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# DISSERTATION

Titel der Dissertation

**The potential role of ribosome heterogeneity in regulation of protein synthesis in *Escherichia coli***

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## 2. SUMMARY

The rate limiting step in prokaryotic translation is the formation of the ternary translation initiation complex, comprising the 30S ribosomal subunit, the fMet-tRNA$_f^{Met}$, and mRNA. The efficiency of the translation initiation complex formation is modulated by the intrinsic features of the 5'-untranslated region (UTR). In contrast, leaderless mRNAs, which are translated in all three kingdom of life, lack ribosomal recruitment signals other than the 5'-terminal AUG-start codon. Previous studies in our laboratory have shown that leaderless mRNAs employ an alternative pathway for translation initiation, which involves 70S monosomes.

In this study I focused on the phenomenon of sustained translation of leaderless mRNAs in the presence of the antibiotic Kasugamycin (Ksg). I found that Ksg treatment induced the formation of stable 61S particles *in vivo*. Furthermore, my data demonstrated, that although the 61S particles were devoid of more than six proteins of the small subunit, including the functionally important proteins S1 and S12, they were still proficient in translation of leaderless mRNAs. Moreover, structural analysis of the 16S rRNA determined by primer extension analyses revealed that the lack of these ribosomal proteins in the small subunit could be attributed to structural changes induced by Ksg binding. These results provide *in vivo* evidence for the functionality of ribosomes devoid of multiple proteins and shed light on the evolutionary history of ribosomes.

The experiments performed in our laboratory revealed that after prolonged treatment with Ksg (up to 120 min) translation of about 5% of *E. coli* proteins was resumed compared to their blocked synthesis during the first 60 minutes of Ksg treatment. Therefore, in the second part of this study, we further studied this phenomenon and the data obtained revealed that a number of *E. coli* mRNAs became leaderless upon antibiotic treatment, which resulted in their selective translation in the presence of Ksg. Moreover, further analysis suggested that the appearance of conditional leaderless mRNAs upon Ksg treatment likely involved the *E. coli* RNA interferase MazF, the toxin component of the MazEF toxin-antitoxin system.

The formation of 70S monosomes *via* association of the 30S and 50S ribosomal subunits has been shown to be assisted by the initiation factor 2 (IF2). This suggests that IF2 can potentially stimulate formation of 61S particles by increasing the pool of 70S ribosomes, which are subsequently converted to 61S particles in the presence of Ksg.

Moreover, the GTP hydrolysis on IF2 induces structural rearrangements in the 30S subunit, which might result or induce the loss of the respective ribosomal proteins to form 61S particles. Therefore, in the third part of this study I investigated whether IF2 is also able to facilitate formation of 61S ribosomal particles during Ksg treatment. Our *in vivo* experiments were able to confirm this hypothesis. Moreover, our data revealed that Ksg treatment additionally leads to accumulation of truncated IF2 polypeptides. Further analysis indicated that formation of the truncated IF2 polypeptides could be attributed to translation of leaderless *infB* mRNA variants lacking 5'-coding regions of this transcript. Primer extension analysis showed that accumulation of leaderless *infB* mRNA variants occur in the wild-type *E. coli* strain treated with Ksg and is stimulated by MazF overexpression but does not occur in a *mazF* mutant lacking the functional RNA endoribonuclese (interferase) MazF. Therefore, our data suggest that truncated forms of the *infB* transcript are likely generated by means of *infB* mRNA processing by MazF during Ksg treatment. Taken together, these observations suggest a novel adaptation mechanism leading to formation of leaderless mRNAs selectively translated by modified ribosomes accumulating upon stress.

S1 is essential for translation in Gram-negative bacteria. It binds to the 30S ribosomal subunit through protein-protein interaction. Biochemical evidence showed the requirement of protein S2 for assembly of S1 to the ribosome. Therefore, in the fourth part of my study, I scrutinized the S1-S2 protein-protein interactions which might serve as a potential target for novel antimicrobial compounds acting selectively against Gram-negative pathogens. I used an NMR-based approach to study the interaction between protein S1 and S2. The results indicate that S1 and S2 can directly interact with each other. Furthermore, the truncated variants of S1, $S1_{106}$ and $S1_{194}$, bind to the ribosome in the same manner as the full-length S1 protein. Moreover, we show that overproduction of $S1_{106}$ inhibits bacterial growth. This effect could be attributed to the ability of this polypeptide to substitute endogenous S1, thus yielding translationally inactive ribosomes.

# 3. ZUSAMMENFASSUNG

Die Ausbildung des ternären Translationsinitiationskomplexes, bestehend aus der 30S Untereinheit des Ribosomes, der fMet-tRNA-fMet und der mRNA, ist der limitierende Schritt in der Proteinbiosynthese in Bakterien. Die Bildung des Initiationskomplexes kann durch verschiedene Merkmale der 5´-untranslatierten Region der mRNA moduliert werden. Diese Merkmale fehlen den sogenannten „leaderless“ mRNAs, die in Bakterien, Archaea und Eukaryoten vorhanden sind. Da diese mRNAs direkt mit dem 5´-terminalen Startkodon beginnen, weisen sie keine spezifischen Translationsinitiationssignale außer dem AUG Startkodon auf. Vorangegangene Studien in unserem Labor haben gezeigt, dass die Initiation an „leaderless“ mRNAs über einen alternativen Initiationsweg -über nicht-dissoziierte 70S Ribosomen- ablaufen kann.

In der vorliegenden Studie wurde die Translation von „leaderless“ mRNAs in Gegenwart des Antibiotikums Kasugamycin näher untersucht. Dieses Aminoglycosid hemmt die Initiation der Translation an kanonischen mRNAs. Im Gegensatz dazu, werden „leaderless“ mRNAs auch in Gegewart des Antibiotikums translatiert. Wir konnten zeigen, dass in Gegenwart des Antibiotikums protein-defiziente Ribosomen gebildet werden, denen mehr als sechs Proteine, darunter die essentiellen Proteine S1 und S21, fehlen. Diese ribosomalen 61S Partikel sind für die selektive Translationder „leaderless“ mRNA in Gegenwart von Kasugamycin verantwortlich. Weiters konnte ich durch Strukturanalysen das Fehlen der ribosomalen Proteine auf eine, durch die Bindung von Kasugamycin an 70S Ribosomen induzierte Konformationsänderung der 16S rRNA der 30S Untereinheit zurückführen. Zusammengefasst geben diese Ergebnisse einen ersten Hinweis auf die Funktionalität von protein-defizienten Ribosomen *in vivo* und eröffnen somit auch einen möglichen Einblick in die evolutionäre Entwicklung des Proteinbiosyntheseapparats.

Eine Funktion des Initiationsfaktor 2 ist die Stimulation der Assoziation der ribosomalen Untereinheiten. Eine erhöhte Konzentration des Faktors in der Zelle führt dadurch vermehrt zur Bildung von 70S Ribosomen in der Zelle. Dadurch könnte IF2 auch die Bildung von 61S Partikeln stimulieren. Zusätzlich kommt es durch die IF2-abhängige GTP-Hydrolyse zu einer Konformationsänderung in der 30S Untereinheit, die

einen zusätzlichen Effekt auf den Verlust der ribosomalen Proteine haben könnte. In der vorliegenden Arbeit konnte ich diese hypothetische Funktion von IF2 durch *in vivo* Studien bestätigen. Interessanterweise haben meine Ergebnisse zusätzlich gezeigt, dass es nach Inkubation mit Kasugamycin zur Akkumulierung von kürzeren IF2 Polypeptiden kommt. Nähere Untersuchungen dieses Phänomens wiesen darauf hin, dass die Ausbildung dieser kuzen Polypeptide nicht auf Proteolyse, sondern auf die selektive Translation von verkürzten, „leaderless" mRNAs zurückgefuhrt werden kann. Diese 5´-terminale Prozessierung der mRNA, die zur Entfernung eines Teiles der kodierenden Sequenz führt, ist abhängig von der Endoribonuclease MazF. MazF ist Teil eines Toxin-Antitoxin-Systems in *Escherichia coli*, das durch verschiedene Stressbedingungen, wie z.B. auch Inkubation mit einem Antibiotikum, induziert wird. Wir konnten zeigen, dass die MazF-Aktivität, die zur Degradierung der Mehrzahl der mRNAs führt, auch zur Ausbildung bestimmter „leaderless" mRNAs führt. Im Fall von *infB* konnten wir auch die Bildung von verkürzten mRNAs nachweisen, deren Translation zur Synthese von N-terminal verkürzten IF2 Polypeptiden führt. Zusammengefasst, weisen diese Ergebnisse auf einen möglichen Stressanpassungs-mechanismus hin, der die Proteinsynthese unter Stressbedingungen durch die Ausbildung von „leaderless" mRNAs, die durch protein-defiziente Ribosomen translatiert werden können, modifiziert.

Im letzten Teil dieser Arbeit habe ich die Bindung des ribosomalen Proteins S1 an die 30S Untereinheit näher untersucht. S1 bindet durch Protein-Protein-Interaktionen an das Ribosom. Da diese Interaktion nur für Gram-negative Bakterien essentiellen ist, könnte ein Molekül, das diese Bindung verhindert, eine selektive antimikrobielle Wirkung auf Gram-negative Bakterien haben. Vorrangegangene Studien in unserem Labor wiesen darauf hin, dass das ribosomale Protein S2 für die Bindung von S1 an das Ribosom notwendig ist. Daher war ein Ziel dieser Arbeit die nähere Charakterisierung der Interaktion zwischen den Proteinen S1-S2. Mit Hilfe einer NMR-basierten Methode konnte ich die direkte Protein-Protein-Interaktion nachweisen. Weiters haben Studien mit verkürzten Protein S1 Varianten gezeigt, dass die N-terminale Domäne für diese Bindung verantwortlich ist, und dass Überexpression dieser Dömäne das Wachstum von *Escherichia coli* hemmt, indem es die Bindung des nativen Proteins S1 an das Ribosom hemmt. Zusammengefasst, unterstützen diese Ergebnisse die Hypothese, dass die S1/S2 Interaktion ein potentielles Angriffsziel für die Entwicklung neuer semi-selektiver antimikrobieller Wirkstoffe darstellt.

# 4. INTRODUCTION

## 4.1. Protein synthesis in the prokaryotic cell

Bacteria have evolved numerous mechanisms to rapidly adapt their growth in response to environmental changes. Many of these mechanisms are tightly controlled at the level of transcription and translation. In the process of transcription, RNA copies of selected DNA sequences are generated, which are translated into the corresponding sequence of amino acid residues covalently linked to each other by a multicomponent ribonucleoprotein complex, the ribosome. As transcription and translation are coupled in bacteria, ribosomes can initiate translation already on a nascent mRNA. Moreover, assembly of transcripts into polyribosomes enables ribosomes to limit the access of endoribonucleases to the translated mRNA by masking potential cleavage sites, and therefore to protect mRNA from degradation (Richards et al., 2008).

As the assembly of the translation initiation complex can take seconds, while the growth of the polypeptide chain (elongation) can occur at the rate of 10 to 20 amino acids per second (Dennis and Bremer, 1974; Rodnina et al., 2007; Wilson and Nierhaus, 2003; Wintermeyer et al., 2004) and with high accuracy (Lovmar and Ehrenberg, 2006), the initiation of translation is believed to be the rate-limiting step of translation (Gualerzi and Pon, 1990).

In addition to ribosomes and mRNAs, the process of translation is dependent on protein factors and transfer RNAs (**tRNAs**).The latter carry activated amino acids to the catalytic site of ribosomes. The charged tRNAs (i.e. tRNAs with attached amino acids) are used by ribosomes to recognize specific codons in the translated mRNA, thereby ensuring that the specific sequence of each mRNA directs the incorporation of the corresponding amino acid residue in the polypeptide chain in accordance with the genetic code. The following parts of this chapter primarily focus on molecular features of protein synthesis in prokaryotes and outline some general principles and mechanisms common for the process of translation and functioning of the protein-synthesizing machinery in all organisms.

## 4.2. The structure of the prokaryotic ribosome

Protein synthesis is performed by ribosomes, large ribonucleoprotein complexes consisting of three non-coding RNA molecules, in prokaryotes namely the 16S, 23S and 5S ribosomal RNAs (**rRNA**s), and more than 50 ribosomal proteins (**r-proteins**). Due to the

universal mechanism of translation, r-proteins and rRNAs are highly conserved across all three kingdoms of life, i.e. archaea, prokarya and eukarya.

In recent years, the structure of the ribosome and its components has been examined at the atomic level. In the year of 2000, remarkable accomplishments in ribosome research were revolutionized by atomic resolution crystal structures. These include the small subunit from eubacteria *Thermus thermophilus*, T30S (Schluenzen et al., 2000; Wimberly et al., 2000) and the large ribosomal subunit of the archaeon *Haloarcula marismortui*, H50S (Ban et al., 2000). The studies of the crystal structures of ribosome were extensively reviewed (Moore and Steitz, 2005; Ogle and Ramakrishnan, 2005; Yonath, 2005) and have being rewarded with Nobel Prize for chemistry 2009. Most of the available ribosomal structures are from the organisms that have adapted to extreme environments as these are more suitable for crystallization (additional crystal structures are summarized in Table 1).

