the binding of tRNA to the P site (Brown *et al*., 1993).

The best-studied antibiotics are the aminoglycosides, such as neomycin, gentamycin, paramycin and kanamycin, which bind to the 30S ribosomal subunit. They disrupt the mRNA-decoding fidelity by displacing two adenosine residues in helix 44 of the 16S rRNA, which stabilize a conformation which only occurs when the correct mRNA-tRNA complex is formed. (Sutclife, 2005)

4.2 Kasugamycin, an inhibitor of translation initiation

Another aminoglycoside antibiotic is kasugamycin (Ksg), a bacteriostatic agent, which has been shown to specifically inhibit the formation of the translation initiation complex in Pro- and Eukaryotes (Okuyama 1971). Like common aminoglycosides it has a universal two-ring structure which includes the 2'-deoxystreptamine moiety (Ogle *et al.*, 2001).

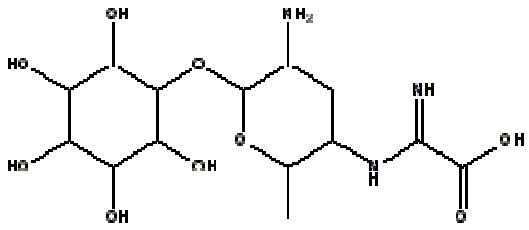


Figure 4: Kasugamycin

Ksg was shown to prevent the formation of the pre-initiation complex by interfering with fMet-$tRNA_f^{Met}$ binding to the ribosomal P-site in *E. coli* ribosomes (Poldermans *et al.*, 1979; Woodcock *et al.*, 1991). Resistance to Ksg is caused by the lack of the di-methylation of two adjacent adenosines positioned at nts 1518/1519 in helix h45 at the 3´-end of 16S rRNA (Helser *et al*., 1971). This post-transcriptional modification was shown to alter the conformation and flexibility of the ultimate stem-loop in 16S rRNA (Rife and Moore, 1998). Very recently, the binding site(s) of Ksg on

the ribosome has been determined. The compound blocks the initiation of protein synthesis by binding to two distinct sites on the 16S rRNA at nucleotides A794 and G926. These nucleotides lie on top of helix 44 (h44), spanning the region between h24 and h28 of the 16S rRNA, within the mRNA path at the P- and E-site (Figure 5, Schluenzen, 2006; Schuwirth *et al.*, 2006).

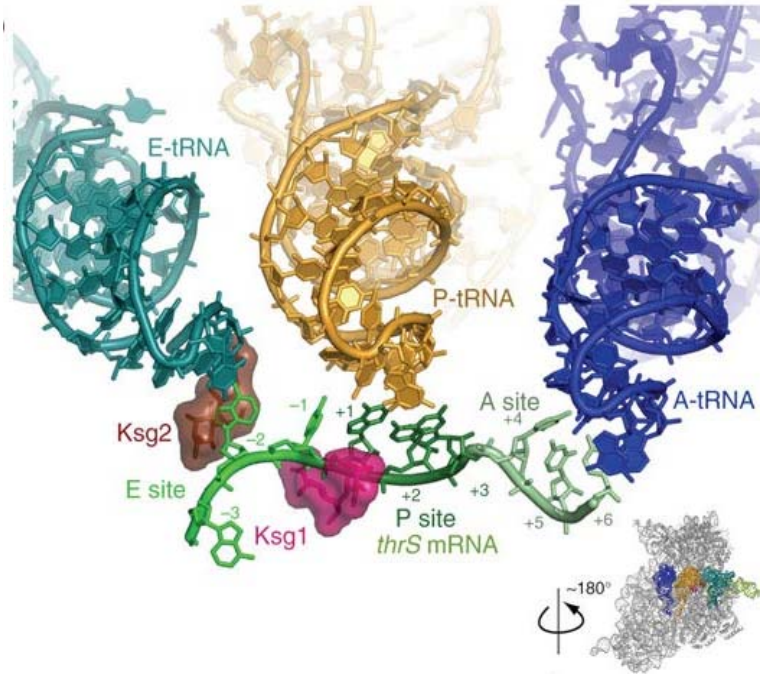
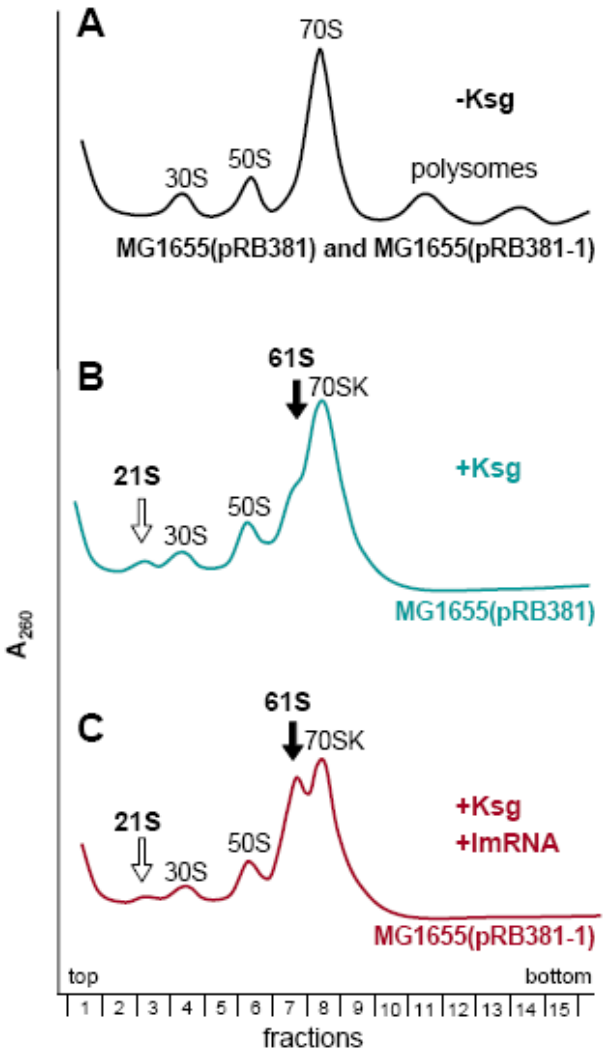


Figure 5: Two binding sites of Ksg within the path of the mRNA at the 30S subunit. Binding sites 1 and 2 (brown and pink) are shown relative to the A, P and E site tRNAs, and overlap with the P site mRNA (Schluenzen 2006).

4.2.1 The 61S particle

In contrast to canonical mRNAs, the antibiotic Ksg does not inhibit translation initiation on leaderless mRNAs (Chin *et al*., 1993; Moll and Bläsi, 2002). As these mRNAs lack a 5´-untranslated region, and are therefore devoid of ribosome recruitment signals other than the AUG start codon, in general, the leaderless mRNAs bind with less affinity to the ribosome. Recent studies performed to scrutinize the ongoing translation of leaderless mRNAs in the presence of the antibiotic

revealed the formation of ribosomal particles that lack several proteins of the 30S subunit upon treatment with Ksg *in vivo* (Figure 6, Kaberdina *et al.,* 2009) According to their sedimentation rate, these protein-depleted ribosomes are termed 61S particles.



D

	21S		61S	
	QI	MS	QI	MS
S1	-	↓	-	-
S2	-	-	-	-
S3	-	↓	↓	↓
S4	+	+	+	+
S5	+	+	↓	↓
S6	+	+	-	-
S7	+	+	+	+
S8	+	+	+	+
S9	+	+	+	+
S10	+	↓	+	+
S11	+	+	↓	↓
S12	-	-	-	-
S13	-	-	+	+
S14	-	-	+	↓
S15	+	n.d.	+	n.d.
S16	+	+	↓	↓
S17	-	n.d.	↓	n.d.
S18	+	n.d.	-	n.d.
S19	n.d.	-	n.d.	+
S20	-	-	+	↓
S21	-	-	-	-

Figure 6: Formation of the 61S particle in *E. coli* in the presence of Ksg *in vivo*. Ribosome sedimentation profiles of *E. coli* grown A) in the absence of Ksg, B) in the presence of 1mg/ml Ksg, and upon overexpression of a leaderless mRNA. Peaks representing 30S and 50S subunits, 70S ribosomes, and polysomes are indicated. Peaks corresponding to 21S particles and 61S particles formed in the presence of Ksg are indicated by an open and closed arrow, respectively. The fraction numbers are indicated on the bottom. D) Protein content of 21S and 61S particles (Kaberdina et al., 2009).

Determination of the protein composition of the 61S particles by Western blot analysis and mass spectrometry revealed the lack of several essential proteins of the small subunit including the functionally important proteins S1 and S12. In addition, five proteins were present only in a strongly reduced amount. However, the protein composition of the 50S subunit remained unaltered (Figure 6D, Kaberdina *et al.*, 2009). Structural analysis of the 16S rRNA suggested the disruption of several helices upon binding of Ksg. In addition, these studies revealed the presence of the di-methylations at positions A1519 and A1518, which are universally conserved in all three kingdoms of life and confer sensitivity to the antibiotic Ksg. Since the KsgA methylase requires a minimal particle of 16SrRNA and eight ribosomal proteins, half of which are missing in the 61S particle for its activity, the authors concluded that binding of Ksg to the fully assembled 70S ribosome leads to the loss of the ribosomal proteins. Furthermore, the presence of leaderless mRNA even stimulated the formation of these particles (Figure 6C; Kaberdina *et al.*, 2009), which supports the hypothesis that binding of Ksg concomitantly with the positioning of the AUG start codon of a leaderless mRNA in the P site of the 70S ribosome induces a conformational change within the central region of the 16S rRNA. This structural rearrangement could lead to the disruption of the 16S rRNA helices in juxtaposition to the Ksg binding site and the subsequent release of the respective ribosomal proteins directly or indirectly attached to this region (Kaberdina *et al.*, 2009).

4.2.2 Functionally important proteins lacking from 61S particles: S1 and S12

S1 is among the few ribosomal proteins for which a function in translation has been determined and the only ribosomal protein that binds to the 30S subunit *via* protein-protein interactions (Boni *et al*. 1982). As protein S1 binds to a pyrimidin-rich

region upstream of the SD-sequence of canonical ribosome binding sites (Sengupta *et al.*, 2001), it is essential for translation of canonical mRNAs in most Gram-negative bacteria (Szer *et a*l. 1975; van Dieijen *et al*. 1976, Sorensen, 1998). The C-terminus of S1 faces towards ribosomal protein S2 (Sengupta, 2001), and it has been shown that S2 is required for binding of S1 to the 30S subunit (Laughrea and Moore 1978, Moll *et al*. 2002). Since protein S1 was shown to be dispensable for the formation of a translation initiation complex on leaderless mRNAs *in vitro* (Tedin *et al.*, 1997*)*, and that both, S1 and S2, are dispensable for the translation of leaderless mRNAs *in vivo* (Moll and Bläsi, 2002), the lack of these proteins from the protein-depleted 61S particles would already explain their selective translation of leaderless mRNAs Kaberdina *et al.*, 2009).

Protein S12 is located at the subunit interface of 70S ribosomes, where it plays a critical role as a control element for translocation by interacting with tRNA and the large ribosomal subunit (Cukras *et al*., 2003). It was shown to make direct contacts with the codon-anticodon helix in the A-site (Ogle *et al*., 2001), and mutations in protein S12 resulted in hyper-accurate ribosomes (Lodmell and Dahlberg, 1997). These results are supported by a recent publication, where S12 has been identified as critical component to distinguish between cognate and near-cognate tRNA species (Sharma *et al*. 2007). A number of S12 residues conserved in all three kingdoms of life are clustered in two loops, the PNSA and the PGVRY loop. The conserved lobe of S12, the universal PNSA sequence, projects into the space between the 530 loop and nucleotides 1492-1493 of the 16S rRNA at the decoding site, completing the floor of the 30S subunit A-site (Figure 7, Sharma *et al*. 2007). Mutations in the protruding S12 lobe could therefore widen the space between the 530-loop and the nucleotides 1492/1493, thereby loosening the interaction between

the tRNA-mRNA complex and the 30S A-site (Yusupov *et al*. 2001), which cause a “restrictive”, hyper-accurate phenotype. Thus, it would be reasonable to assume that a ribosome which harbors not only a mutant protein S12, but lacks the protein completely, would perform hyper-accurate protein synthesis.

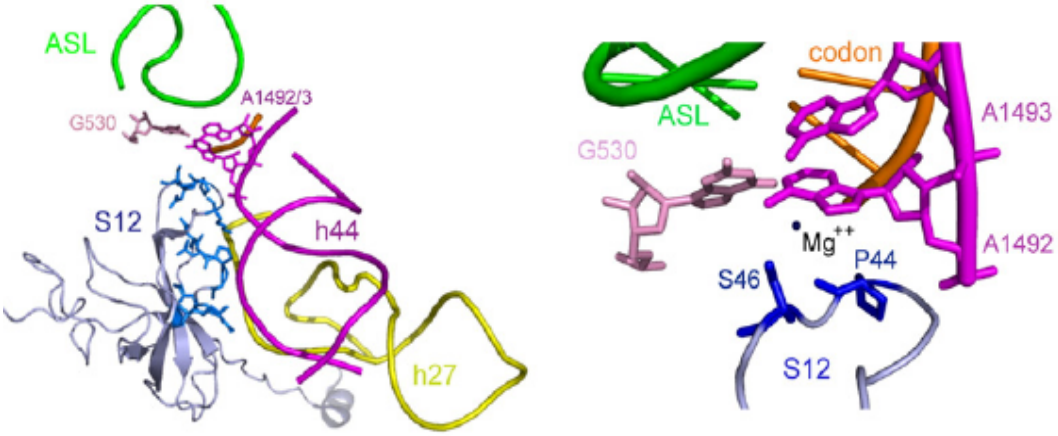


Figure 7: Position of S12 on the 30S subunit. The conserved lobe of S12 (light blue), the universal PNSA sequence, projects into the space between the 530 loop and nucleotides 1492-1493 of the 16S rRNA at the decoding site, completing the floor of the 30S subunit A-site. Neighboring helices are shown: h27 in yellow and h44 including A1492 and A1493 in magenta, A-site codon in orange and ASL in green. Hyperrestrictive phenotype: mutations cluster in a region that interacts with helices 18, 27 and 44 of the 16S rRNA (taken from Sharma *et al*.

5. Aims of the study

As outlined before, the lack of protein S12 from the 61S particles formed in the presence of Ksg suggested that these particles might display an increased translational fidelity when compared to 70S ribosomes. Therefore, the aim of this study was to determine the translational accuracy of the protein-depleted particles, employing a leaderless green fluorescent protein (GFP) construct. The GFP gene allows the concomitant determination of the total and active protein synthesized under different conditions. Since the fluorescence activity of GFP is sensitive to incorporation of incorrect amino acids, the ratio of total versus active fraction of GFP reflects the overall rate of misincorporation (Dinos *et al*., 2004).

During the course of this study, the restored translation of specific proteins upon prolonged Ksg treatment has been observed. Since Ksg inhibits translation of canonical mRNAs, solely the translation of leaderless mRNAs is resistant to the antibiotic. However, hitherto no leaderless mRNAs have been described for *E. coli*. Therefore, the second aim of this study was to further investigate this observation. As we anticipated that the corresponding mRNAs are leaderless, I first identified the respective proteins employing pulse labeling and 2D-gel analysis and subsequent mass spectroscopy. Subsequently, the 5´-termini of the respective mRNAs have been determined by primer extension using total RNA purified from cells treated with Ksg.

6. Results and Discussion

6.1 The translational fidelity of 30S/50S ribosomes is not affected by Ksg *in vitro*

As described in 4.2.1., the 61S particles were anticipated to exhibit increased translational fidelity when compared to 70S ribosomes, due to the lack of protein S12, which was shown to make direct contacts with the codon-anticodon helix in the A-site (Ogle *et al*.,2001). In order to address this question, a leaderless GFP construct was placed under control of a constitutive *lac* promoter. The green fluorescent protein (GFP) was originally isolated from the jellyfish *Aequorea victoria*, and fluoresces green when exposed to UV light. It consists of 238 amino acids which build up an 11 strand β-barrel (Figure 8). The central α-helix of this cylinder auto-catalytically forms a fluorophore from a tri-peptide sequence (Iskakova *et al*. 2006). This protein is particularly well suited for the fidelity assays since the activity of GFP is easy to measure and quantify. Additionally, GFP was found to be sensitive to mutations. Consequently a decrease in translational fidelity, which leads to the incorporation of incorrect amino acids, will reduce the fluorescence activity of the protein (Dinos *et al*., 2004)

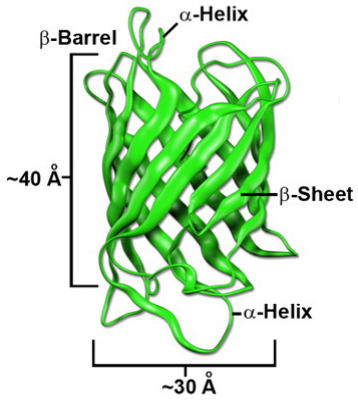


Figure 8: The GFP protein; the central α-helix autocatalytically forms a fluorophore. The protein is extremely mutation-sensitive. (Shaner *et al*., 2007)

To verify previous observations, that Ksg does not induce frame shifting, misreading or read through (van Buul *et al*., 1984), and to exclude the effect of Ksg on translation by intact 30S/50S ribosomes, a canonical GFP mRNA was translated *in vitro* employing 30S/50S ribosomes as described in Material and Methods. Upon incubation in the absence and in the presence of increasing concentrations of Ksg, the reaction mixtures were applied to a 12.5% SDS gel, to measure the amount of total protein synthesized, as well as onto a native protein gel, where the activity of GFP could be quantified. The activity of GFP without addition of Ksg was set to 100%. To calculate the translational fidelity, the total amount of GFP was divided by the amount of active GFP in each aliquot. This ratio can be used as a direct measure for the translational fidelity (Dinos *et al*., 2004).

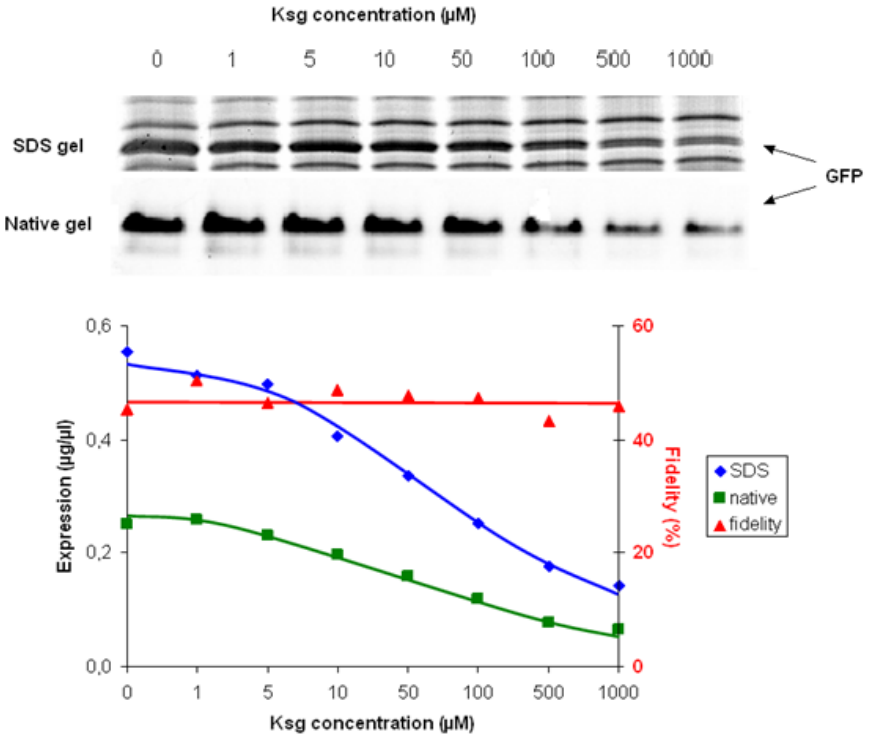


Figure 9: Ksg does not influence translational fidelity of canonical GFP *in vitro*. Translational fidelity of a canonical GFP construct in the presence of Ksg: With increasing concentrations of Ksg, the amount of translated protein drops (blue). However, the amount of active protein also decreases (green), thus maintaining the translational fidelity at a constant level (red).