**Table 1. Studies on crystal structures of the ribosome.**

| Ribosome complexes | | Organism | References |
|---|---|---|---|
| 50S | | *Haloarcula marismortui* | (Ban et al., 2000) |
| | | *Deinococcus radiodurans* | (Harms et al., 2001) |
| 30S | | *Thermus thermophilus* | (Wimberly et al., 2000) (Schluenzen et al., 2000) |
| 70S | | *Thermus thermophilus* | (Yusupova et al., 2001) |
| | | *Escherichia coli* | (Schuwirth et al., 2005) |
| 70S | mRNA and tRNA | *Thermus thermophilus* | (Selmer et al., 2006) |
| | tRNA | *Thermus thermophilus* | (Korostelev et al., 2006) |
| | mRNA | *Thermus thermophilus* | (Yusupova et al., 2006) |
| | | | (Jenner et al., 2007; Jenner et al., 2005) |
| 70S | Release factor 2 | *Thermus thermophilus* | (Weixlbaumer et al., 2008) |
| | Ribosome Recycling factor | *Escherichia coli* and *T. thermophilus*) | (Pai et al., 2008) |
| | Release factors RF1 And RF2 | *Thermus thermophilus* | (Petry et al., 2005) |
| 30S | mRNA, SD interaction | *Thermus thermophilus* | (Kaminishi et al., 2007) |
| | tRNAs with an expanded anticodon loop | *Thermus thermophilus* | (Dunham et al., 2007) |
| | tRNAs | Thermus thermophilus | (Weixlbaumer et al., 2007) |
| 70S | Substrate analogs | *Haloarcula marismortui* | (Nissen et al., 2000) |
| | | *Deinococcus radiodurans* | (Bashan et al., 2003) |
| | | *Haloarcula marismortui* | (Schmeing et al., 2005a; Schmeing et al., 2005b) |
| 50S | Trigger factors(N-terminal) | *Deinococcus radiodurans* | (Baram et al., 2005) |
| | | | (Schlunzen et al., 2005) |
| | Trigger factors (*E. coli*) | *Haloarcula marismortui* | (Ferbitz et al., 2004) |
| | Domain I of ribosome recycling factor RRF (*E. coli*) | *Deinococcus radiodurans* | (Wilson et al., 2005) |

One of the most significant conclusions from these studies is that the ribosome is a ribozyme (Ban et al., 2000) (Steitz and Moore, 2003) (Cech, 2000), as the catalytic function of this ribonucleoprotein complex, peptide transfer and peptide release are both catalyzed by the peptidyl-transfer centre (**PTC**), which consists solely of the 23S rRNA in the 50S subunit (Petry et al., 2005; Trobro and Aqvist, 2005).

The bacterial ribosome has a mass of approximately 2.6-2.8 MDa and a diameter of 200-250 angstrom (**Å**) and possesses a relative sedimentation constant of 70S. It is composed of two smaller particles, the 50S and 30S subunits. The *E. coli* 50S ribosomal subunit with a mass of 1.5 MDa consists of the 5S rRNA (120 nucleotides (nt) in length), the 23S rRNA (2,904 nt in length) and 33 r-proteins. The 30S ribosomal subunit with a mass of 0.8 MDa, consists of 16S rRNA (1542 nt in length) and 21 r-proteins (Moore and Steitz, 2002; Ramakrishnan and Moore, 2001). Both rRNAs and r-proteins are required for optimal ribosome structure and function.

Cryo-electron microscopic (cryo-EM) and X-ray maps of 70S ribosomes show a number of highly conserved bridges connecting the subunits (Cate et al., 1999; Frank et al., 1995; Gabashvili et al., 1999). Initially, two tRNA binding sites were mapped on the ribosome: the **A site** named after its function to bind incoming aminoacyl-tRNAs and the **P site** hosting peptidyl-tRNA molecules. At the early 80's, a third tRNA binding site has been also discovered (Grajevskaja et al., 1982; Lill et al., 1984; Rheinberger et al., 1981), the **E site** (E stands for the "exit"), from which the deacylated tRNA leaves the ribosome.

The shape of the 30S subunit is greatly dependent on the folding of 16S rRNA, which has four structural domains (Wimberly et al., 2000). The tertiary structure of this rRNA is stabilized by $Mg^{2+}$ bridges, RNA-RNA and RNA-protein interactions (Noller and Woese, 1981). Recent studies revealed that RNA-protein interactions in the ribosome often engage the sugar-phosphate backbone of rRNAs. Therefore, these interactions are sequence-independent and mainly rely on specific structural motifs within rRNAs (Ban et al., 2000; Leontis and Westhof, 1998). Interestingly, many of the ribosomal proteins have highly conserved non-globular extensions. These extra sequences (containing approximately 26% arginine and lysine residues) were proposed to penetrate into the ribosome to fill the gaps between RNA helices (Ban et al., 2000; Steitz and Moore, 2003). Based on its shape, the structure of the 30S subunit has been subdivided into the **head**, **body** and the connecting neck structure that includes a **shoulder** and **platform**. The lower part of the body has a protrusion called the **spur**. The front portion of 30S subunit faces the 50S subunit, whereas its back is exposed to the solvent (Fig.1).

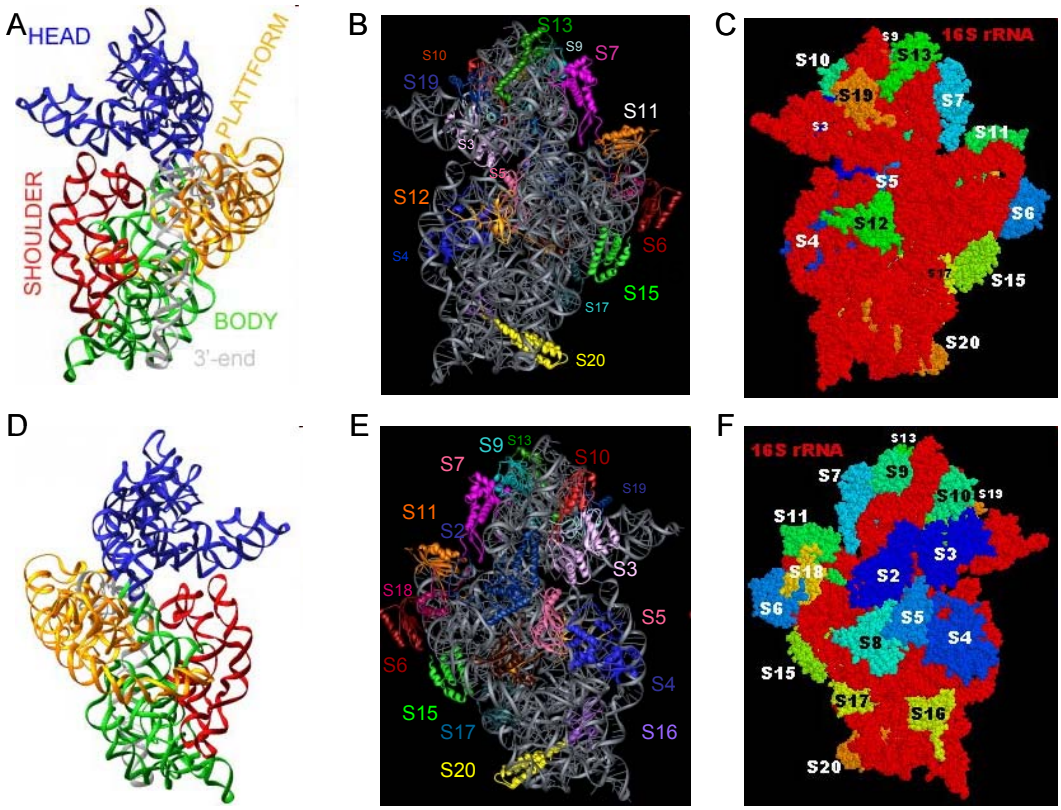


***Figure 1. The structure of the 30S ribosomal subunit from* Thermus thermophilus *shown from different views.*** The distribution of the 16S rRNA domains (panel A and D) and r-proteins (panels B, C, E and F) within the 30S ribosome. (A, B and C) Views from the front facing the 50S subunit. The **Head** and **Body** of the 30S subunit are connected through a single rRNA-helix (H28), which permits small independent movements. The small gap between the **Head** and the **Shoulder** serves as a kind of clamp, which allows for fix the position of the mRNA during the decoding process. (D, E and F) Views from the back facing the solvent (taken from Schluenzen at al., 2000).

The 30S subunit is known to mediate codon-anticodon interactions (i.e. involved in the process of decoding) between mRNAs and tRNA. The **decoding centre** is located in the upper part of the body and in the lower part of the head of the 30S subunit and is nearly entirely composed of 16S rRNA (Schluenzen et al., 2000). It is part of the ribosomal A site and is situated at the end of the 16S rRNA helix 44 which monitors the codon-anticodon pairing after the aminoacylated tRNA has been placed in the A-site. Several conformational changes within the 16S rRNA were found to play an active role in tRNA selection by enabling interaction of the 30S subunit with the first and second base pairs of the codon-anticodon helix. When the mRNA codon and the tRNA anticodon fit cognately, their ribose-phosphate backbone favours hydrogen bonding with A1492 and 1493 causing their flipping out of the helix 44. An additional conformational change in G530 of the 16S rRNA is induced by tRNA

binding to the A site, which in turn supports the flipped-out conformation of A1492 and A1493 in 30S subunit (Ogle et al., 2003; Ogle et al., 2002). These conformational changes ensure the precise codon-anticodon interaction, which triggers peptide bond formation and leads to GTP hydrolysis by elongation factor (**EF**) EF-Tu about 75 Å away. In addition, crystal structures of the intact *E. coli* 70S ribosome also indicate a movement of the small subunit body during the decoding step (Vila-Sanjurjo et al., 2003). Even though the decoding centre is primarily composed of rRNA, a number of studies have demonstrated that tRNA selection is also assisted by several r-proteins of the 30S subunit (i.e. S4, S5 and S12) (Ogle and Ramakrishnan, 2005) and by r-protein L9 belonging to the 50S subunit (Sharma et al., 2007).

Likewise, the movement of the mRNA through the ribosome is assisted by r-proteins. The entrance of the mRNA tunnel is formed by r-proteins S3, S4 and S5, referred to as the **helicase centre**, and the exit of the tunnel, which is flanked by r-proteins S7, S11 and S18, known as the **platform centre**. The r-proteins S3, S4 and S5 form a ring structure downstream of the A site between the head and shoulder of the 30S subunit and S3 and S4 are able to unwind the structured mRNA during translation elongation (Kurkcuoglu et al., 2008; Noller et al., 2001). The platform r-proteins S7, S11 and S18 are involved in assuring translation fidelity by constraining the mRNA at the mRNA exit channel together with S2, thus making the platform centre responsible for the binding and docking of the structured mRNA during translation initiation (Jenner et al., 2005; Marzi et al., 2007) (Fig. 2). In the tunnel, the mRNA also interacts with the 3‘ end of the 16S rRNA to form the Shine-Dalgarno (SD) / anti-SD duplex during the initiation of translation (Kurkcuoglu et al., 2008).

Six secondary-structure domains of the 23S rRNA form a monolithic structure in the 50S subunit, whereas the 5S rRNA is considered as the separate seventh RNA domain of the subunit (Steitz and Moore, 2003). Surprisingly, the active site of the 50S subunit (also known as the peptidyl-transferase centre) is completely devoid of protein moiety, namely, it is composed of rRNA similar to several functional regions in the 30S subunit. The structure of the 50S subunit is defined by three protuberances: the **L1 protuberance** (**L1 stalk**), the **central protuberance** (**CP**), and the **L7/L12 stalk** (Harms et al., 2001; Mueller et al., 2000; Penczek et al., 1999; Wilson and Nierhaus, 2003) (Fig. 3). The L1 stalk includes helices H75-H78 and protein L1. The L1 stalk interacts with the elbow of the E site bound tRNA (**E-tRNA**) and together with r-protein S7 blocks the exit path of the E-tRNA (Yusupov et al., 2001). The movement of one or both of these structural elements can release the deacylated tRNA from the ribosome. The L7/L12 stalk consisting of H42-H44 and protein L7/L12 and L10 is bound to the ribosome through r-protein L11 (Diaconu et al., 2005). The C-terminal domain of proteins L7/L12 have been shown to interact with translational factors, like initiation factor (**IF**) IF2, EF-Tu, EF-G and release factor (**RF**) RF3 (Helgstrand et al., 2007), and to be involved in factor-dependent GTPase activity (Chandra Sanyal and Liljas, 2000). The N-

terminus of protein L7/L12 is responsible for its dimerisation and binding to the ribosome by interacting with r-protein L10 (Bocharov et al., 2004).

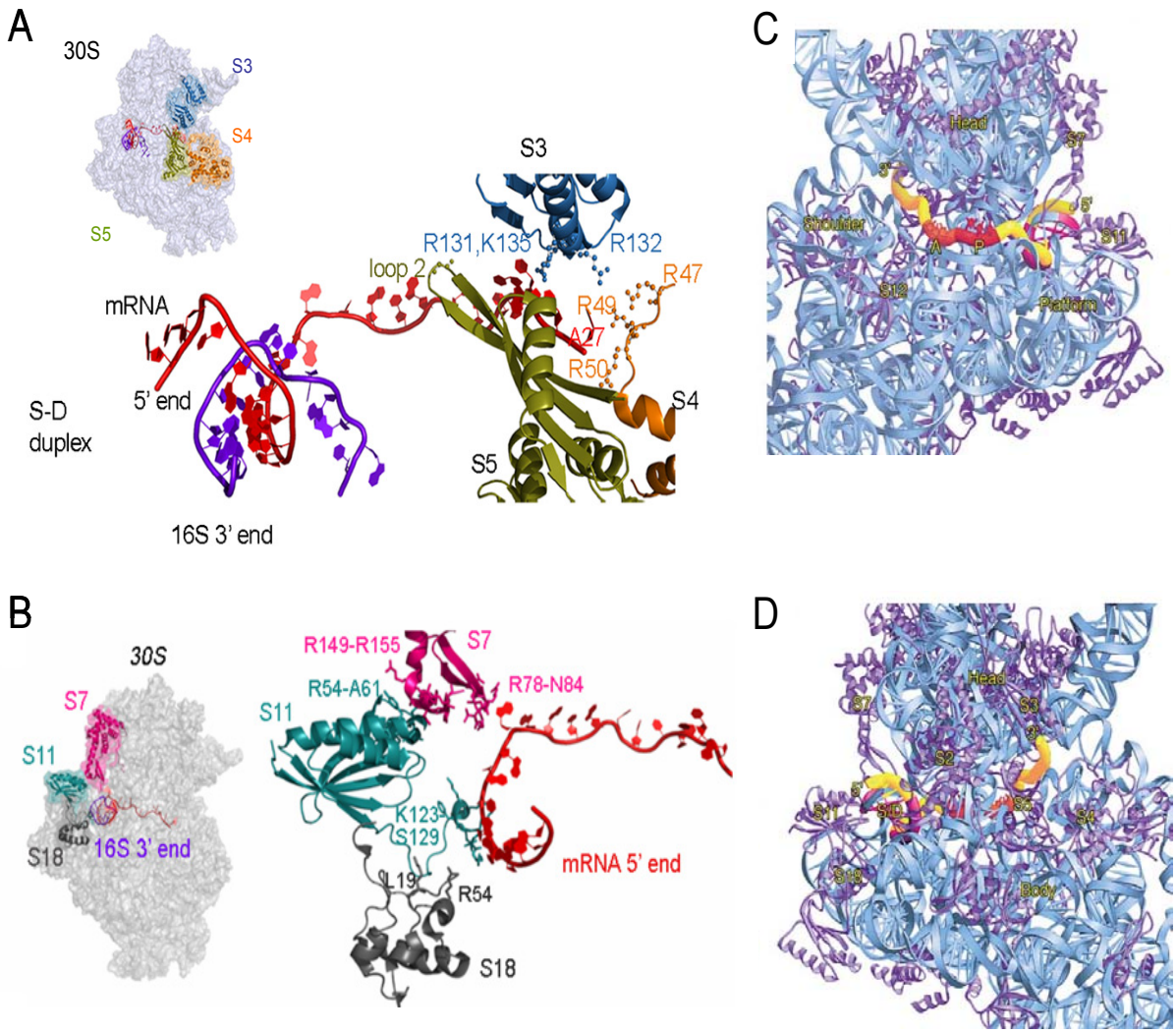


***Figure 2. The mRNA track in the 30S ribosomal subunit.*** *(A)* The mRNA entrance tunnel and the helicase centre. The mRNA (red) 3' end surrounded by ribosomal proteins S3 (blue), S4 (orange) and S5 (green) and its 5' end interacting with the 16S 3' end (purple). Residues reported to be important for the helicase activity and the translation fidelity of the ribosome, are shown on proteins S3, S4 and S5. (B) The 5´ end of the mRNA (red) is surrounded by ribosomal proteins S7 (magenta), S11 (turquoise) and S18 (gray) at the exit channel, the platfoem centre, shown in two views. The important residues reported in various studies are shown on the right in a stick form (taken from Kurkcuoglu et al., 2008). (C) Interface and (D) solvent side views of the mRNA in the 30S ribosomal subunit. A, P; the A- and P-site codons. 5´, 3´; the 5´ and 3´ correspond to positions -15 and +15 of the mRNA model. The head, platform, shoulder, and body of the subunit, and ribosomal proteins S2, S3, S4, S5, S7, S11, S12, and S18 are indicated. The ribosomal proteins are shown in dark blue, 16S rRNA in cyan and the mRNA is depicted in yellow, the Shine-Dalgarno (SD) helix is shown in magenta and the A- and P-site codons are highlighted in orange and red, respectively (taken from Yusupov et al., 2001).

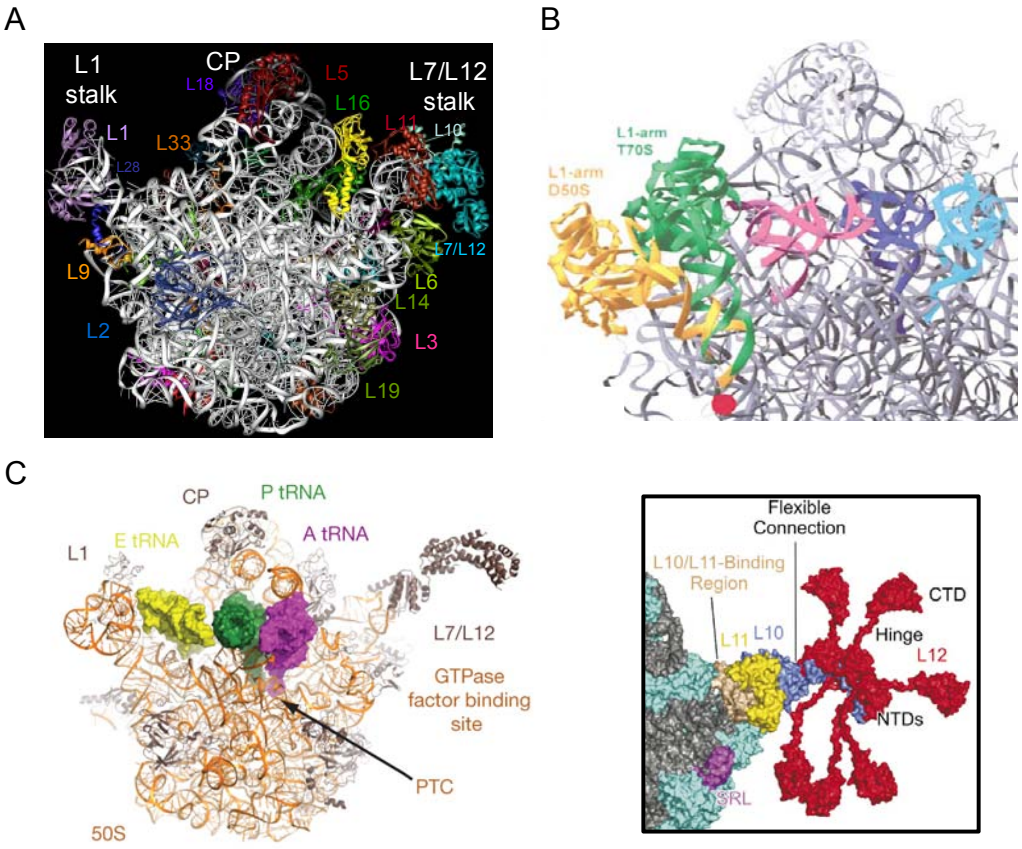


***FIGURE 3. The 50S ribosome subunit. (A) The crown view representation of the 50S structure from Deinococcus radiodurans (D50S), shown from the side facing the small subunit within the 70S ribosome.*** The RNA chains are shown as silver ribbons and the protein main chains in different colors. The L7/L12 stalk is on the right, the L1 stalk is on the left and the central protuberance (CP), and including the 5S RNA is in the middle of the upper part of the particle. The semitransparent proteins are less well resolved. **(B) A possible movement about L1 stalk.** Part of the 50S structure (D50S, as gray ribbons). The L1-arm of D50S is highlighted (in gold). Also shown is the L1-stalk of 70S from Thermus thermophilus (T70S, green) and protein L1 of T70S (dark green) and the potential location of protein L1 in D50S (yellow-gold). In T70S, the L1-arm and protein L1 block the exit of the E-tRNA (magenta), whereas in D50S, the L1-arm swings around a pivot point (marked by a red dot) by around 30° (taken and modified from Harms J. et al.,2001(Harms et al., 2001). (C) **Exploded view of the 50S subunit.** The structure of the L7/L12 arm (Diaconu et al., 2005) was fit onto the 70S ribosome (Voorhees et al., 2009) (taken from Schmeing and Ramakrishnan, 2009). In the left, presenting the model of the L7/L12 stalk based on cryo-EM and X-ray crystal structures, viewed from the interface side of the 50S subunit. Shown are the L10/L11-binding region (beige), L11 (yellow), L10 (blue), and the N-terminal domains (NTDs) and C-terminal domains (CTDs) of L12 (red). The locations of the flexible connection and hinge regions are also shown (taken from Berk and Cate, 2007).

Likewise, the interface between 50S and 30S subunits, and especially the mRNA and tRNAs binding domains, are formed by rRNAs, again pointing to the ribozyme nature of the protein-synthesizing machinery. An approximately 100 Å-long tunnel (corresponding to a polypeptide length of 30-40 amino acids) starts at the PTC at the interface cavity and

extends to the P-site, which allows the nascent polypeptide to exit at the base side of the 50S subunit, thus facing the cytoplasm (Ban et al., 2000; Voss et al., 2006). The interior surface of the tunnel is highly irregular in shape and in the upper portion (the one closest to the subunit interface) is mainly composed of rRNA. The narrowest portion occupying the last one-third of the tunnel is made of r-protein L4 and L22 merged with rRNA. This part of the tunnel acts as a sensor for metabolic control by pausing the synthesis of certain peptides (Gong and Yanofsky, 2002; Nakatogawa and Ito, 2004; Tenson and Ehrenberg, 2002). In addition to guiding the nascent polypeptide chain and protecting it from proteases, the tunnel is also a place where early steps of protein folding occur (Basu et al., 2008; Das et al., 1992; Noller, 1991).

## *4.3. Protein synthesis in bacteria*

The process of protein synthesis is conventionally divided into three consecutive phases (Fig. 4): initiation, elongation and termination. Each stage is assisted by translational factors of initiation (IFs), elongation (EFs) and termination (TF) and recycling (RFs), respectively. Briefly, after IF3 and IF1 bind to the 30S subunit, mRNA and formyl-methionine charged initiator tRNA (**fMet-tRNA$_f^{Met}$**) together with IF2·GTP join to form the pre-initiation 30S initiation complex (**30SIC**). This process occurs in a highly cooperative manner(Antoun et al., 2006; Celano et al., 1988; Wintermeyer and Gualerzi, 1983), but in a random order (Gualerzi and Pon, 1990). The initiator tRNA binding at the P-site is facilitated by IF2 and by the codon-anticodon interaction with mRNA. Following the 50S subunit docking to the 30SIC, which is also assisted by IFs, the 70S initiation complex (**70SIC**) is formed (Antoun et al., 2006). In the elongation phase, aminoacyl-tRNAs enter the A site and the process is assisted by EF-Tu. When the cognate codon-anticodon interaction takes place, the EF-Tu-bound GTP is rapidly hydrolyzed enabling aminoacyl-tRNA accommodation in the A site, followed by peptide bond formation catalyzed in the PTC of the 50S subunit (Rodnina et al., 2007). As a result, the peptidyl-tRNA in the A site acquires nascent peptide chain elongated by one amino acid and the deacylated tRNA resides in the P site. In the next step, EF-G binds to the A-site bound peptidyl-tRNA and induces the translocation of the A site bound tRNA to the P site and the P-site bound tRNA to the E site, which corresponds to a single forward move of the mRNA by one codon in its reading frame (Frank et al., 2007). Ribosomes consequently move along the mRNA to produce the polypeptide chain until a stop codon of mRNA enters the A-site. This serves as a signal for translation termination assisted by release factors 1, 2 or 3 (RF1, RF2 and RF3) and finally leads to peptide release. The stop codons UAA and UAG are recognized exclusively by RF1 and codons UAA and UGA by RF2. After the ester bond linking the polypeptide with the P site bound tRNA is hydrolyzed (Kisselev and

Buckingham, 2000), RF1 and RF2 dissociate from the ribosome in the presence of RF3 in a GTP-dependent manner (Zavialov et al., 2002). Subsequently, the ribosome dissociates into subunits, which is assisted by RRF, EF-G and IF3(Karimi et al., 1999; Petrelli et al., 2001). This mechanism is likewise used by bacteria to reactivate idled ribosome during recovery from stress or slow-growth conditions(Janosi et al., 1998; Wilson and Nechifor, 2004).

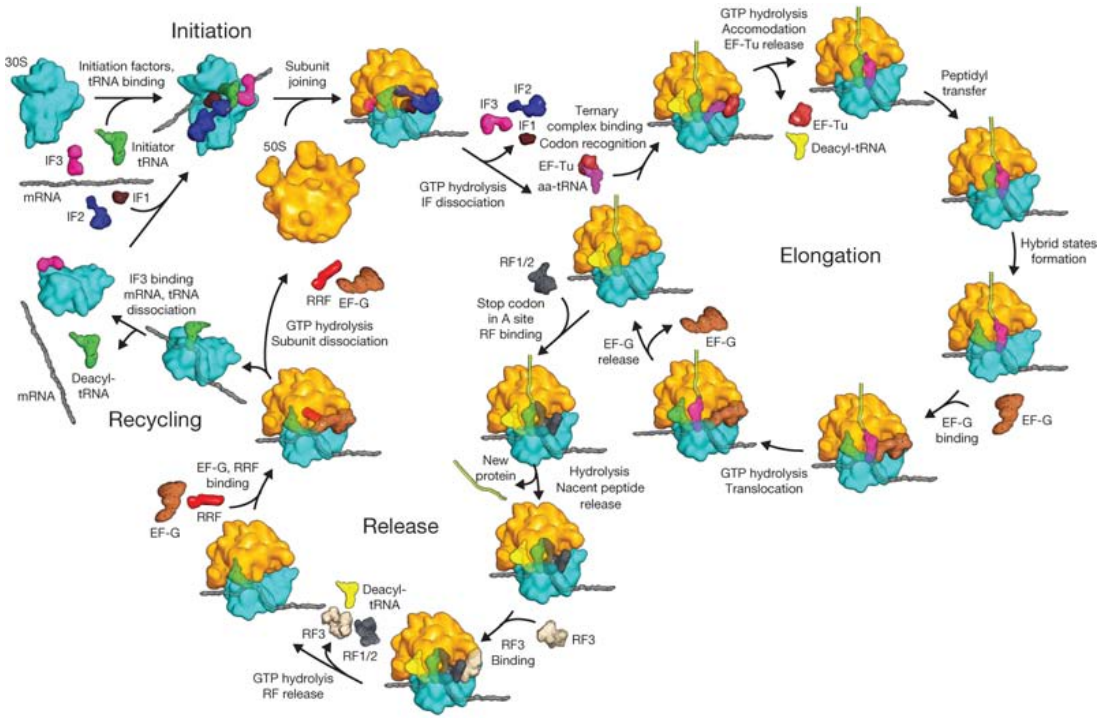


***Figure 4. Schematic view of the protein translation process.*** Translation initiation, elongation and termination (release and recycling) are depicted. 30S and 50S subunits are highlighted in cyan and gold color, respectively. mRNA, tRNAs, and translational factors are shown. Adopted from Schmeing and Ramakrishnan, 2009 (Schmeing and Ramakrishnan, 2009).

## *4.4. Initiation of protein synthesis in bacteria*

Translation initiation, which is the rate-limiting step of protein synthesis, is a sequence of events that leads to the assembly of the initiation complex, which consists of the ribosomal subunits, ancillary protein factors, the mRNA and the initiator tRNA positioned in the P-site (Fig. 5). In contrast to elongation and termination, the initiation of translation significantly

differs in prokarya, archaea and eukarya (Pestova and Hellen, 2000; Roll-Mecak et al., 2001).