The antibiotic inhibited the translation of GFP increasingly with higher concentrations of the antibiotic. However, the amount of active protein measured by the fluorescence decreased with the same ratio, indicating that the accuracy of translation of canonical GFP was not affected by Ksg *in vitro* (Figure 9, red line). Therefore, it was feasible to use the canonical GFP translation and activity measurements as a baseline for the determination of the fidelity of the 61S ribosomes.

6.2 Translational accuracy of 61S ribosomes *in vivo*

In order to determine the translational accuracy of the 61S ribosomes *in vivo*, *E. coli* strain MG1655 harboring either plasmid pRB-llGFP or plasmid pRB-canGFP, encoding the leaderless and the canonical GFP constructs, respectively, was treated with Ksg (Figure 10). Before addition of 1mg/ml Ksg (time point 0), and 30, 60, 90, 120 and 150 minutes after the addition of the antibiotic, two samples were withdrawn and subjected to protein separation on a SDS-PAGE, and concomitantly on a native protein gel, as described in Material and Methods. The translational accuracy at each time point was calculated by dividing the relative total amount of GFP with the relative activity of the GFP. As mentioned above, the canonical GFP was employed to determine the baseline for the inhibition of translation by intact 30S/50S. Since translation of the leaderless GFP construct is more than 10 times less efficient than translation of canonical mRNAs, for a direct comparison the translational efficiency at time point 0 was set to 1, and the relative ratios are given.

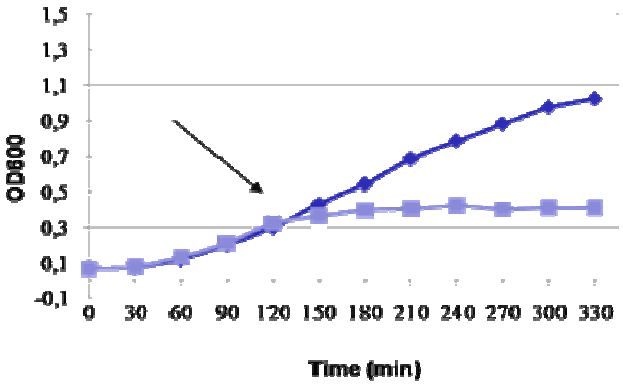


Figure 10: Growth of *E.coli* MG1655 pRB-lIGFP in LB-medium the absence (dark blue line) and upon addition of Ksg at OD_{600} of 0.3 (indicated by an arrow; light blue line). Growth ceases upon the addition of the antibiotic.

6.3 Kasugamycin increases the translational fidelity of a leaderless cI-GFP construct *in vivo*

Determination of the total amount of GFP present in the cells by Western blot analysis revealed that in the absence of the antibiotic, the amount of total GFP protein per cell remained constant in both cases, when the leaderless and the canonical constructs were employed (Fig 11A). Likewise, the GFP activity was not affected in the absence of Ksg (Figure 11B), indicating that the fidelity of translation of leaderless and canonical mRNAs did not change during growth (Figure 11C). In contrast, upon addition of Ksg at an OD_{600} of 0.3, growth of *E. coli* ceases (Figure 10) and canonical mRNA translation is inhibited. Therefore, the total amount of GFP per cell encoded by the canonical mRNA remained constant (Figure 11C). However, as shown in Figure 11D, the activity of the protein decreased as determined by native gel analysis, indicating the loss of activity during bacteriostasis. The amount of GFP encoded by the leaderless *c*I-*gfp* fusion gene revealed only a slight increase, which is in contrast to the ongoing translation of leaderless mRNAs in the presence of the antibiotic. Nevertheless, the activity of the GFP protein encoded by the leaderless

mRNA did not decrease in the presence of Ksg (Figure 11E). It is conceivable, that this result could be attributed to protein degradation under bacteriostatic conditions and the concomitant translation of the leaderless *c*I-*gfp* mRNA by the 61S ribosomes at a low level but with increased fidelity. This would result in a constant amount of protein, however, the *de novo* translated active fraction would increase, as shown by the ratio between the active fraction to the total amount of GFP, indicating an increase in translational accuracy of leaderless mRNAs after the addition of Ksg, which might be attributed to the lack of protein S12 (Figure 11F).

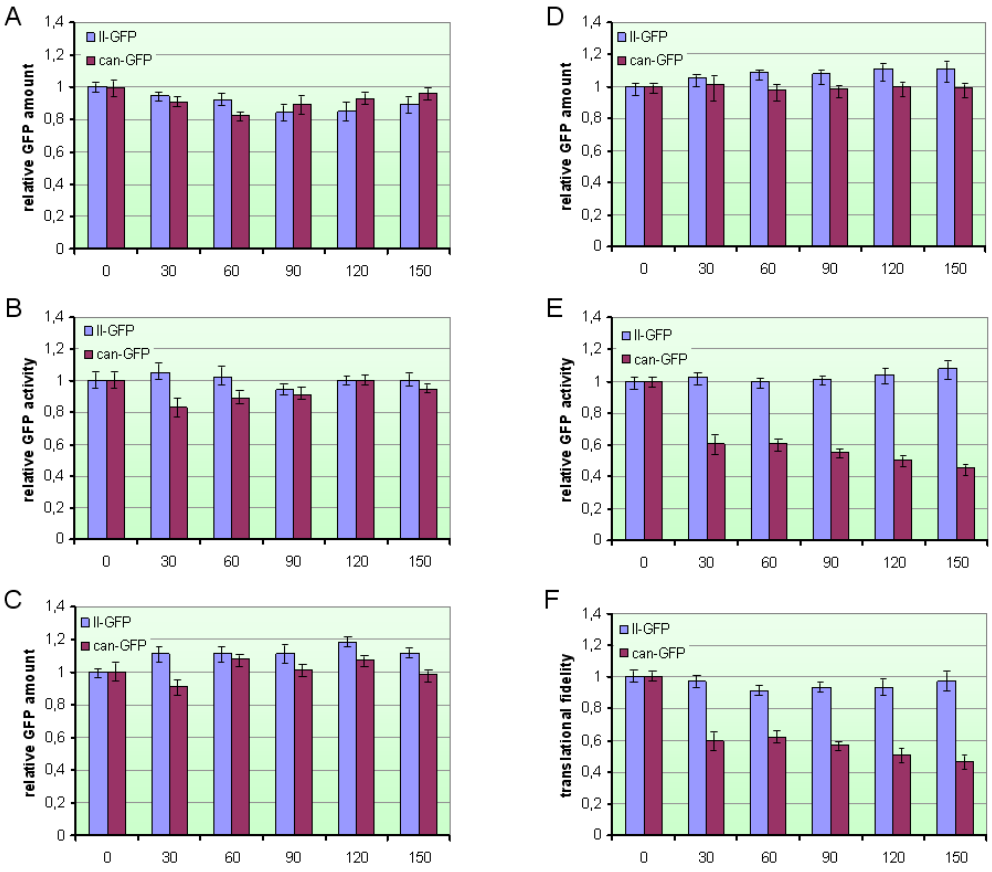


Figure 11: Determination of translational fidelity of 61S ribosomes in the absence (A, B, and C) or in the presence (D, E, and F) of Ksg *in vivo*. Blue and red bars indicate the values for GFP encoded by the leaderless mRNA and the canonical mRNA, respectively. A and D: determination of the total amount of GFP by Western blot analysis. B and E: Determination of the active GFP fraction by measuring the fluorescence on native gels. C and F: The ratio of the active GFP to the total GFP provides a measure of the translational fidelity of the system. The experiment has been performed in triplicate, standard deviations are given. To directly compare the IIGFP with the canGFP, the values determined for the time point 0 were set to 1, and relative values are given. For further explanation, see text.

Taken together, these facts indicate that the formation of leaderless mRNA could be a mechanism to ensure gene expression even under adverse conditions, and that increased fidelity would compensate for the lower level of translation by ribosomes with an altered protein complement. However, to unequivocally demonstrate the increased translational fidelity of the protein-deficient ribosomes, further *in vitro* translation studies have to be performed employing purified 61S ribosomes.

6.4 Selective translation of specific mRNAs in the presence of Ksg *in vivo*

As Ksg inhibits translation of canonical mRNA, and no endogenous leaderless mRNA is described in *E.coli,* a complete inhibition of translation is expected. This anticipation was verified by pulse labeling experiments performed 30´, 60´, and 90´ after the addition of Ksg (Kaberdina, *et al*., 2009), which showed selective translation of a leaderless mRNA. However, in the experiments described above as well as in previous experiments performed in this group, the restoration of translation of certain proteins was observed upon prolonged incubation with Ksg (Figure 12). To investigate this phenomenon and to answer the question how these proteins are synthesized in the presence of Ksg, *E.coli* strain MG1655 pRBIIGFP was grown until an OD_{600} of 0,3. Then, the culture was divided and 1mg/ml Ksg was added to one half. Ksg has been shown to exert its bactericidal effect on *E.coli* from a concentration of >500µg/ml (Levitan, 1967), and at a concentration of 1mg/ml, growth of *E.coli* is drastically reduced (Figure 12A).

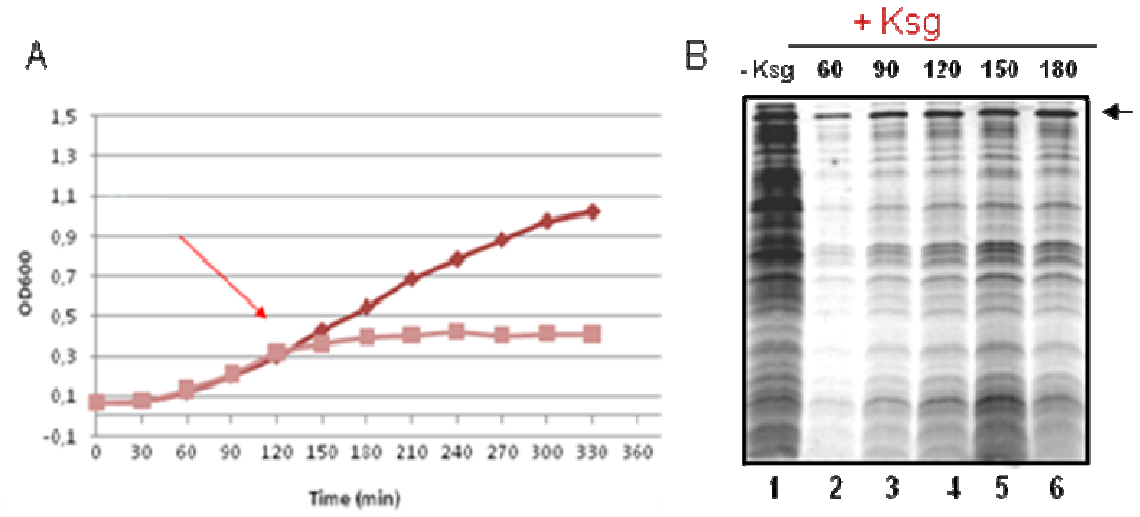


Figure 12: A) Growth of *E.coli* MG1655 pRB-llGFP in M9-medium the absence (dark red line) and upon addition of Ksg at OD_{600} of 0.3 (indicated by an arrow; light red line); Growth ceases upon the addition of the antibiotic. B) Selective protein synthesis in the presence of the translation initiator inhibitor Ksg. Lane 1 pulse labeling of *E.coli* strain MG1655 harboring a plasmid borne *cI-lacZ* fusion gene in the absence of Ksg. Lanes 2-5, pulse labeling of the same strain 60, 90, 120, 150 and 180 minutes respectively upon addition of 1mg/ml Ksg. The position of the CI-LacZ fusion protein is indicated by an arrow.

Pulse labelling experiments revealed that the translation of bulk mRNA was strongly diminished for approximately 60 minutes upon addition of Ksg, whereas *de novo* translation of leaderless mRNA continued in the presence of the drug (Figure 12B). However, upon further incubation in the presence of Ksg, the synthesis of distinct proteins resumed. Moreover, the selective translation of the respective mRNAs continued even upon addition of new Ksg, which excluded the possibility that degradation of the antibiotic would allow restart of translation. This result put forward the question, which mRNAs are translated under these conditions?

6.5 Determination of the *de novo* translated proteins after addition of Ksg

To identify the proteins synthesized under Ksg conditions pulse labelling experiments of *E.coli* strain MG1655 harbouring the plasmid pRB-llGFP either untreated or grown in the presence of 1mg/ml Ksg have been performed. The labelled proteins were separated using two dimensional gels (Figure 13). Selected spots were cut out and the respective proteins were determined by mass spectrometry. The prominent spots (Table 1, #1, #6, #7, #8, #9) were identified by comparison of the respective position in the 2D gels with standard gels (Expasy Proteomics server). It is intriguing to note that most of the proteins synthesized in the presence of the drug were stress response proteins, encoding heat shock as well as cold shock proteins, ribosomal proteins and ribosome modifying enzymes (Table 1). Since the presence of Ksg leads to the inhibition of canonical mRNAs, we expected the mRNAs encoding the respective proteins to be leaderless. However, as shown in Table 1, the mRNAs harbour a long 5'-UTR. Since this fact is difficult to reconcile with the activity of Ksg in inhibiting translation initiation of canonical mRNAs, these results could imply that translation in the presence of the antibiotic might be attributed to the removal of their 5´-UTRs.

	Protein	Function	5´-UTR	Reference
1	Enolase	enzyme of glycolysis, phosphoprotein; component of the RNA degradosom	76 nts	Gama-Castro et al., 2008
2	L7/12	ribosomal protein	plJ-rplL-operon	Post et al., 1979
3	CspA	cold-shock protein A	109 nts	Goldstein J., et al., 1990
4	RplI	ribosomal protein L9	rpsF-rplI-operon	Gama-Castro et al., 2008
5	YfiD	pyruvate formate lyase homolog	74 nts	Green et al., 1998
6	GroES	chaperone	72 nts	Nonaka et al., 2006
7	UspA	universal stress protein	111 nts	Nystrom T., et al., 1992
8	DnaK	Heat-shock chaperone	115 nts	Cowing et al., 1985
9	DnaJ	Heat-shock chaperone	*dnaK-J operon*	Cowing et al., 1985
10	RimL	L12 modifying enzyme	196 nts	Tanaka et al., 1989
11	ClpB	ATP-dependent protease	32 nts	Nonaka et al., 2006
12	GroEL	chaperone	409 nts	Nonaka et al., 2006
13	RbfA	Ribosome binding factor A	*infB*-operon	Ishii et al., 1984

Table 1: Proteins synthesized in the presence of Ksg and the 5´-UTR of the respective mRNAs

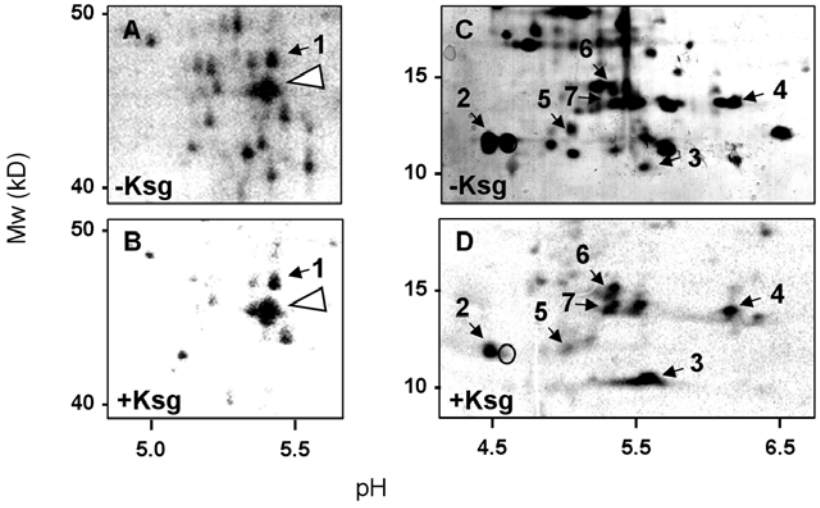


Figure 13: 2D gel analysis of pulse labeled proteins of *E. coli* strain MC4100 before (A and C) and upon incubation with 1mg/ml Ksg for 120min (B and D). The proteins identified are numbered according to Table 1. Elongation factor 2 is indicated by an arrow head and the unmodified protein RplL (L12) is encircled. The sections of the autoradiographs showing identified proteins #1-7 are depicted. The molecular weight and the pH values are indicated to the right and on the bottom, respectively.

6.6 Conditionally leaderless mRNAs are formed in the presence of Ksg *in vivo*

To test whether translation in the presence of the antibiotic might be due to the lack of a 5´-UTR under these conditions, four mRNAs, *dnaK*, *clpP*, *eno* and *yfiD*, have been selected and primer extension analyses have been performed to determine their 5´-termini in the absence and in the presence of Ksg. Therefore, total RNA was purified from *E. coli* MC4100 cells grown either in the absence of Ksg or upon incubation for 180 min in the presence of the antibiotic. Subsequent primer extension analysis has been performed using primers which bind to a short sequence 50-100nt downstream of the AUG start codon of the selected mRNAs.

DnaK is a heat-shock protein, with a mass of 70kDa, and is thus also called Hsp70 (Schröder *et al.,* 1993). It interacts with protein substrates in an ATP-dependent manner, to prevent protein aggregation and misfolding upon heat shock

(McCarty *et al.,* 1995) and is one of the central components of the cellular network of molecular chaperons and folding catalysts (Mayer *et al.,* 2000). Primer extension performed with the *dnaK*-specific primer revealed the occurrence of a short-leadered transcript upon Ksg-treatment due to the presence of an alternative transcriptional start site 19 nucleotides (nts) upstream of the AUG-start codon (Figure 14A and B), which has been determined before (Cowing *et al.,* 1985). Since it has been shown that a short-leadered *ompA* mRNA containing a 21 nts long 5´-UTR is likewise translated in the presence of Ksg *in vivo* (I. Moll, unpublished data), the truncation of the *dnaK* 5´-UTR by alternative transcription could likewise allow the translation of the mRNA in the presence of the antibiotic. Similar results have been observed, when primer extension analysis was performed with a primer specific for the *clpP* mRNA. ClpP is a serine-type protease (Maurizi *et al*., 1990) identified in a wide range of bacteria (Porankiewitz et al., 2002) Proteases such as ClpP play a vital role in the cell by regulating the level of proteins and removing damaged ones which could interfere with the cell metabolism. This function is crucial for cell homeostasis and optimal metabolic activities (Gottesman, 1996). The protein is present in low levels during normal growth, but is inducible by various forms of stress (Kroh and Simon, 1990; Damerau and St.John, 1993)