In eukarya, translation initiation is dependent on the “cap” structure located at the 5’-end of eukaryotic transcripts. The start codon is identified by a scanning mechanism, where the small ribosomal subunit loaded with the tRNA binds to the 5’ end of the mRNA and scans the 5’ untranslated region to identify an AUG start codon. This process is of higher complexity and involves more than 12 initiation factors (Lopez-Lastra et al., 2005; Pestova and Hellen, 2000; Sonenberg and Hinnebusch, 2009).

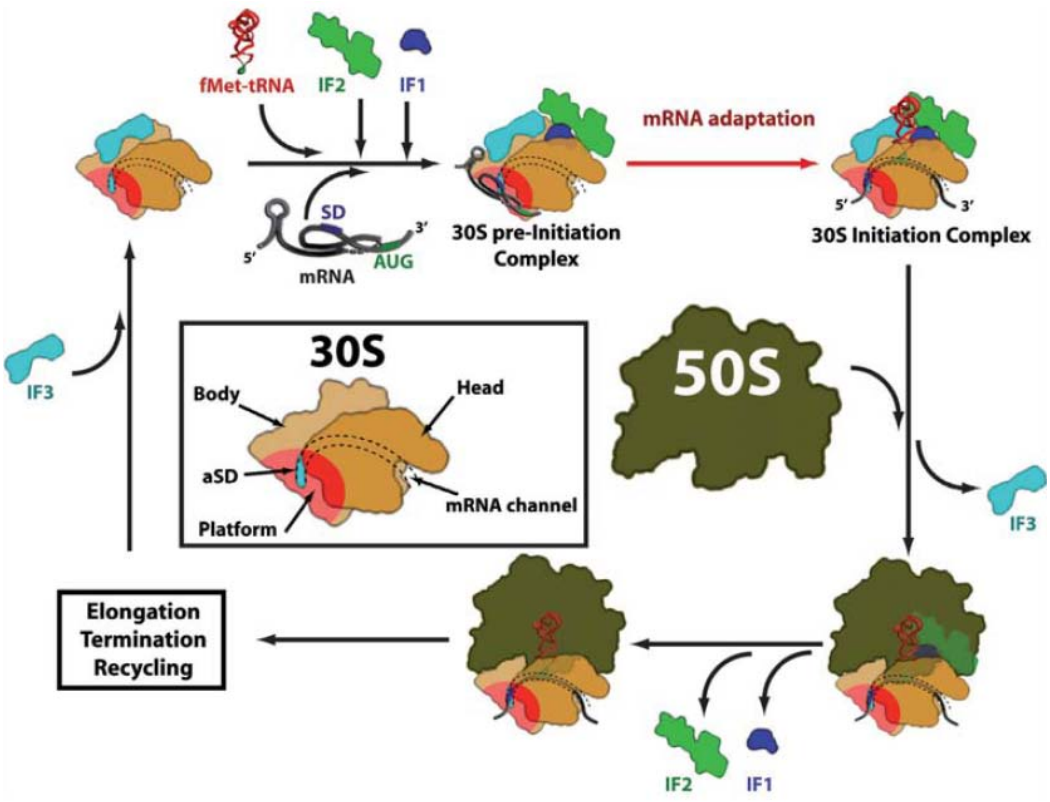


***Figure 5. Schematic view of the initiation process.*** Formation of 30S (30SIC) and 70S (70SIC) translation initiation complexes, containing ribosomes (30S subunit in orange, 50S in brown), initiator fMet-tRNA$^{fMet}$, mRNA, initiation factors IF1 (in blue), IF2 (in green) and IF3 (in light blue). View of 30S ribosomal subunit and ribosome from the top. The platform of the 30S is in red with the anti-Shine-Dalgarno (aSD) sequence in cyan. Structured mRNA binds to 30S in two distinct steps: the docking of the mRNA on the platform of the 30S subunit forms the pre-initiation complex that is followed by the accommodation of the mRNA into the normal path to promote the codonanticodon interaction in the P site (Marzi et al., 2007). The resulting 30SIC engages the 50S subunit to form the 70SIC from which the initiation factors are expelled and the synthesis of the encoded protein can proceed through the elongation, termination and ribosome recycling phases (Marzi et al., 2008) (taken from Simonetti et al. 2009).

In contrast, translation initiation in prokaryotes requires only three initiation factors, namely IF1, IF2 and IF3, which assist the formation of an intermediate initiation complex consisting of mRNA, initiator tRNA and the 30S ribosomal subunit. In bacteria, mRNA recognition involves base-pairing of the **Shine-Dalgarno (SD)** sequence located upstream of the start codon on the mRNA with the **anti-Shine-Dalgarno** (**anti-SD**) sequence located at the 3'-end of the 16S rRNA (Gold et al., 1981). Hence, modulating the accessibility of the SD sequence is one of the main mechanisms to regulate translation. Furthermore, the strong interaction between initiator tRNA and IF2 is another feature specific to prokaryotes. Therefore, both, mRNA and IFs, play an important role to ensure the fidelity of initiation. The chronology of the molecular events during translation initiation as well as the exact function of the IFs is still controversial (Laursen et al., 2005; Simonetti et al., 2009).

### *4.4.1. Initiation factor 1*

*E. coli* initiation factor 1 (IF1) (encoded by the *infA* gene) is the smallest translation initiation factors (Mw ~ 8.2 kDa) in bacteria. The solution structure of *E. coli* IF1 has been determined by NMR (Sette et al., 1999). The protein is composed of a rigid five-stranded β-barrel flanked by flexible and disordered sequences. Similar five-stranded β-barrel folds are also found in ribosomal proteins (r-proteins) S1, S17, L2 and L7 (Ramakrishnan and White, 1998), the cold shock proteins CspA and CspB (Bycroft et al., 1997), the aspartyl-tRNA synthetase (Ruff et al., 1991), eIF2α (Nonato et al., 2002) and eIF5A (Kim et al., 1998).

Recent analysis of the crystal structure of the complex of IF1 with the 30S subunit have shown that IF1 binds to the 30S subunit in proximity to the A site, in a niche created by ribosomal protein S12 and the penultimate stem loop of 16S rRNA (Carter et al., 2001). IF1 interacts with two adenines (A1492 and A1493) of helix 44 in the 16S rRNA and forces these two nucleotides to flip out, thus altering the 30S subunit conformation and affecting the association-dissociation equilibrium of initiator tRNAs in the P site (Carter et al., 2001). Furthermore, IF1 binding to 30S subunit was shown to prevent the initiator tRNA binding to the A site and promotes tRNA binding to the P site (Gualerzi et al., 1989; Milon et al., 2008) and stimulates the activities of IF2 and IF3 by promoting their binding to the 30S subunit (Pon and Gualerzi, 1984; Wintermeyer and Gualerzi, 1983). In contrast to the results of cross-linking experiments (Boileau et al., 1983), the structural data obtained for the 30S initiation complex containing IF1 and IF2 revealed that there is no direct interaction between IF1 and IF2 (Fig 6) (Simonetti et al., 2008).

### 4.4.2. Initiation factor 2

IF2 is the only initiation factor that remains attached to ribosomes throughout the entire translation initiation stage (Boelens and Gualerzi, 2002; Laursen et al., 2005). Therefore, specific interaction of IF2 with the initiator tRNA is the most important step during translation initiation.

The *E. coli* IF2 is composed of six domains (Domains I-VI) (Mortensen et al. 1998), as shown in Figure 7. The N-terminal domain (**NTD**) consists of three sub-domains (Domains I–III) (Steffensen et al. 1997; Sørensen et al. 2001). NMR results show that the first 50 residues form a compact globular domain that is present in most bacterial and plastid IF2 proteins (Laursen et al. 2003). Domains II and III are not conserved in sequence or length among bacteria. The residues 51–97 are structurally disordered, whereas residues 98–157 form a helix containing a repetitive sequence of mainly hydrophilic amino acids (Laursen et al. 2003). Domain II has been shown to interact with the ribosome (Moreno et al., 1999; Moreno et al., 1998), Domains I and II interact with the *infB* mRNA (Laursen et al. 2002b). The conserved C-terminal region (CTD) of IF2 consists of domains IV, V and VI. (Roll-Mecak et al., 2000; Spurio et al., 2000) which are involved in binding and hydrolysis of GTP, as well as binding of initiator tRNA. Amino acid sequence homology predictions reveal similar structures for domains IV–VI for bacterial IF2 and aIF5B from the archaea *Methanobacterium thermoautotrophicum*, whose structure has been solved by X-ray analysis (Roll-Mecak et al., 2000). However, it is interesting, that IF2 homologues from many extremophilic species such as *Thermus* (Bacteria), or *Sulfolobus* and *Methanococcus* (Archaea) genera lack the NTD region (Moreno et al., 2000) and contain only CTD domains. The *E. coli infB* gene encodes three isoforms of IF2, termed IF2-α (97.3 KDa), IF2-β (79.7 KDa) and IF2-γ (78.8 KDa) (Laursen et al., 2002; Nyengaard et al., 1991).

Translation of smaller isoforms (IF2-β and IF2-γ) starts from independent (but in-frame) alternative initiation codons that are just upstream of the sequence coding for domain II. Although under normal growth conditions, the cellular levels of all three isoforms are similar (Howe and Hershey, 1983; Sacerdot et al., 1992), the abundance of the smaller isoforms (IF2-β and IF2-γ) is higher under cold shock conditions (Giuliodori et al., 2004).

Similar to the elongation factors EF-Tu, EF-G and termination factor RF3, IF2 belongs to the family of the GTP/GDP-binding proteins (Gavini and Pulakat, 1991). IF2-GTP binding to the 30S subunit promotes initiator tRNA binding and stimulates the interaction of the ternary complex (30S-tRNA-mRNA) with the 50S subunit. This process is accompanied by conformational changes of IF2 and the entire ribosome (Antoun et al., 2003; Luchin et al., 1999; Severini et al., 1990). After formation of the 70S initiation complex (70SIC), GTP hydrolysis occurs and the low-affinity IF2-GDP complex dissociates from the ribosome.

A recent study shows that ppGpp binds to IF2 at the GTP binding site, thereby preventing IF2 dependent formation of the 30SIC and thus inhibits translation initiation. The authors further propose that IF2 serves as a metabolic sensor, which coordinates protein synthesis with stress condition in a ppGpp dependent manner (Milon et al., 2006).

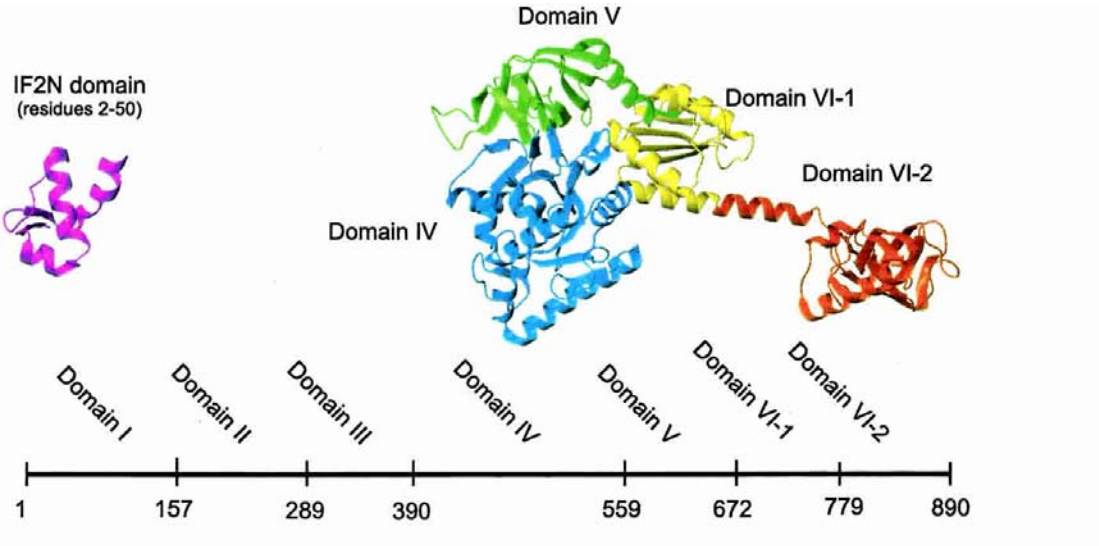


***Figure 6. Schematic representation of IF2 from E. coli.*** (Top) A representation of the structure of IF2 from *E. coli* with the domains indicated in different colors. The cartoon models of the regions of known structure are derived from PDB entry 1ND9 for the N-terminal IF2N domain and PDB entry 1G7T for the C-terminal region. The cartoons were prepared using the program MOLMOL (Koradi et al. 1996) taken from Laursen (2003).

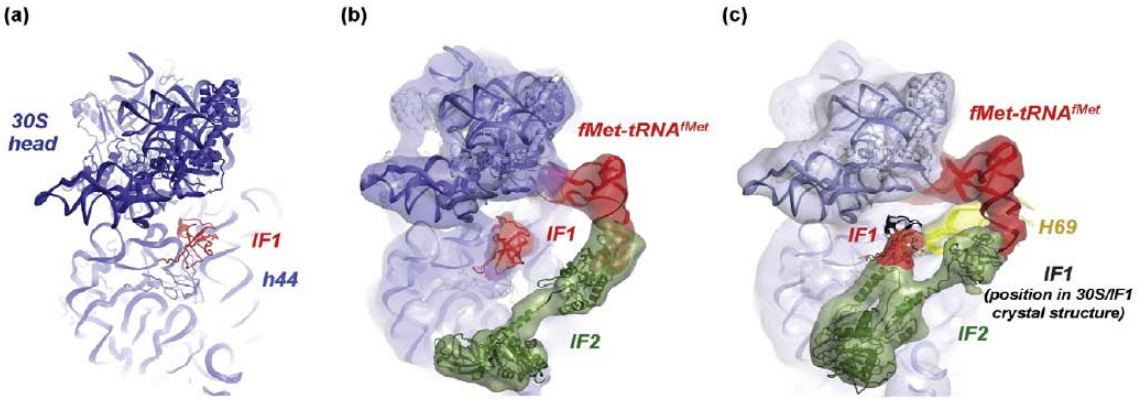


***Figure 7. Localization of translation initiation factors IF1 and IF2 in prokaryotic ribosome complexes.***
*(a) Crystal structure of the 30S–IF1 complex (Carter et al., 2001). 30S is in blue, IF1 is in red. (b) Cryo-EM structure of the 30SIC (Simonetti et al., 2008). 30S density is blue with manually fitted 30S structure, IF1, and fMet-tRNAfMet are red and IF2 is green. (c) Cryo-EM structure of a 70SIC (Allen et al., 2005). View of the 30S side from which the density of the 50S subunit has been masked out for clarity with the exception of helix H69 (yellow; occupies the density annotated as IF3 in Ref. (Allen et al., 2005). The position of IF1 (red) differs from the one observed in the structures of panel (a) and (b), here represented in black (taken and modified from Myasnikov et al., 2009).*

### 4.4.3. Initiation factor 3

IF3 is encoded by the *infC* gene (Olsson et al., 1996), which is essential for the cell viability due to its key role in protein synthesis. IF3 consists of two distinct domains connected by a 20-residue-long (45 Å) lysine-rich spacer (Moreau et al., 1997). *In vitro,* the polypeptide corresponding to the C-terminal domain of IF3 (IF3C, residues 90 to 176) can accomplish all the functions of the full-length IF3 in translation initiation and ribosome recycling (including binding to 30S subunit, shift of the mRNA, stimulation or inhibition of mRNA translation, dissociation of the 70S ribosomes and of pseudo-initiation complexes), whereas the IF3N domain (residues 11 to 77) is only needed for increasing the thermodynamic stability of the IF3-30S complexes (Petrelli et al., 2003). Although the linker is also essential for IF3 function, its length and composition can be varied (de Cock et al., 1999). Cryo-EM analyses have shown that the IF3C domain is positioned within the interface of the 30S and 50S subunits (McCutcheon et al., 1999), In contrast, X-ray data demonstrated that, in the presence of tetracycline and edeine, IF3C can alternatively be found on the opposite site of the platform of the 30S subunit (Pioletti et al., 2001).

IF3 is involved in the discrimination between the initiator and other aminoacyl tRNAs, thereby permitting the presence of only the initiator tRNA at P-site during translation initiation (Hartz et al., 1989; Risuleo et al., 1976), thereby ensuring translation fidelity (Hartz et al., 1989; Sussman et al., 1996). Furthermore, IF3 dissociates the pseudo-initiation complexes and non-canonical initiation complexes (including the initiator tRNA bound ribosome programmed with a leaderless mRNA or a mRNA with non-canonical initiation codon) and acts as a translational fidelity factor (Gualerzi et al., 2001). However, IF3 was originally identified as ribosome disassembly factor as it has high affinity to 30S subunit (Weiel and Hershey, 1981) and prevents the re-association of free 30S and 50S subunits when they are not involved in translation (Grunberg-Manago et al., 1975; Subramanian and Davis, 1970). This property of IF3 is known to play an important role in recycling of stalled ribosomes. It has been demonstrated that RRF and EF-G can recycle the ribosome only when IF3 is additionally attached to the ribosome (Hirokawa et al., 2005; Singh et al., 2005). IF3 can also accelerate dissociation of deacylated tRNA from the post-termination complexes, thus facilitating 70S ribosome disassembly into subunits (Hirokawa et al., 2002).

### 4.4.4. Factors controlling ribosome recruitment to bacterial mRNA

Binding of the mRNA to the ribosome is the most critical step for translation initiation. First, the short duplex between the SD sequence (GGAGG) located within the 5'-untranslated region (UTR) of the mRNA, and the anti-SD sequence, positioned at the 3' end of the 16S rRNA has to be formed. This interaction ensures the selection of the appropriate

initiation codon and open reading frame (Shine and Dalgarno, 1974). Previous work has shown that the SD-anti-SD interaction is accelerated by the presence the initiation factors and the initiator tRNA (Studer and Joseph, 2007). In general the efficiency of the SD-anti-SD interaction depend on several sequence elements present in the translation initiation region (**TIR**), such as the composition of the spacer region located between the initiation codon and the SD sequence, the non-random distribution of the nucleotides upstream of the SD (e.g. pyrimidine-rich regions, which is the r-protein S1 recognition motif (Boni et al., 1991; Komarova et al., 2002)), the sequence downstream of the initiation codon and the secondary structure of the TIR (Darfeuille et al., 2007; de Smit and van Duin, 2003; Marzi et al., 2008; Marzi et al., 2007; Ringquist et al., 1993).

In the 70SIC about 30 nucleotides of the mRNA encompassing the SD sequence and the AUG start codon are positioned around the platform and the shoulder of the 30S subunit. The sequence region downstream of the SD sequence of the mRNA passes through the small tunnel composed by the C-terminal α-helix of r-protein S7 and the h23 and h28 of the 16S rRNA; the start codon is placed in the decoding tunnel and within the A site mRNA; the 3´ end of the 16S rRNA interacts with r-protein S21 (Korostelev et al., 2007; Odom et al., 1984), which is the only r-protein present in the interface region (Yusupova et al., 2006). R-protein S21 is essential for the translation initiation on SD-containing mRNAs in *E.coli* (Van Duin and Wijnands, 1981), this suggesting its function may be to modulate interactions between the SD-helix and the 16S rRNA (Korostelev et al., 2007).

The structures located in the TIR of both prokaryotic and eukaryotic mRNAs can undergo induced conformational changes, in turn affecting the efficiency of translation initiation. These conformational changes can be induced by binding of trans-acting factors such as small metabolites, noncodinging RNAs, regulatory proteins or occur upon temperature downshifts (Kaberdin and Blasi, 2006). An interesting example of this type of regulation includes autoregulation of ribosomal operons by the *E coli* r-proteins S15 and S4. These r-proteins repress translation of their own mRNAs by the **entrapment** mechanism: blocking the mRNA accommodation by stabilized unfolded structured mRNA on the platform region of the ribosome (see Fig. 8) (Boehringer and Ban, 2007; Ehresmann et al., 2004; Schlax and Worhunsky, 2003). In the presence of these repressor r-proteins, the pre-accommodated mRNA is bound on the platform of the 30S ribosome, directly contacting (or being in the close proximity) to several ribosomal proteins S2, S7, S11 and S18. Intriguingly, the same structural features were discovered for initiation complexes formed by several prokaryotic and eukaryotic mRNAs including *E. coli rpsO* and *thrS* mRNAs, the 5' poly (A) - or poly (U) - rich extensions and the hepatitis C virus (HCV) and cricket paralysis virus (CrPV) RNAs with the Internal Ribosome Entry Site (**IRESs**) (Boehringer et al., 2005; Spahn

et al., 2004), thereby suggesting the existence of a common platform docking site for structured mRNA during pre-initiation.

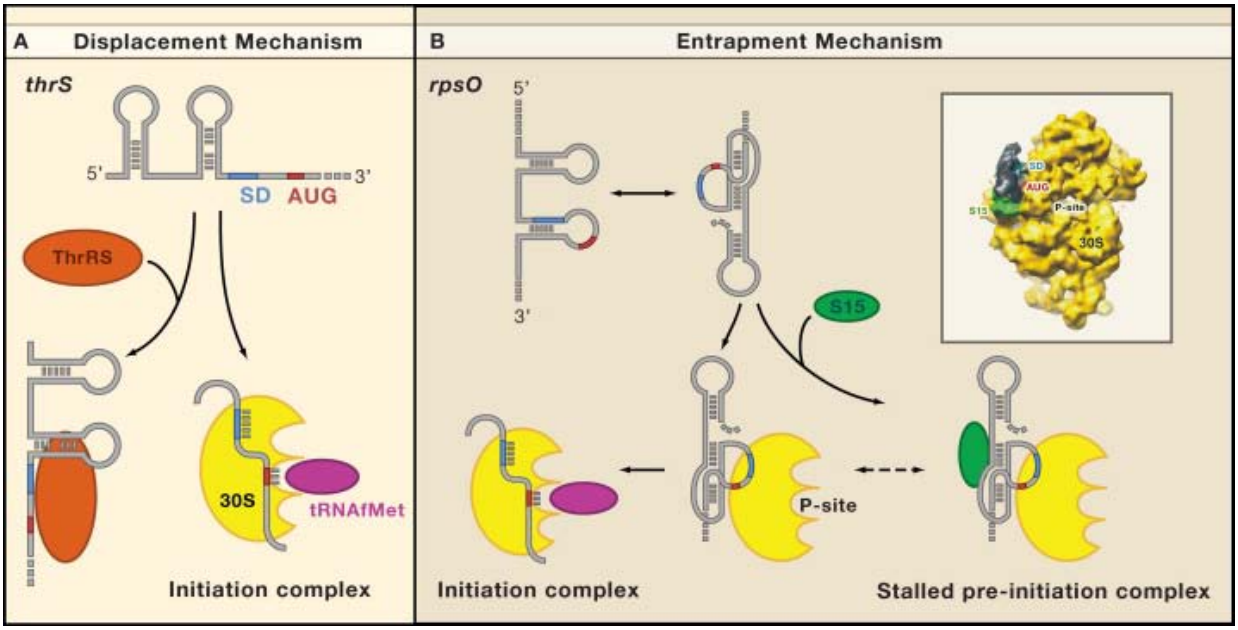


***Figure 8. Mechanisms of Translational Repression in Prokaryotes.*** *(A)* The expression of Thr-tRNA synthetase (ThrRS) is autoregulated by the displacement mechanism (Worhunsky et al., 2003). The ThrRS protein competes with the ribosome for binding to the translation initiation site of its own *thrS* mRNA 5′ untranslated region. (B) The entrapment mechanism is exemplified by E. coli ribosomal protein S15, which represses the translation of its own *rpsO* mRNA. Two conformations of *rpsO* mRNA exist in equilibrium. The pseudoknot is bound by the small ribosomal subunit (30S) by base pairing with the Shine-Dalgarno sequence (SD, blue) in the preinitiation complex (Marzi et al., 2007). In the absence of S15 protein, the pseudoknot is rapidly unwound resulting in the formation of a productive initiation complex with the AUG start codon (red) base-paired to tRNAfMet in the P site. When S15 protein is present in excess it binds and stabilizes the mRNA pseudoknot trapping the ribosome in the preinitiation stage.(Inset) Cryo-EM structure of the stalled preinitiation complex. The repressor protein S15 (green) binds in complex with its own rpsO mRNA (black) to the platform of the 30S ribosomal subunit (yellow). The region of the AUG start codon and the SD sequence are highlighted in red and blue respectively (taken from Boehringer and Ban 2007).

As translation initiation regions of some naturally occurring transcripts can potentially be occluded by stable secondary structures, their translation can be impeded *in vivo*. Recent studies have shown that docking of structured mRNAs on 30S ribosomal subunits and formation of translation initiation complexes may be facilitated by several r-proteins (S7, S2, S11, S18, S21 and S1) which are located at the platform binding site (Myasnikov et al., 2009;

Simonetti et al., 2009). These proteins possess helicase activities enabling the unfolding of the secondary structures within mRNAs thus facilitating their binding to the mRNA channel. The platform of the 30S subunit may be required for the interaction with other ribosome associated factors involved in mRNA binding as suggested by recent cryo-EM derived-structures of proteins Era and S1 bound to the ribosome (Sharma et al., 2005).

### *4.4.5. Re-initiation: the application of the ribosome scanning model*

In eubacteria, many genes are part of polycistronic operons. In *E. coli,* more than 25% of all operons are polycistronic and around 9% of the open reading frames (ORFs) have a start codon overlapping with a stop codon from the upstream ORF (Blattner et al., 1997). To carry out translation of the components of multiprotein complexes or enzymes that catalyze different reactions within the same metabolic pathway, bacteria usually co-express these proteins from polycistronic operons such as operons coding for ribosomal proteins (Sor et al., 1987), ATP sythetase (Rex et al., 1994), photosynthetic complexes (Choudhary and Kaplan, 2000) and phage components (Ivey-Hoyle and Steege, 1989). In this case, the co-ordinated expression of proteins is achieved *via* translational coupling (or re-initiation), if the downstream translation start site overlaps with (or in close proximity to) the termination codon of the upstream gene. Therefore, the scanning ribosome can dissociate from mRNA and re-initiate at the nearby start codon of a downstream gene on polycistronic mRNAs (Adhin and van Duin, 1990).

Both 30S and 70S ribosomes have been suggested to be involved in translation re-initiation (Adhin and van Duin, 1990; Das and Yanofsky, 1984; Inokuchi et al., 2000; Janosi et al., 1998; Karamyshev et al., 2004; Martin and Webster, 1975; Moll et al., 2004). IF2 is required for efficient re-initiation, whereas overexpression of IF3 decreases re-initiation efficiency (Yoo and RajBhandary, 2008). It has also been shown that the SD sequence upstream of the re-initiation start codon enhances re-initiation efficiency (Das and Yanofsky, 1984; Ivey-Hoyle and Steege, 1992; Spanjaard et al., 1989), whereas an increase in distance between the stop codon of an upstream gene and the start codon of the downstream gene decreases the efficiency of this process (Inokuchi et al., 2000; Ivey-Hoyle and Steege, 1992; Karamyshev et al., 2004). The role of other factors in this process is less understood (Janosi et al., 1998).