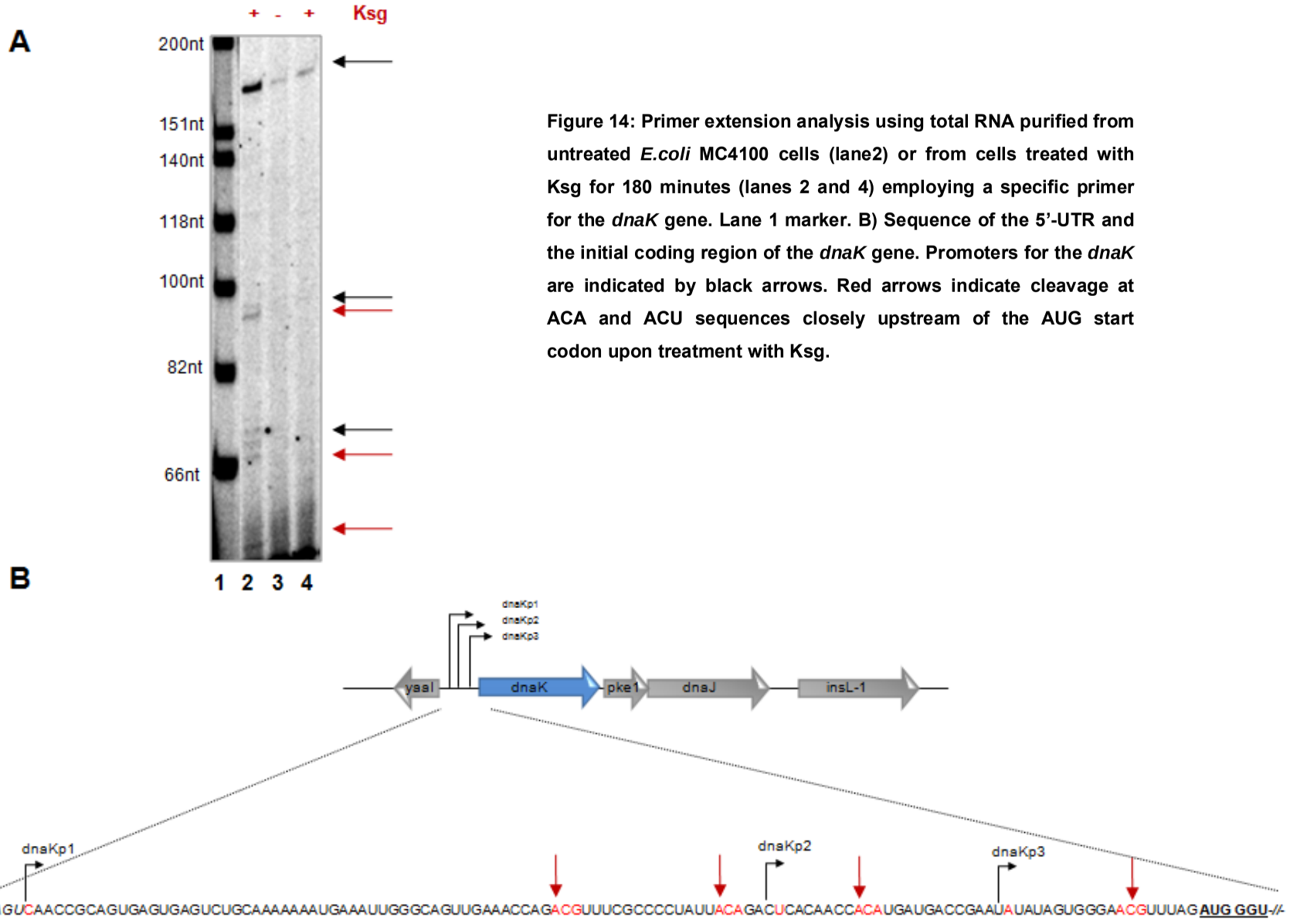


Figure 14: Primer extension analysis using total RNA purified from untreated *E.coli* MC4100 cells (lane2) or from cells treated with Ksg for 180 minutes (lanes 2 and 4) employing a specific primer for the *dnaK* gene. Lane 1 marker. B) Sequence of the 5'-UTR and the initial coding region of the *dnaK* gene. Promoters for the *dnaK* are indicated by black arrows. Red arrows indicate cleavage at ACA and ACU sequences closely upstream of the AUG start codon upon treatment with Ksg.

Total RNA extracted from untreated *E.coli* cells yielded an extension signal that corresponds to the transcription start at the *clpPp1* promoter (Figure 15A, lanes 2 and 4 and Figure 15B; Maurizi *et al.,* 1990). However, primer extension performed with total RNA from cells treated with Ksg revealed two additional stop signals corresponding to the *clpPp3* promoter and the *clpPp2* promoter (Figure 15A, lane 3). Taken together, these results indicate a possible mechanism for the aberrant translational pattern after addition of the antibiotic Ksg. Under stress conditions, i.e. upon antibiotic treatment, the transcription starting at the main promoters, *dnaKp1* and *clpPp1* is turned off. Instead, promoters, which are closer to the AUG start codon, are recognized by the RNA polymerase, which is equipped with alternative sigma factors. Taken together, the results indicate that alternative transcription might be one potential mechanism generating short-leadered variants of the respective mRNAs, which might be translated by protein-depleted 61S ribosomes.

Surprisingly, further analysis revealed the removal of the 5´-UTR from several mRNAs by a post-transcriptional processing event within their 5´-UTRs (Figures 16 and 17). Primer extension analysis was performed with a primer specific for the *yfiD* gene (Figure 16A and B). YfiD is a homologue of the pyruvate formate lyase, which is strongly induced at low pH (Blankenhorn *et al*., 1999). Without Ksg-treatment of the cells, primer extension of total mRNA yielded a signal corresponding to the identified transcriptional start 74 nts upstream of the AUG start codon, corresponding to the determined promoter (Green *et al.,* 1998), and a second signal which is 10 nts shorter (Figure 16A, lane 2). Using total mRNA purified from cells incubated in the presence of Ksg, the extension signals revealed two different 5´-ends of the *yfiD* mRNA, containing 32 nts and 1 nt, respectively, upstream of the start codon (Figure 16A, lane 3 and Figure 16B).

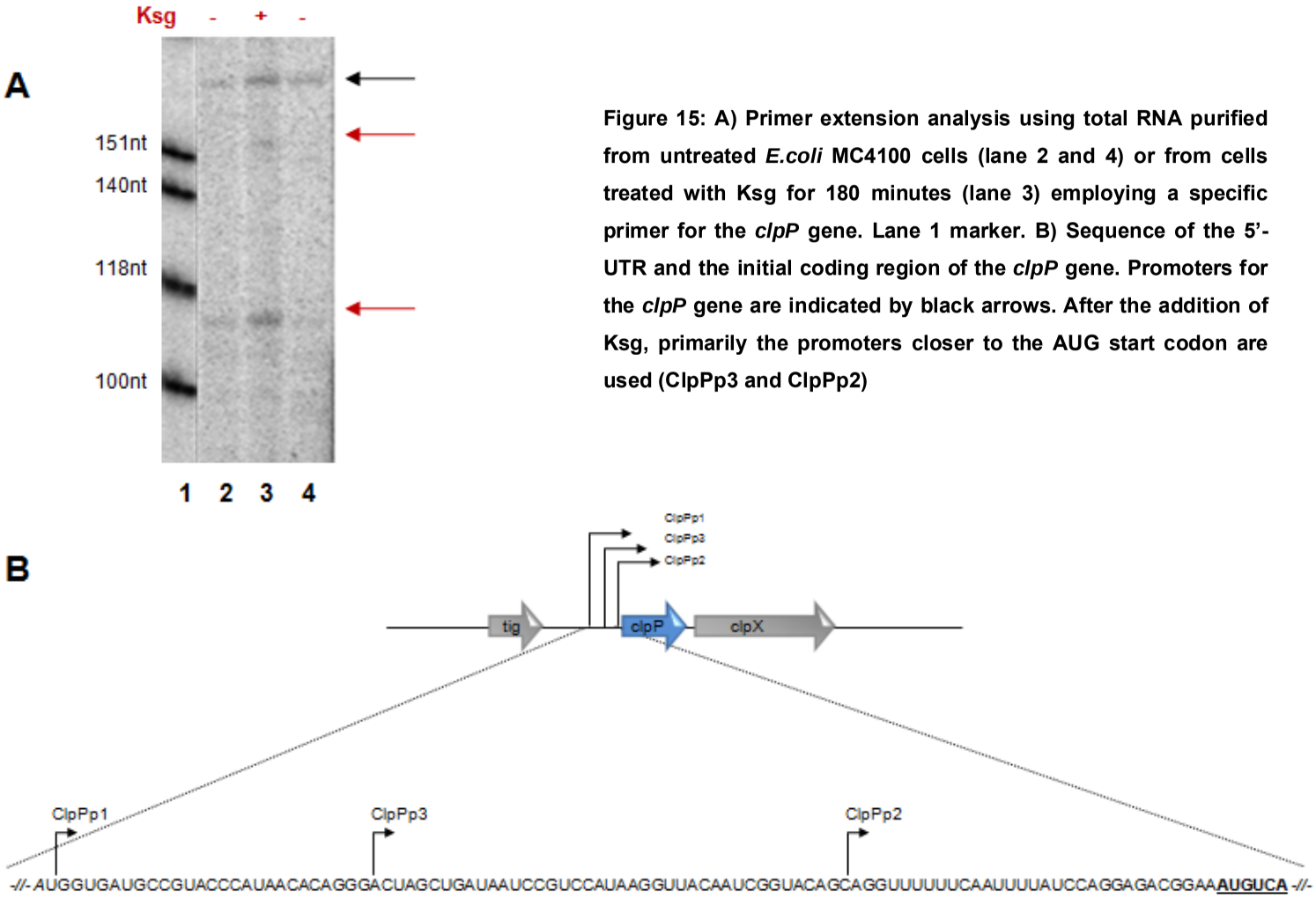


Figure 15: A) Primer extension analysis using total RNA purified from untreated *E.coli* MC4100 cells (lane 2 and 4) or from cells treated with Ksg for 180 minutes (lane 3) employing a specific primer for the *clpP* gene. Lane 1 marker. B) Sequence of the 5'-UTR and the initial coding region of the *clpP* gene. Promoters for the *clpP* gene are indicated by black arrows. After the addition of Ksg, primarily the promoters closer to the AUG start codon are used (ClpPp3 and ClpPp2)