### *4.4.6. Translation initiation on Leaderless mRNAs: the alternative 70S ribosome initiation pathway*

Although some bacterial, archaeal and eukaryal mRNAs are known to lack 5'-leader regions (i.e. belong to the family of leaderless mRNAs (lmRNAs)) including ribosome recruitment sequences, they can still be translated efficiently (Moll et al., 2002b). For instance, transcription of 13 genes in the mammalian mitochondria yields mRNAs lacking both 5' and 3' untranslated regions. The specialized mitochondrial ribosome is able to preferentially dock their unstructured 5' sequences within the mRNA entrance site to initiate translation (Jones et al., 2008). The translation of proteins encoded by single genes or genes that are first in polycistronic operons of the hyperthermophilic crenarchaeon *P. aerophilum* is also initiated by ribosome binding to leaderless transcripts (Slupska et al., 2001). The translation of leaderless mRNAs was additionally documented for several *Streptomyces* species (Horinouchi et al., 1987; Janssen et al., 1989; Winzeler and Shapiro, 1997) and bacteriophages (Shean and Gottesman, 1992). The use of leaderless transcripts by bacteriophages can be rationalized by implicating the existence of selective pressure linked to the limited size of their genomic DNAs. The use of leaderless mRNAs in the case of *Streptomyces* and *Caulobacter* might provide means to differentiate mRNAs that are transcribed under different physiological conditions (e.g. when cells grow in rich or minimal media) and from constitutive and developmentally regulated promoters.

Several lines of evidence have shown that initiation of translation on lmRNA is distinct from the canonical mechanism and involves the un-dissociated ribosome (70S particles) rather than 30S subunits initiation complex (Andreev et al., 2006; Moll et al., 2004; O'Donnell and Janssen, 2002; Udagawa et al., 2004). First, the translation initiation of lmRNA is promoted by IF2, which accelerate the 70S ribosome assembly (Grill et al., 2000; Grill et al., 2001) and stimulates binding of the initiator tRNA to the P site (Boelens and Gualerzi, 2002; Laursen et al., 2005). Second, this process is negatively affected by overexpression of IF3 that triggers ribosome dissociation into 30S and 50S subunits (Grill et al., 2001; Moll et al., 2004). Third, the S1/S2-deficient ribosomes, which form particularly stable 70S ribosomes, selectively translate the lmRNA (Moll et al., 2004).

### *4.4.7. In-phase translation initiation of polycistronic mRNAs*

Another translation initiation mechanism is linked to the phenomenon of the in-phase translation of two or more proteins from the same cistron. This mechanism is known to operate in bacteria, yeast, eukaryotic viruses and bacteriophages. Several mRNAs reported to be translated according to this mechanism include the mRNAs coding for IF2 (Nyengaard

et al., 1991; Plumbridge et al., 1985), ClpB (Squires et al., 1991), McrB (Ross et al., 1989) and CheA (Smith and Parkinson, 1980). This translation initiation mechanism is responsible for the production of three IF2 variants suggested to exist in all bacterial species including the following genera: *Salmonella, Serratia, Proteus, Aeromonas, Pseudomonas, Streptococcus, Sarcina* and *Bacillus* (Howe and Hershey, 1984). It remains still unclear why ribosomes prefer the intracistronic initiation sites within the *infB* transcript (encodes IF2) when the upstream initiation site should be more favourable. One model suggests that the efficient intracistronic translation initiation requires two mRNA features: (1) an single-stranded region to facilitate ribosome binding and (2) the presence of rare codons upstream from the intracistronic initiation site to stall the elongating ribosome (see Figure 9) ( Laursen et al., 2002). This idea is consistent with the observation that the cellular levels of IF2 isoforms are strongly dependent on growth conditions (Giuliodori et al., 2004; Howe and Hershey, 1983; Laalami et al., 1991), but does not exclude that unknown trans-regulators may also participate in this process. Other interesting examples include bacterial caseinolytic peptidase B (ClpB) and yeast heat-shock protein 104 (Hsp 104) that are involved in protein disaggregation and are essential for cell survival during extreme stress condition. Under heat shock conditions, translation of *clpB* mRNA produces two polypeptides (84.1 kDa and 68.5 kDa, respectively), corresponding to the full-length polypeptide and its truncated variant, which is 147 amino acid residues shorter suggested to most likely originate due to initiation of translation from a second translational start found in the *clpB* mRNA (Ross et al., 1989).

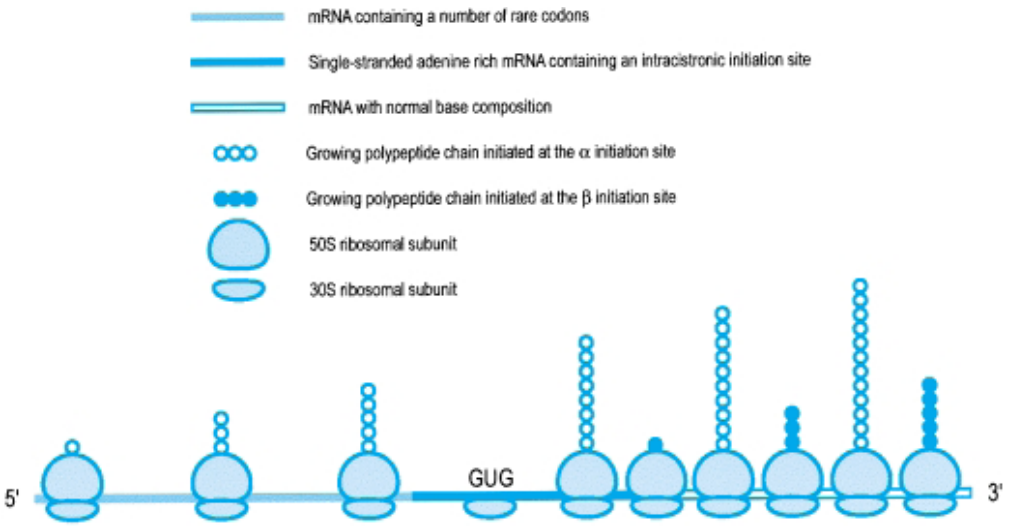


***Figure 9. Schematic view of the translation process on* infB *mRNA.*** The 5'-end of the *infB* mRNA contains at least two intracistronic translation initiation codons (ITICs). The sequences upstream of the ITICs carry several rare codons responsible for ribosome stalling. The latter provides an open single-stranded region of the mRNA which is a necessary for the intracistronic translation initiation of the *infB* mRNA (adopted from Laursen et al. (2002)).

Overviewing the mechanism applied in canonical initiation, re-initiation, lmRNA initiation and in-phase initiation also covers the main mechanisms that function in translation initiation in Eukaryotes. The existence of the IRES dependent initiation and the ribosome scanning mechanisms in eukaryotic cells not only enables to consider bacterial translation initiation mechanisms as possible prototype of their eukaryotic variants but also shed some light on the diversity of regulatory mechanisms regulating translation in Bacteria.

## *4.5. The ribosomal proteins*

Although rRNAs are responsible for the catalysis of ribosomal functions such as the decoding and peptidyl-transferase activity (Ramakrishnan, 2002; Steitz and Moore, 2003; Wilson and Nierhaus, 2003), ribosomal proteins also play essential roles in protein synthesis by facilitating rRNA folding, controlling ribosome assembly and its optimal activity. They also have important functions by being involved in interactions taking place at the mRNA entrance pore, during translation-factor binding and at the tunnel exit (as mentioned in 3.2, the 30S ribosome structure and Fig. 2). There is extensive cooperation between r-proteins when they exert their ribosome functions, and therefore it is normally difficult to determine the individual contribution of each r-protein during translation. The existence of this cooperation is supported by several observations. Alterations in r-proteins L4 and L22 in the 50S subunit lead to the antibiotics microcline erythromycin resistance, through perturbation of the surrounding 23S rRNA, rather than through a direct interaction with the antibiotic (Gregory and Dahlberg, 1999). Furthermore, the erythromycin resistance conferred by mutations in L4 is modulated by additional mutations in the 30S subunit (Saltzman and Apirion, 1976). *Vice versa*, alterations in r-proteins of the large subunit can affect properties of the small subunit, e.g. some changes in r-protein L6 inhibit binding of the aminoglycoside antibiotic gentamycin, which can induce misreading, to the decoding centre on the small ribosome subunit (Davies et al., 1998). Mutations occurring in r-proteins that belong to the *E. coli* 50S subunit can induce an antibiotic resistance phenotype associated with the 30S subunit. For instance, mutations in S12 and S8 can confer ribosome resistance to streptomycin (Dabbs, 1978). Thus, r-proteins together with rRNAs constitute a perfect regulation network controlling both ribosomal functions and ribosome biogenesis.

About 30% of r-proteins of *E. coli*, Eukarya and Archaea have orthologous counterparts in two other kingdoms of life (Lecompte et al., 2002). Moreover, 30% of the r-proteins that are shared by eukaryotic and archaeal ribosomes are normally absent in bacteria. This indicates that phylogenetically conserved r-proteins of Bacteria, Archaea and Eukarya represent a minimal set of r-protein initially selected to carry out ribosome functions, whereas all extra r-proteins were added to bacterial, archaeal and eukaryal ribosomes later to further optimize ribosome structure and function with the needs of

translation specific for each particular kingdom of life (Table 1 and Fig 10). This idea is consistent with the presence of specific elements (e.g. zinc fingers and helix-turn-helix motifs) in the structure of the kingdom-specific r-proteins that indicate their capacity to bind to the naked rRNAs during their selective acquisition by ribosomes of different origin at the later steps of ribosome evolution (Mears et al., 2002). The above features of the kingdom-specific r-proteins are also present in six r-proteins (S1, S6, S16, S18, S20 and S21) found only in *E. coli* and other bacteria, but not in Archaea or Eukarya. Finally, the existence of minimal, phylogenetically conserved set of r-proteins also correlates with the composition of the translationally active 61S ribosome, which was characterized in this study (Kaberdina et al., 2009).

**Table 2. Concordance between the universally conserved ribosomal proteins from the three kingdoms of life**

| Small Subunit | | | | Large Subunit | | | |
|---|---|---|---|---|---|---|---|
| Bacteria: *E.coli** | Archaea *H. Ma** | Low Eukarya: *Yeast** | High Eukarya: *Rat** | Bacteria: *E.coli** | Archaea *H. Ma** | Low Eukarya: *Yeast** | High Eukarya: *Rat** |
| S2 | S2 | S0 | Sa | L1 | L1 | L1 | L10a |
| S3 | S3 | S3 | S3 | L2 | L2 | L2 | L8 |
| S4 | S4 | S9 | S9 | L3 | L3 | L3 | L3 |
| S5 | S5 | S4 | S2 | L4 | L4 | L4 | L4 |
| S7 | S7 | S5 | S5 | L5 | L5 | L11 | L11 |
| S8 | S8 | S22 | S15a | L6 | L6 | L9 | L9 |
| S9 | S9 | S9 | S16 | L10 | L10 | P0(A0) | P0 |
| S10 | S10 | S20 | S20 | L11 | L11 | L12 | L12 |
| S11 | S11 | S14 | S14 | L12 | L12 | P1/P2 | P1/P2 |
| S12 | S12 | S28 | S23 | L13 | L13 | L16 | L13a |
| S13 | S13 | S18 | S18 | L14 | L14 | L23 | L23 |
| S14 | S14 | S29 | S29 | L15 | L15 | L28 | L27a |
| S15 | S15 | S13 | S13 | L16 | L10e | L10 | L10 |
| S17 | S17 | S18 | S11 | L18 | L18 | L5 | L5 |
| S19 | S19 | S15 | S15 | L22 | L22 | L17 | L17 |
| | | | | L23 | L23 | L25 | L23a |
| | | | | L24 | L24 | L26 | L26 |
| | | | | L29 | L29 | L35 | L35 |
| | | | | L30 | L30 | L7 | L7 |

**E.coli, Escherichia coli; H. Ma., Haloarcula marismortur; Yeast, saccharomyces cerivisiae; Rat, Rattus norvegicus.*
Source:http://www.expasy.org/cgi-bin/lists?ribosomep.txt

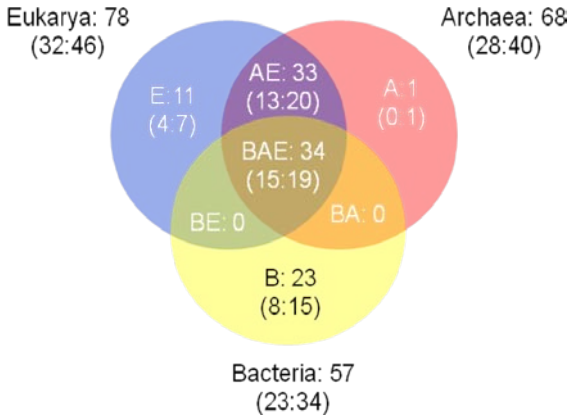


***Figure 10. Ribosomal proteins shared by the ribosomes from the three kingdoms of life: bacteria, archaea and eukarya (adopted from Lecompte et al. (2002)).*** B, A, and E denote ribosomes from bacteria, archaea and eukarya, respectively. AE:33 (13:20) means that the archaeal and eukaryotic ribosomes have 33 r-proteins in common, 13 in small and 20 in large subunit ribosomal subunits. Moreover, all three kingdoms have 34 r-proteins in common, 15 in small ribosome and 19 in large subunit.

Ribosomes of phylogenetically distant organisms can significantly differ in the size, composition and rRNA : r-protein ratio. For example, two thirds of *E. coli* ribosomal mass is represented by rRNA and one third by r-proteins, whereas in the case of mitochondrial ribosomes, the ribosome mass is distributed between rRNAs and protein in an opposite manner with the ratio of rRNAs to r-proteins of approximately 1 to 3.

In *Caenorhabditis elegans*, mitochondrial ribosomes lack 5S rRNA, whereas the remaining rRNAs are shorter (697 and 953 nt, respectively) when compared to their *E. coli* counterparts (1542 and 2904 nt, respectively). Moreover, r-proteins of *Caenorhabditis elegans* are larger and more diverse (Mears et al., 2002). Likewise, the existence of 77 r-proteins, some of which are species-specific, in bovine mitochondrial ribosomes significantly exceed their number in bacteria resulting in larger ribosomes (Cavdar Koc et al., 2001; Suzuki et al., 2001a, b). The presence of additional and/or larger r-proteins in the mitochondrial ribosome may be required to compensate the lack of some structural domains that are possibly absent in shorter mitochondrial rRNAs. Indeed, this may explain why some mitochondrial r-proteins occupy new positions within the ribosome such as their new locations within intersubunit bridges largely composed of r-proteins (but not of rRNAs) in mitochondria as well as their role in formation of a gate-like structure at the mRNA entrance area (Sharma et al., 2003).