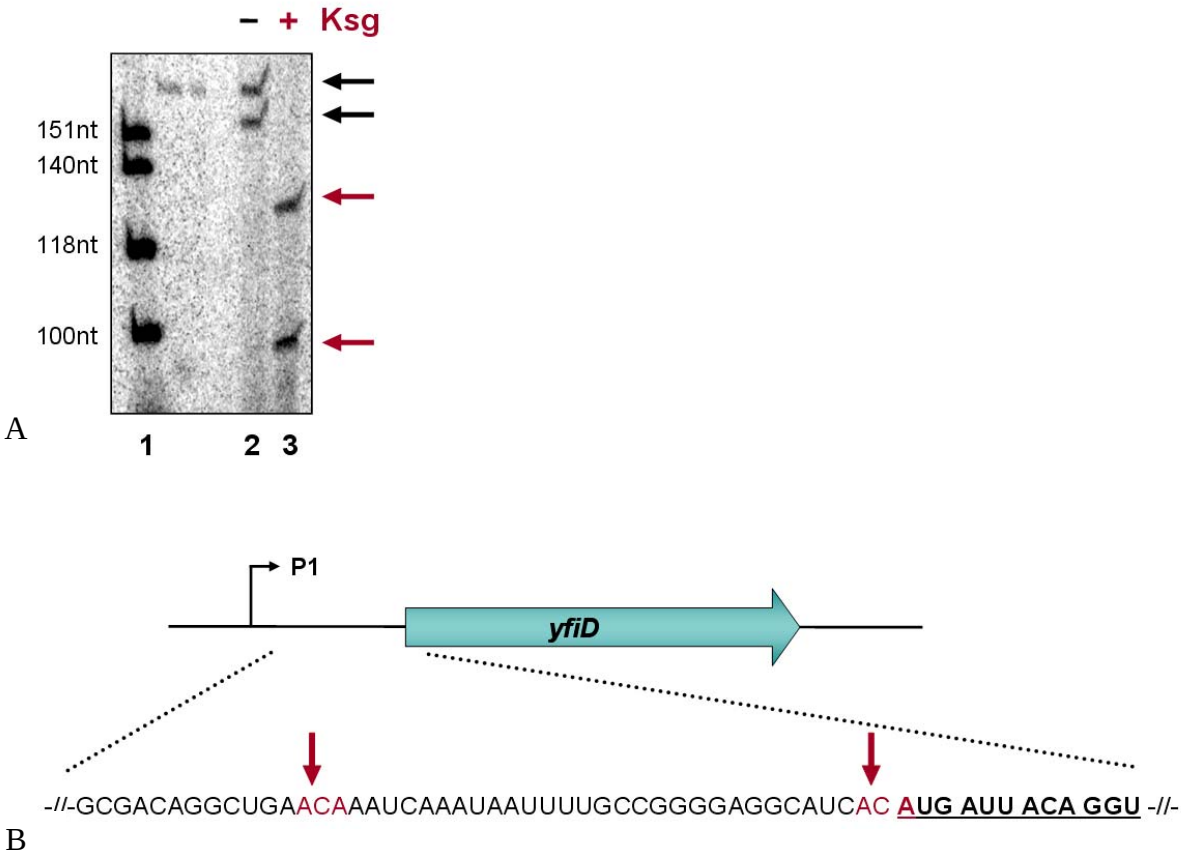


Figure 16: (A) Primer extension analysis using total RNA purified from untreated *E. coli* MC4100 cells (lane 2) or from cells treated with Ksg for 180 min (lane 3) employing a specific primer for the *yfiD* gene. Lane 1, marker (B) Sequence of the 5´-UTR and the initial coding region of the *yfiD* gene. Promoter for the *yfiD* gene is indicated. Red arrows indicate signals corresponding to cleavage at ACA codons closely upstream of the AUG start codon upon treatment with Ksg.

Similar results have been obtained upon primer extension reactions using a specific primer for the *eno* gene encoding the phosphoprotein enolase, which is involved in glycolysis and furthermore, represents a component of the degradosome, a multi-protein complex involved in RNA processing and degradation (Liou *et al*., 2001). In the absence of the antibiotic we obtained an extension signal which corresponds to the transcriptional start at the promoter within the *pyrG* gene preceding the *eno* gene (Figure 17A, lane 3, black arrow; Shimada *et al.,* 2005). However, upon addition of Ksg, a faint signal corresponding to the alternative

transcription start 76 nts upstream of the *eno* gene appeared (Figure 17A, lane 2 and Figure 17B, pink arrow; Gama-Castro *et al.,* 2008). In addition, primer extension yielded distinct bands corresponding to 5´-UTRs with a length of 16 nts or 23 nts, respectively, indicating alternative 5´-ends of the mRNA (Figure 17A, lane 2 and Figure 17B, open and closed red arrow, respectively). Again, since no promoters are present upstream of these nucleotides, which could allow alternative transcription, these results indicate a processing event within the 5´-UTR of the respective genes, generating leaderless mRNAs.

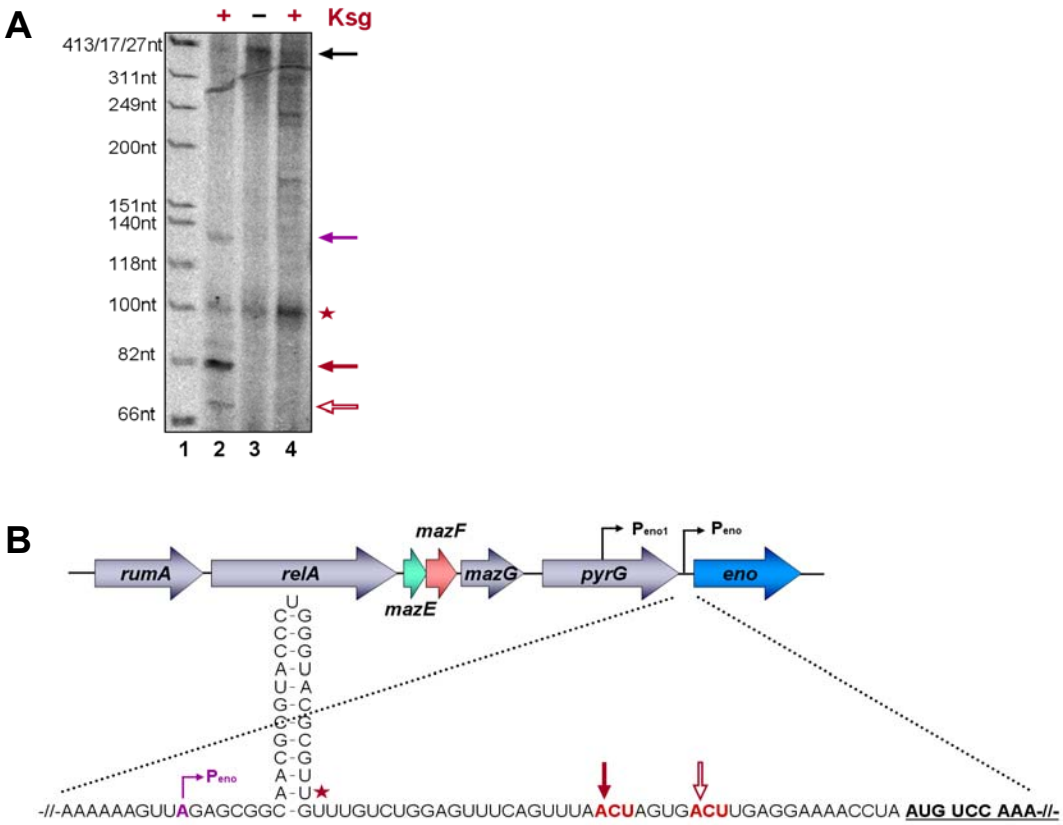


Figure 17: A) Primer extension analysis using total RNA purified from *E. coli* MC4100 cells treated with Ksg (lane 2), from untreated MC4100 cells (lane 3), or from the MC4100ΔmazEF strain treated with Ksg (lane 4) employing a specific primer for the *eno* gene. The signal corresponding to the transcription start within the upstream gene *pyrG* is indicated by a black arrow. The red star indicates an unspecific signal due to a secondary structure. Lane 1, marker. (B) Sequence of the 5´-UTR and the initial coding region of the *eno* gene. Promoter for the *eno* gene is indicated. Red arrows indicate cleavage at ACU codons closely upstream of the AUG start codon.

6.7 MazEF, a toxin-antitoxin system responsible for the removal of the 5´-UTR of specific mRNAs?

What mechanism(s)/factors might be employed for cleavage of the mRNAs? As shown in the *eno* and *yfiD* mRNA sequences (Figure 16B and 17B), the signals coincide with a possible cleavage 3´ of the first A in ACU and ACA sequences, respectively. As toxin-antitoxin (TA) modules are known to be induced by antibiotics affecting the protein synthesis machinery (Figure 18; Engelberg-Kulka *et al*., 2006), and the ChpBK and MazF toxins were shown to cleave specifically either 5´ or 3´ of the A residue in ACY sequences (Y is U, A, or G; Zhang *et al.,* 2005) and ACA sequences (Zhang *et al.,* 2003), respectively, we hypothesized that the endonucleolytic activity of the TA loci might contribute to the appearance of conditionally leaderless or "short-leadered" mRNAs upon antibiotic treatment. Bacteria encode multiple copies of TA modules, which consist of two consecutive and translationally coupled genes encoding an unstable anti-toxin and a stable toxin, respectively (Gerdes, 2000; Christensen *et al.,* 2003). Primarily, they have been identified as addiction modules which are located on a single plasmid to prevent growth of plasmid-free bacteria by a mechanism termed "post-segregational killing". If the bacterium loses the plasmid, or the transcriptional/translational machinery is affected, the antitoxin is degraded, and can no longer prevent the toxin from killing the cell. In that sense, the bacterium is "addicted" to the plasmid. One of the best studied TA modules in *E.coli* is the *mazEF* system. It encodes the 9kDa MazE protein, the labile antitoxin, and 12kDa MazF protein, the stabile toxin, which is a sequence-specific single strand RNA endoribonuclease (Zhang et al. 2003). *mazEF* is a stress-induced suicide module that can be triggered by stress conditions, which affect the transcriptional/translational machinery (Sat et al., 2001). In that case, a

specific ATP-dependent serine protease, ClpAP, degrades the antitoxin, and thereby frees the endoribonuclease to cleave the mRNA (Aizenman et al., 1996). The preferred sequence of the sequence-specific endoribonuclease is ACA, however, cleavage at ACU and ACG has also been observed. This in turn blocks protein synthesis, leading to a reduction in growth and even cell death. The trigger for this system can be cellular stress, including, but not limited to extreme amino acid starvation leading to the production of the starvation signaling molecule ppGpp (Aizenman *et al.* 1996), antibiotics that inhibit transcription or translation (Sat *et al.* 2001), heat shock, oxidative stress or DNA damage (Hazan *et al*. 2004) (Figure 18).

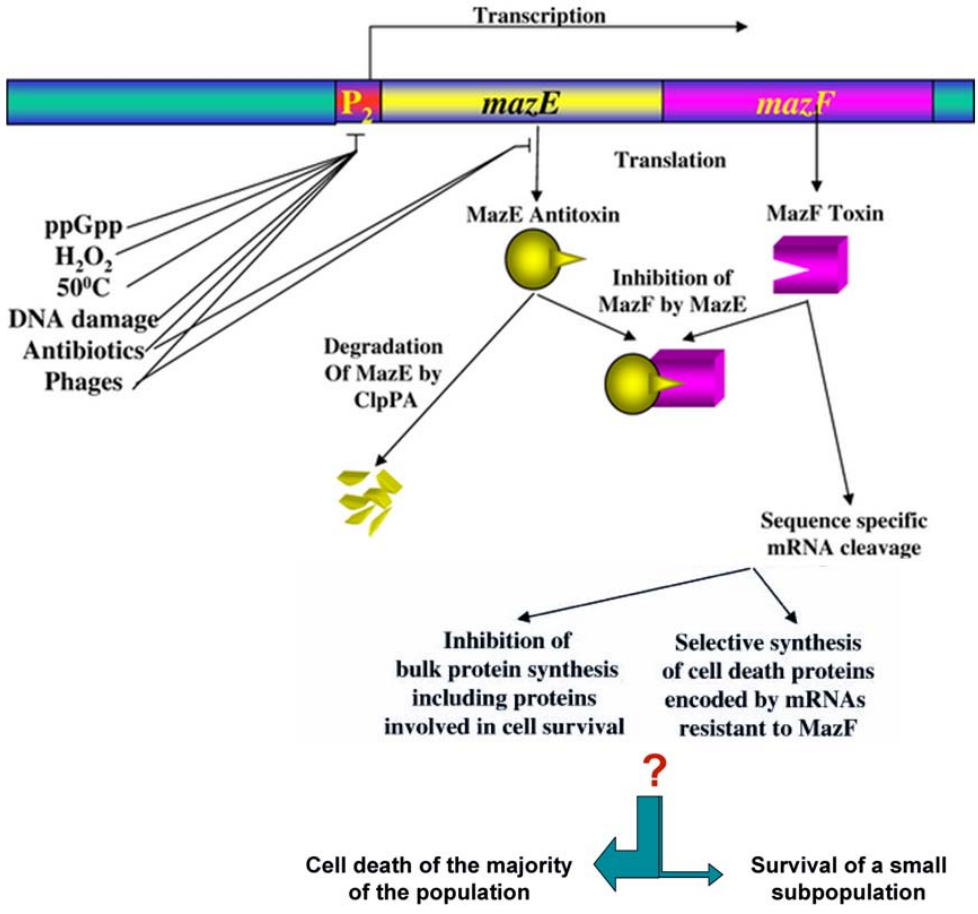


Figure 18: Induction and function of the *E. coli* toxin-antitoxin module *mazEF*. For details, see text (taken from Engelberg-Kulka (2006); with minor modifications)

To test for the hypothesis, whether the removal of the 5´-UTR might be attributed to a cleavage reaction of the RNA interferase MazF at the ACA site(s) closely upstream of the AUG start codon of the *eno* mRNA, again primer extension analysis has been performed. The total RNA has been purified from an *E. coli* strain which is deficient for *mazEF* (Engelberg-Kulka *et al.,* 1998; MC4100Δ*mazEF*) upon treatment with Ksg for 180 min. As shown in Figure 17A (lane 4), we did not observe the signals corresponding to the processing/cleavage at the ACU-sites proceeding the AUG-start codon of the *eno* gene, indicating the involvement of the MazF interferase in the formation of conditionally leaderless mRNAs under stress conditions.

6.8 A novel stress response pathway in *E. coli*?

In the presence of Ksg, which mimics stress conditions targeting the translational machinery, the ribosomes loose several ribosomal proteins from the 30S subunit, which are essential for translation of canonical mRNAs. In contrast, these protein-deficient 61S particles are still competent to translate leaderless mRNAs (Chin *et al.,* 1993; Moll and Bläsi, 2002; Kaberdina *et al.,* 2009). The results presented in this study indicate, that these ribosomal particles translate the leaderless mRNAs with an increased accuracy, which might be attributed to the absence of S12 (Figure 10). This higher translational accuracy might as well contribute to the survival of the bacteria under adverse conditions, since no energy resources are wasted. Furthermore, I present evidence that a small population of mRNAs, translation of which supports the survival of the bacteria, becomes leaderless under these stress conditions by two distinct molecular mechanisms.

First, the preferential usage of alternative promoters in the presence of Ksg has been determined to contribute to the formation of short-leadered mRNAs, as shown for the *dnaK* and *clpP* mRNAs (Figure 14 and 15). These transcription start points closely upstream of the start codon are recognized upon encountering stress conditions, when alternative sigma factors bind to the catalytic core of the RNA polymerase and change their promoter specificity (Boor, 2006).

The second possibility for the formation of leaderless mRNAs is an RNA processing event, which can be attributed to the endoribonuclease activity of so-called toxin-antitoxin systems. In the present study, the data indicate the involvement of the toxin MazF. This enzyme is activated when translation is inhibited or stalled by antibiotic treatment, amino acid starvation or heat and oxidative stress conditions. Under these conditions most of the mRNAs are degraded by MazF cleavage at ACA sites present in single stranded regions and therefore would lead to the inhibition of translation of most mRNAs (Zhang *et al*., 2003). However, a small subgroup of mRNAs are cleaved in the 5´-UTR closely upstream of the AUG-start codon, making the mRNAs leaderless, as shown for the *eno* and *yfiD* mRNAs.

Taken together, these results point towards a potential hitherto undescribed stress response mechanism in *E. coli*, which is based on the formation of leaderless mRNAs. Since several lines of evidence indicate the presence of heterogeneous ribosome populations and/or the formation of protein-deficient aberrant ribosomes under adverse conditions (Deusser and Wittmann, 1972; Kaberdina et al., 2009), the lack of a 5´-UTR of the respective mRNAs could allow the selective translation of stress proteins, which could support the bacterial population to survive.

7. Materials and methods

7.1 Bacterial strains and plasmids

Strains and Plasmids	Description	Reference
MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1	Blattner et al. (1997)
MC4100	araD139 D(*argF-lac*)205 *flbB5301 ptsF25 rpsL150 deoC1 relA1*	Casadaban (1976)
MC4100 Δ*mazEF*	Δ*mazE* Δ*mazF* derivative of MC4100 KanR, TetR	Engelberg-Kulka et al. (1998)
pIVEX2.2.GFP	canGFP, Amp^R	Roche
pUH 21-2	Amp^R, lac-promotor	Lanzer M, Bajard H (1988)
pRB381	Amp^R	Brückner (1992)
pKTplaccI	λ-cI, lac promoter, Tet^R	Grill *et a*l. (2000)
pRB-llGFP	pRB381 derivative, Amp^R, carries the leaderless *c*I-*gfp* fusion gene	This work
pRB-canGFP	pRB381 derivative Amp^R, carries the canonical *gfp* gene	This work
pSA1	Derivative of pQE30 (Qiuagen) carrying the $lacI^q$ gene, *mazF* gene under T5 promoter and lac operator control	Amitai et al. (2009)

Table 2: Strains and plasmids used in this study

If not indicated otherwise, the strain was grown in Luria-Bertani medium (Miller, 1972). For plasmid maintenance 100µg/ml ampicillin was added. Growth of liquid cultures was monitored photometrically by measuring the optical density at a wavelength of 600nm (OD_{600}).