Compared to the average isoelectric point (pI) of most of the translation factors (pI 4 to 5), many r-proteins are usually basic (pI ~ 10.0), and therefore their interaction with rRNA enables to counteract the negative charges naturally conferred by rRNA backbone's

phosphate groups. However, some r-proteins such as S1 (pI 4.61), S6 (pI 5.07), and L7/L12 (pI 4.31) are acidic proteins, thus indicating that these r-proteins may be involved in different types of interaction. Although the role of ribosomal proteins is still poorly understood some of their ribosomal (or extraribosomal) functions can be related to their ability to function as RNA chaperones (Semrad et al., 2004). In this respect, further work will be needed to study their potential contribution to other RNA-based mechanisms likely involving RNA-binding or RNA chaperone properties of r-proteins.

### *4.5.1. Specific functions of r-proteins within the ribosomes*

Although r-proteins have a clear and essential function in the assembly of ribosomal subunits, some r-proteins additionally play important roles in assisting the translation process after the ribosome is assembled. For instance, it has been shown that r-protein S1 binds to the mRNA and brings it close to the ribosome during the phase of translation initiation. R-protein S2 might be involved in protecting and stabilizing the SD helix docking (Kaminishi et al., 2007) and binding the SD duplex at the post-initiation step (Yusupova et al., 2006). R-proteins S3, S4 and S5 form the mRNA entry pore on the platform and unwind mRNA secondary structures (Kurkcuoglu et al., 2008; Noller et al., 2001). The action of these r-proteins is important during the decoding process, as they control the fidelity of translation. Namely, the correct binding of cognate tRNAs is controlled at the step of disruption of multiple interactions at the interface between S4 and S5 and establishment of the salt-bridges between S12 and either h44 or h27 of the 16S rRNA to stabilize the closed form of the 30S subunit (Ogle et al., 2002). In addition, S7 and S11 influence the translational fidelity by forming part of the binding site of the anticodon loop of the E site tRNA and indirectly influencing the accuracy of the A-site decoding and the maintenance of ribosome reading frame (Geigenmuller and Nierhaus, 1990; Marquez et al., 2004; Robert and Brakier-Gingras, 2003).

Several r-proteins of the 50S subunit play also important roles in translation. L1 and its rRNA binding region constitute the landmark structure of the 50S subunit, the so-called L1 stalk controlling removal of deacylated tRNA from the E site of the ribosome (Agrawal et al., 2000; Harms et al., 2001; Yusupov et al., 2001). L9 with its two globular RNA-binding domains, separated by a long and invariant nine-turn α-helix, appears to influence mRNA movement through the ribosome, which is especially critical during ribosome bypassing documented for T4 gene 60 mRNA, (Adamski et al., 1996). This ribosomal protein also stabilizes the tRNA at the P-site through binding to the base of the L1 stalk (Adamski et al., 1996; Yusupov et al., 2001). Nevertheless, since the L9 is absent in archaea and eukaryea and L9 deletion mutants exist in *E. coli* (Herr et al., 2001), the effect of L9 on tRNA docking

at the P-site probably is a fine-tuning mechanism specific for bacteria (Wilson and Nierhaus, 2005). In *E. coli* L11 and L10 X $(L7/L12)_4$ compose the L7/L12 stalk region (Brot and Weissbach, 1981; Wahl and Moller, 2002), which is involved in EF-G and EF-Tu binding to ribosome and stimulates their GTPase activity (Dey et al., 1995; Kischa et al., 1971). On the back of ribosome, the exit of the peptide tunnel is surrounded by a ring of r-proteins, including L22, L23, L24, L29. L23 directly interacts with the trigger factor, the first component of a cascade chaperone system, which directs the nascent chains to ensure that they adopt their native conformation by means of the proper folding (Ferbitz et al., 2004). In bacteria, L23 is also involved in an interaction with the signal recognition particle. This interaction directs the translating ribosome to the corresponding docking site on the membrane (Raine et al., 2004). Finally, L16 influences the translation initiation in an IF2-dependent manner (Belova et al., 2001), whereas the action of L27 is likely related to ribosome recycling (Wilson et al., 2005).

### *4.5.2. Extra-ribosomal functions of r-proteins*

Apart from their multiple roles as components of ribosomes, some r-proteins can additionally interact with non-ribosomal RNAs or proteins and thus exert other cellular functions (Warner and McIntosh, 2009). The extraribosomal function of *E. coli* r-proteins was first demonstrated in the late 70's using a bacteriophage system. This study revealed that several host proteins including the r-protein S1 and translation factors EF-Tu and EF-Ts interact with one of the polypeptides encoded by bacteriophage Qβ gene to act as an RNA helicase necessary to facilitate replication of a phage genome (Blumenthal and Dennis, 1978). Previous studies revealed that r-proteins S9 and L14 are involved in DNA repair and replication (Yancey and Matson, 1991).

The most common non-ribosomal function of r-proteins is to serve as feedback translational regulators of the polycistronic mRNAs that encode these proteins. Several *E. coli* r-proteins that function as autogenous regulators are S1, S2, S4, S7, S8, S15, L1, L4, L10 and L20 (Boni et al., 2001; Guillier et al., 2005; Guillier et al., 2002; Johnsen et al., 1982; Mathy et al., 2004; McCormick et al., 1994; Skouv et al., 1990; Stelzl et al., 2003) and S13, S14, S28, L2, L10 and L30, in eukaryotic cells (Malygin et al., 2007; Mitrovich and Anderson, 2000). These proteins recognize similar RNA patterns on the rRNA and their cognate mRNAs. Such control is known to be exerted by S2, a r-proteins that joins ribosome assembly at one of the final steps of this process (Culver, 2003). Recent work has shown that the S2-mediated autogenous control involves S2 cooperation with r-protein S1 (Aseev et al., 2008). Autogenous regulation is a general strategy of balancing ribosomal protein synthesis involving extra-ribosomal functions of r-proteins but not extra-ribosomal factors.

Another extra ribosomal function was discovered for S4 and S10 (NusE) known to participate in transcriptional anti-termination (Das et al., 1985). Both of them exert their regulatory functions by binding directly to the RNA polymerase (Mason and Greenblatt, 1991; Mason et al., 1992). S10 has been shown to form heterodimers with NusB *in vitro* and the latter binds to the *rrn boxA* sequence. The r-protein S1 can also bind *boxA*, interfering with NusB–E binding (Mason et al., 1992; Mogridge and Greenblatt, 1998; Nodwell and Greenblatt, 1993) suggesting a negative regulatory role for S1 in *rrn* antitermination *in vivo*.

Some r-proteins can function as sensors of changing physiological conditions. During the condition of starvation L11 can detect the presence of the uncharged tRNA on the A site of the stalled ribosome and signals this event to RelA, the stringent factor, which responds and catalyzes the synthesis of the alarmone (p)ppGpp (Wendrich et al., 2002).

## *4.6. Ribosome heterogeneity*

Kurland and co-workers have suggested that ribosomes can have various composition in different phase of translation, which in turn determines the overall heterogeneity of the ribosome population (Kurland et al., 1969). Consistently, another work has shown that the amount of the ribosomal protein S1 increases on the ribosome during translation initiation, which in turn can stimulate mRNA binding to the ribosome but also greatly decreases the amounts of S21 r-protein on the free 30S ribosome subunit (Van Duin and Kurland, 1970; van Duin et al., 1972). This finding suggests that minor proteins can transiently be associated with ribosomes (e.g. only in specific translation phases) and subsequently affect the amount of other r-proteins in the ribosome. Independent analysis of ribosome heterogeneity with respect to their protein content reveal that, although many *E. coli* ribosomal proteins are present in nearly equimolar amounts in ribosomes, still one fourth of the r-proteins is present in less than one copy per ribosome (Deusser, 1972; Voynow and Kurland, 1971). Consequently, it has been demonstrated that ribosome variants differing in the content of S6, S21 and L7/L12 can be formed in *E. coli* in response to different growth rates or specific growth conditions (Deusser, 1972; Deusser and Wittmann, 1972; Milne et al., 1975).

In addition to the protein content, there are additional factors that contribute to ribosomal heterogeneity. For instance, previous studies have shown that many fungi express different forms of 5S rRNA and two different forms of this rRNA were found to exist in S. cerevisiae (Selker et al., 1985). Apart from this, numerous ribosomal proteins in bacteria, rat, human and yeast have been found to be subjected to a variety of posttranslational modifications including phosphorylation, methylation, ubiquitination, and acetylation (Arnold and Reilly, 1999, 2002; Lee et al., 2002; Louie et al., 1996; Odintsova et al., 2003; Vladimirov

et al., 1996). Many of these modifications can impact the translational activity of the ribosomes and therefore play important roles in regulation of ribosome function *in vivo* (Bachand et al., 2006; Mazumder et al., 2003).

These founding are fully consistent with the ribosome filter theory, which was proposed by Vincent P. Mauro and Gerald M. Edelman. According to their hypothesis, control of gene expression lies in part with the ribosome itself (Mauro and Edelman, 2002).Namely, the ribosome structure and ribosome ability to initiate translation of selected mRNAs can differentially affect translation of particular subsets of mRNAs and thereby affect patterns of protein production. For example, and interesting mechanism consistent with the above theory has recently been described for the S1/S2-depleted *E. coli* ribosomes shown to be able to selectively translate leaderless mRNAs in contrast to canonical mRNAs (Moll et al., 2002a).

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## *5. AIMS OF THE STUDY*

The enigma of ribosome heterogeneity and its role in regulation in gene expression raise a number of interesting questions related to adjustment of ribosome composition and structure in response to environmental stresses such as exposure of bacterial cell to antibiotics. Addressing these questions will enable to gain fundamental insights into molecular mechanisms that are used by bacteria to adopt the protein synthesizing machinery to stress and will reveal new potential targets for antibacterial drug design.

As mentioned before that the S1/S2-depleted *E. coli* ribosomes are able to selectively translate leaderless mRNAs in preference to canonical mRNAs. However, the aminoglycoside antibiotic Kasugamycin (Ksg) inhibits translation initiation on canonical mRNAs, but does not inhibit translation initiation on leaderless mRNAs *in vivo*. Therefore, the first aim of this study is the characterization of the molecular mechanisms underlying the formation of ribosome heterogeneity under stress condition, i.e. under antibiotic treatment.

The second aim of this study is to further elucidate the affect of the adjustment of the ribosome composition and structure in response to antibiotic treatment on the post-transcriptional and translational levels. As initiation factor 2 (IF2) selectively stimulates translation of leaderless mRNAs, the potential roles of IF2 on the formation of heterogeneous ribosomes will be addressed in this work.

Ribosomal protein S1 binds to the 30S subunit through protein-protein interaction *via* ribosomal protein S2. Since r-protein S1 is essential for translation in Gram-negative bacteria, the interaction between the two ribosomal proteins S1 and S2 has the potential to represent a target site for antimicrobial activity. Furthermore, no functional homologue of protein S1 is present in Gram-positive bacteria nor in mitochondria, and eukarya employ a different mechanism for translation initiation. Therefore, the third aim is to design an antimicrobial compound affecting ribosome assembly by disruption of the S1/S2 interaction. Such a compound could act semi selective against Gram-negative pathogens and would not affect beneficial Gram-positive flora nor cause severe side effects. Here I will verify the interaction of proteins S1 and S2 by performing NMR-based analysis.

# *6. PUBLICATIONS AND MANUSCRIPTS*

## *6.1. AN UNEXPECTED TYPE OF RIBOSOMES INDUCED BY KASUGAMYCIN: A LOOK INTO ANCESTRAL TIMES OF PROTEIN SYNTHESIS?*

Molecular Cell
Article

# An Unexpected Type of Ribosomes Induced by Kasugamycin: A Look into Ancestral Times of Protein Synthesis?

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## SUMMARY

**Translation of leaderless mRNAs, lacking ribosomal recruitment signals other than the 5′-terminal AUG-initiating codon, occurs in all three domains of life. Contemporary leaderless mRNAs may therefore be viewed as molecular fossils resembling ancestral mRNAs. Here, we analyzed the phenomenon of sustained translation of a leaderless mRNA in the presence of the antibiotic kasugamycin. Unexpected from the known in vitro effects of the drug, kasugamycin induced the formation of stable ~61S ribosomes in vivo, which were proficient in selectively translating leaderless mRNA. 61S particles are devoid of more than six proteins of the small subunit, including the functionally important proteins S1 and S12. The lack of these proteins could be reconciled with structural changes in the 16S rRNA. These studies provide in vivo evidence for the functionality of ribosomes devoid of multiple proteins and shed light on the evolutionary history of ribosomes.**

## INTRODUCTION

The rate limiting step and major checkpoint of translational regulation in Bacteria is the formation of the translation initiation complex, comprising the 30S ribosomal subunit, fMet-tRNA$_f^{Met}$, and mRNA (Gualerzi and Pon, 1990). The efficiency of formation of this ternary complex is modulated by intrinsic features of the 5′-untranslated region (UTR) of the mRNA, including the Shine and Dalgarno (SD) sequence and a region rich in pyrimidines, which is recognized by ribosomal protein (r-protein) S1 (reviewed in Laursen et al., 2005). In contrast, leaderless mRNAs (lmRNAs), which are present in Bacteria, Archaea, and Eukarya, start directly with the AUG-initiation codon, and thus lack these recognition motifs. As there is no evidence for ribosome recruitment signals in the 5′-coding region of lmRNAs (reviewed in Moll et al., 2002b), the 5′ terminus, with its start codon, represents the only constant recognition element for the ribosome.