7.2 Construction of plasmids

As it has been shown that the 5' end of the leaderless GFP mRNA folds into a stable stem loop (Figure 18b), making it impossible for the translational machinery to form an initiation complex, the first 198 nucleotides of the λ-cI gene coding for a leaderless mRNA were fused upstream of the GFP coding sequence. This did not alter the activity of the protein (Figure 18c). The plasmid pRB381-llGFP containing the leaderless *cI-GFP* fusion gene is a derivative of plasmid pRB381 (Brückner, R**.** 1992), and was constructed as follows. A PCR was performed using primers M41 and E39 (Table 3) and plasmid pIVEX2.2.GFP (Roche) as template. The resulting PCR product containing the coding region of GFP from was cleaved with ScaI and was fused to a second PCR product using primers C10 and D6-1 (Table 3) and plasmid pKTplaccI (Table 2) as template containing the first 198 nucleotides of λ-cI, which was cleaved with SmaI. The ligation of these two fragments was used as a template with primer C10, containing a XbaI-site and primer E39, harboring an EcoRI-site. The resulting PCR product was cleaved with XbaI and EcoRI, and inserted into the same sites of plasmid pRB381.

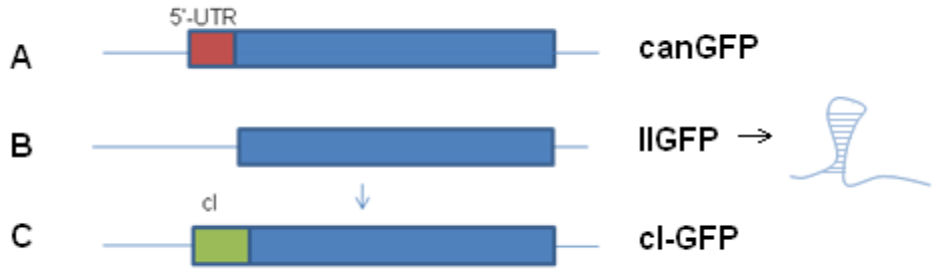


Figure 18: Schematic depiction of the A) canonical GFP, carrying a 5´-UTR, B) a leaderless GFP construct, devoid of the 5´-UTR, which forms a secondary structure, and C) the leaderless construct, where the 5'-terminal coding region (63 codons) of the λ-*c*I gene are fused to the 5'-terminus of the GFP gene, preventing the formation of the stem loop.

The plasmid pRB381-canGFP is a derivative of plasmid pRB381. Plasmid pRB381 was cleaved with EcoRI and BamHI, treated with Klenow polymerase to obtain PstI cleavage sites, and then cleaved with PstI to accommodate the insert. A PCR fragment containing the canonical GFP gene from plasmid pIVEX2.2.GFP, amplified with primers L41 and E39, was inserted into the EcoRI/EcoRI site of plasmid pUH21-2 (Lanzer M, Bajard H 1988). The resulting plasmid was used as a template with PstI forward and reverse primers C10 and E39. The PCR product was cleaved with PstI and inserted into the same sites of pRB381. All DNA manipulations were verified by sequencing.

Primers	Sequence 5'-3'
C10	AAATCTAGACTGCAGCCCTTTCGTCTTCACCTCGAG
D6-1	TTTTGCAAGCAACCCGGGGTTATAAGC
E39	AGGAATTCATGGCGACCACACCCGTCCTG
L41	CCGGAATTCTAATACGACTCAGTATAGGGAGACCAC
M41	ATTATAAGTACTAGCTGGAGCCACCCGC

Table 3: Sequences of primers used for the construction of plasmids

7.3 Translation fidelity assays

E.coli strain MG1655 harboring either plasmid pRB381-llGFP or pRB381-canGFP was grown in Luria Bertani medium. At an OD_{600} of 0.3 either no antibiotic or Ksg was added (1 mg/ml). 30, 60 and 90 minutes after the addition of the antibiotic, two 1,5 ml aliquots were withdrawn from each culture. From each time point, one aliquot was collected by centrifugation at maximal speed, resuspended in sterile

water according to their OD_{600}, and subjected to three cycles of freezing and thawing in liquid nitrogen. This aliquot was used to determine the amount of active GFP as described below. The second aliquot was collected by centrifugation at maximal speed, and the resulting pellet was resuspended in SDS gel loading buffer (100 mM Tris–Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol), according to their OD_{600} and heated at 95 °C for 10 min. 15 µl of each sample containing equal amounts of total cellular protein were separated on 12% SDS–polyacrylamide gels under denaturating conditions (Laemmli and Favre, 1973). The proteins were then transferred to Immobilon-P (Millipore) membranes by electro-blotting. The membranes were blocked in 10% dried milk suspension in 1x TBS (50mM Tris -HCl, 2,7mM KCl, 137mM NaCl, pH 7.4) overnight at 4˚C and probed with an antibody specific against GFP derived from mouse. The secondary antibody was labeled with alkaline phosphatase and binding of the antibody was visualized using NBT and BZIP. The western blots were scanned and the GFP bands quantified to determine the total amount of GFP in each aliquot. To determine the active GFP present at the same time points, 10µl of the aliquots resuspended in H_2O were measured for the fluoresce-nce of the GFP at 430-580nm using an ultraviolet transilluminator. To calculate the translational fidelity, the amount of active GFP was compared to the total amount of GFP in each aliquot. The activity of GFP at the time point before the addition of Ksg was set to 100%. As the activity of the GFP molecule is sensitive to the incorporation of incorrect amino acids, the reduction of GFP activity compared to the total amount of GFP, determined by Western blot analysis, can be used as a measure for the decrease in translational fidelity (Dinos et al., 2006).

7.4 Pulse labelling

E. coli cells (MC4100 and MC4100 Δ*mazEF*) were grown at 37°C in M9 medium (minimal medium, 10µg/ml of each amino acid (Miller, 1972)) without methionin. At time point 0, when the OD_{600} reached 0.3, the cultures were divided and 1mg/ml of Ksg was added to one part. Samples for pulse labeling were taken at OD_{600} 0,7, which was 30 minutes after time point 0 in the case of the culture which did not receive the antibiotic, and approximately 2h in the case of the culture growing in the presence of Ksg. 1ml of culture was withdrawn and 5µl of methionine ^{35}S was added. The cells were incubated in a thermo-mixer at 37°C for 5 minutes. The reaction was stopped by addition of 100µl cold methionine (20mg/ml), and incubation in a shaker at 37°C for another 5 minutes. Then, the proteins were precipitated with 5% TCA on ice for 15 minutes, and pelleted by centrifugation for 15 min at 15000 rpm at 4°C. Upon resuspension in 1 x Laemmli buffer the samples were applied onto a 12% SDS-PAGE to separate the proteins. The gel was vacuum-dried, incubated on a phosphorImager screen and the labeled proteins were visualized using a Molecular Dynamics PhosphoImager.

7.5 2D Gel electrophoresis

To identify the proteins, which are translated in the presence of the antibiotic, total cellular protein extracts were analyzed upon pulse labeling by two-dimensional gel electrophoresis. Equal amounts of cell material were dissolved in lysis buffer (8 M urea, 4%, w}v, CHAPS and 40 mM Tris-base) and the cells were disrupted by repeated freezing in liquid N_2 and thawing at 37°C. For the first dimension the Immobiline Dry strip pH 3-10 (18 cm) (Amersham Pharmacia Biotech) was used with the following IEF program: 12 h rehydration, 1 h 500 V, 1 h 1000 V, 4 h 8000 V

(IPGphor isoelectric focusing system). Resolution in the second dimension was performed on 12% SDS polyacrylamide gels for 15 min at 10 mA and then for 4 h at 20 mA. Buffers and conditions were used according to the manufacturer's instructions. The gels were dried and then exposed to a Molecular Dynamics Phosphor Imager screen and analyzed with PDQuest software (Bio-Rad). For further analysis of the protein spots by Mass spectrometry, unlabelled proteins were separated likewise on a two-dimensional gel and the proteins were visualized by silver-staining as described (Blum et al. 1987).

7.6 Primer extension

The *E. coli* cells (MC4100, MC4100Δ*mazEF*) were grown in LB media until they reached an OD_{600} of 0.3. Then, the cultures were divided, and 1mg/ml Ksg was added to one half. The total RNA was purified from samples that were withdrawn at approximately OD_{600} 0.7 in case of the culture with no antibiotic added and 2h after the addition of Ksg in case of the Ksg-treated culture. Alternatively, MC4100 *relA1* cells harboring plasmid pSA1 carrying the IPTG-inducible *mazF* gene was used. These cells were grown in a minimal M9 medium up to an OD_{600} of 0,5, then expression of *mazF* was induced by addition of 1mM IPTG, and the cells were incubated for 15 minutes at 37°C without shaking. The RNA from all bacterial strains was extracted with the QUIAGEN RNeasy Kit according to the manufacturer's instructions.

For the primer labeling, 50pmol of primer (sequences Table x) was 5´-terminally labeled with γ-ATP using PNK (Promega) in a 30µl reaction. The mixture was incubated at 37°C for one hour, precipitated and resuspended in 22x hybridization buffer (0,45M KCl, 0,22M Hepes)

RNA	Sequence
dnaK	CGCTACACAAGAGTTGG
eno	GGGTTACCACGGGAG
clpP	CCGCGTGAGGTCTGTTCA
yfiD	GATGCAACGCGCTTCGC

Table 4: Primers used in the primer extension assays

2µl of labeled primer (~0,5µmol) were taken for each primer extension reaction, and mixed with 2.5µl of the total RNA (concentration ~1µg/µl). For primer annealing the mixture was heated to 90°C for one minute, and then cooled to 42°C using a thermomixer. Primer extension has been performed using the AMV reverse transcriptase (Promega) in a volume of 10µl. Buffer conditions were used according to the manufacturer's instructions. The reactions were incubated at 42°C for 30-45 minutes. To stop the reactions the same volume of loading dye (10M Urea, 2xTBE, traces of Bromphenolblue and Xylencyanol) was added to the reaction. Alternatively, the reaction was precipitated with ethanol, and then resuspended in 13µl AMV loading dye. The samples were heated to 95°C for 5 min prior to loading on a 8% polyacrylamide - 8M urea gel and the extension signals were visualized using the Molecular Dynamics Phosphoimager.

8. References

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