Translation of lmRNAs was stimulated by translation initiation factor 2 (IF2) (Grill et al., 2000, 2001), which facilitates fMet-tRNA$_f^{Met}$ binding to the ribosome. These data were interpreted as showing that the 5′-terminal start codon of lmRNAs is recognized by fMet-tRNA$_f^{Met}$ bound to ribosomes. In addition, IF2 stimulates 30S-50S subunit association (La Teana et al., 2001; Antoun et al., 2003), which offers an additional explanation for the stimulating effect of IF2 via the 70S initiation pathway (Moll et al., 2004). A number of observations support the view that 70S ribosomes initiate the translation of lmRNA: (1) recent experiments revealed that intact 70S monosomes are competent for translation initiation of lmRNAs (Moll et al., 2004; Udagawa et al., 2004), whereas (2) overexpression of IF3, triggering dissociation of the ribosomal subunits, was shown to exert a negative effect on lmRNA translation (Moll et al., 2004; Grill et al., 2001). (3) The 70S ribosome translation initiation pathway of lmRNAs was supported by a set of experiments using ribosomes devoid of the r-proteins S1 and S2. These S1/S2-deficient monosomes were shown to be particularly stable and to perform selective translation of lmRNA (Moll et al., 2004).

The aminoglycoside antibiotic kasugamycin (Ksg) inhibits translation at the step of initiation complex formation in Prokaryotes and Eukaryotes (Okuyama et al., 1971). Recently, the binding sites of Ksg on the *Thermus thermophilus* 30S ribosomal subunit as well as on the *Escherichia coli* 70S ribosome were identified by X-ray crystallography (Schluenzen et al., 2006; Schuwirth et al., 2006). These studies located a primary Ksg-binding site on top of helix 44 (h44), spanning the region between h24 and h28 of 16S rRNA, which contacts the conserved nucleotides A794 and G926. This position coincides with the mRNA track at the P- and E-site codon. Binding experiments indicated that Ksg indirectly induces dissociation of P-site-bound fMet-tRNA$_f^{Met}$ from 30S subunits through perturbation of the mRNA, thereby interfering with translation initiation (Schluenzen et al., 2006; Schuwirth et al., 2006).

In contrast to the inhibitory effect of Ksg on the translation of canonical mRNAs, the translation of lmRNAs appeared to prevail in the presence of the drug in vivo (Chin et al., 1993; Moll and Bläsi, 2002). In this study, we have scrutinized the molecular mechanism(s) underlying the translation of lmRNA in the presence of Ksg. Our data revealed that during lmRNA expression Ksg induces in vivo the formation of ribosomal particles lacking several proteins of the small subunit, hereafter termed "61S particles." We demonstrate that these protein-deficient ribosomes selectively translate lmRNAs in the presence of the

drug in vivo and in vitro, which provides evidence for the in vivo functionality of ribosomes lacking several proteins. In addition, chemical probing studies are presented, which suggest a model for the formation of these protein-deficient particles in the presence of the drug.

## RESULTS

### Differential Effects of Ksg on the Translation of lmRNA In Vivo and In Vitro

We and others (Chin et al., 1993; Moll and Bläsi, 2002) observed that the activity of λ ClΦLacZ fusion proteins encoded by lmRNAs continued to increase in the presence of Ksg. To verify the apparent resistance of lmRNA translation to Ksg, we first performed a pulse labeling experiment in the presence of 750 μg/ml Ksg by using *E. coli* strain MG1655, harboring plasmid pRB381-1, which encodes a leaderless *cI-lacZ* fusion gene. This concentration of Ksg drastically reduced growth (data not shown). As shown in Figure 1A, lanes 5–7, the translation of bulk mRNA was strongly diminished upon addition of Ksg, whereas, in striking contrast, the de novo synthesis of the ClΦLacZ fusion protein continued in the presence of the drug. However, we were unable to recapitulate these results in vitro by programming an *E. coli* in vitro translation system with both the leaderless λ *cI* mRNA and *E. coli ompA* mRNA, the latter of which contains a 135 nt long 5′ UTR, containing a canonical ribosome-binding site (Rosenbaum et al., 1993). In contrast to the in vivo experiment (Figure 1A), increasing concentrations of Ksg inhibited in vitro translation of both mRNAs (Figure 1B, lanes 2–5).

70S initiation complexes on lmRNA rather than 30S ternary complexes were shown to be comparatively resistant to Ksg (Moll and Bläsi, 2002) (see Figure 4). Considering the established 70S translation initiation pathway for lmRNAs (Moll et al., 2004; Udagawa et al., 2004), we next tested whether the observed resistance of lmRNA translation to Ksg (Figure 1A) could be attributed to translation initiation of the lmRNA by intact 70S ribosomes. An in vitro translation assay was performed with crosslinked 70S ribosomes, which were previously shown to be competent in translating lmRNA (Moll et al., 2004). In contrast to our expectation, in vitro translation of λ *cI* mRNA by crosslinked 70S ribosomes was likewise inhibited in the presence of increasing concentrations of the antibiotic (Figure 1C, lanes 2–5), indicating that the observed selective translation of lmRNA in the presence of Ksg in vivo (Figure 1A) cannot be attributed to 70S monosomes.

### Ksg Induces the Formation of Protein-Deficient 61S Particles In Vivo

The conflicting in vivo and in vitro results shown in Figures 1A and 1B together with our previous observation that the r-proteins S1 and S2 are dispensable for lmRNA translation (Moll et al., 2002a) prompted us to study whether in vivo translation of *cI* mRNA is accomplished by a particular population of ribosomes formed in the presence of Ksg. *E. coli* strain MG1655(pRB381) was grown to an $OD_{600}$ of 0.3 and then treated with Ksg (750 μg/ml). The ribosomal profiles were analyzed by sucrose density centrifugation 60 min after the addition of Ksg. In contrast to the ribosome profile obtained in the absence of the

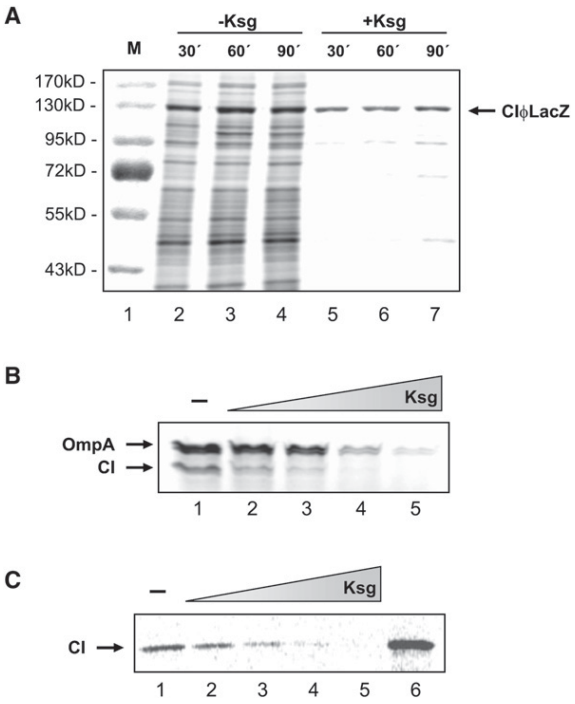


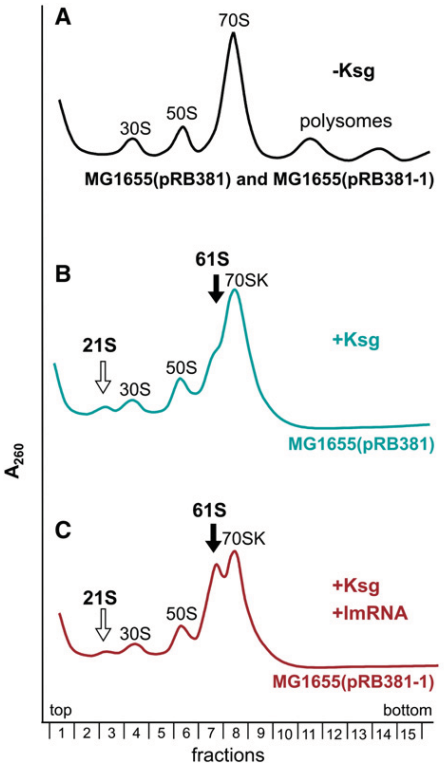

**Figure 1. Differential Inhibition of lmRNA Translation by Ksg In Vivo and In Vitro**

(A) De novo synthesis of a ClΦLacZ fusion protein in the presence of Ksg. Lane 1, protein marker (M). Lanes 2–4, pulse labeling of strain MG1655(pRB381-1) at 30, 60, and 90 min. Lanes 5–7, pulse labeling of strain MG1655(pRB381-1) at 30, 60, and 90 min after addition of Ksg as specified in the Experimental Procedures. The molecular masses of marker proteins and the position of the ClΦLacZ fusion protein are indicated on the left and on the right, respectively.

(B) Kasugamycin inhibits translation of canonical and lmRNA in vitro. In vitro translation of equimolar amounts of *E. coli ompA* mRNA and leaderless λ *cI* mRNA, respectively, with 30S/50S ribosomes and an *E. coli* S100 extract. Lane 1, translation of both mRNAs in the absence of Ksg. Lane 2–5, translation of both mRNAs in the presence of a 10-fold (lane 2), a 100-fold (lane 3), a 1000-fold (lane 4), and a 10,000-fold (lane 5) molar excess of Ksg over ribosomes. The positions of the OmpA and the CI proteins are indicated.

(C) Ksg inhibits in vitro translation of λ *cI* lmRNA by 70S crosslinked ribosomes. Lane 1, translation of *cI* mRNA in the absence of Ksg; lanes 2–5, translation in the presence of a 10-fold (lane 2), a 100-fold (lane 3), a 1000-fold (lane 4), and a 10,000-fold (lane 5) molar excess of Ksg over crosslinked 70S ribosomes. Lane 6, translation of *cI* mRNA with 30S/50S ribosomes. The position of the CI protein is indicated.

antibiotic (Figure 2A), no polysomes were detected in the presence of Ksg, i.e., bulk translation was inhibited (Figures 2B and 2C). Similar to the results by Champney and colleagues obtained with other aminoglycoside antibiotics (Mehta and Champney, 2002; Foster and Champney, 2007), a small 21S peak, corresponding to protein-deficient pre-30S particles, accumulated (Figures 2B and 2C; see Figure S2C available online). During the course of our experiments, we observed a slight shoulder of the 70S peak in ribosome profiles of the control strain MG1655(pRB381) (Figure 2B). However, it was difficult to reproduce the appearance of the shoulder, unless the leaderless *cI-lacZ* mRNA was simultaneously expressed from plasmid pRB381-1. The presence of the lmRNA resulted in the formation




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 2. Addition of Ksg Leads to the Accumulation of Protein-Deficient 61S and 21S Particles In Vivo**

(A) Ribosome sedimentation profiles of strains MG1655 (pRB381) and MG1655(pRB381-1) obtained in the absence of Ksg.

(B and C) Ribosome sedimentation profiles of the control strain MG1655(pRB381) and MG1655(pRB381-1), respectively, 60 min after addition of 750 μg/ml Ksg. 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. The r-proteins were extracted from purified 70S ribosomes, 21S particles, and 61S particles and were identified by quantitative immunoblotting (QI) by using antibodies against all r-proteins, except S19, as described in the Experimental Procedures and shown in Figure S2, and, in addition, were subjected to mass spectrometry (MS). The relative abundance of all r-proteins was then normalized with respect to the amount of proteins present in 70S ribosomes. (+) and (–) indicate the presence or absence, respectively, of r-proteins. (↓) indicates the significant reduction (<50%) of the respective protein. Proteins S15, S17, and S18 were not detected by MS.

of a distinct 61S peak (Figure 2C). These conditions enabled the purification and biochemical characterization of these 61S particles as specified in the Experimental Procedures (Figure S1). The 61S protein composition was determined by western blot analysis of the respective particles with antibodies directed against r-proteins (Figure S2B) as well as by mass spectrometry (MS). Both methods gave nearly identical results, with the exception of S14 and S20, which were found to be slightly underrepresented in MS analysis. Furthermore, S15, S17, and S18 were not detectable by MS even in 70S ribosomes. The data are summarized in Figure 2D, showing the protein content of the small subunit of the 61S particles compared to that of the 30S precursor 21S. The 61S particles were deficient in r-proteins S1, S2, S6, S12, S18, and S21 and contained a significantly reduced amount (>50%) of proteins S3, S5, S11, S16, and S17. The group of absent proteins was only partially overlapping with the set of proteins absent in the 21S precursor of the 30S subunit (Figure 2D). In contrast, the protein composition of the 50S subunit was found to be unaltered (data not shown).

### 61S Particles Efficiently Translate ImRNA In Vivo

Next, we tried to obtain evidence for the functionality of the 61S particles in ImRNA translation in vivo. Protein S12, which was among the proteins absent in the 61S particles, is involved in binding of the antibiotic streptomycin (Ozaki et al., 1969; Carter et al., 2000). We therefore anticipated that 61S particles, if functional, would be resistant to streptomycin (Sharma et al., 2007). As a means to inhibit the translational activity of residual wild-type ribosomes, we added streptomycin to strain MG1655 (pRB381-1) after Ksg-induced formation of the 61S particles. In the absence of Ksg and in the presence of streptomycin, a complete shut off of translation was observed (Figure 3A, lanes 2–4). However, when 61S particle formation was induced prior to the addition of streptomycin, the *cI-lacZ* mRNA continued to be translated in the presence of both drugs (Figure 3A, lanes 5–7) with an efficiency comparable to that seen in the presence of Ksg only (Figure 1A, lanes 5–7). These results indicated that translation of the ImRNA in the presence of both antibiotics, Ksg and streptomycin, is performed by ribosomal particles devoid of S12, and thus most likely by the 61S particles.

In the presence of Ksg, no polysomes were detected by densitometry (Figure 2C). However, the 61S particles, if active in ImRNA translation, as well as the ImRNA ought to be present in the polysome fractions. Therefore, we analyzed the corresponding fractions obtained from strain MG1655(pRB381-1) for the presence of the leaderless *cI-lacZ* and *ompA* (canonical control) transcripts. After purification of the RNA of the respective fractions of the sucrose gradients (see Figures 2A and 2C, fractions 11–15), we attempted to detect both mRNAs by primer extension. Both mRNAs were present in the polysome fraction in the absence of Ksg (Figure 3B, lanes 6–10). In contrast, in the presence of Ksg only the *cI-lacZ* ImRNA (Figure 3B, lanes 1–5) was present in elongating ribosomes. In parallel with the RNA preparation, we analyzed the protein composition of the ribosomes present in polysomes of MG1655(pRB381-1) upon Ksg treatment by immunological means. With the exception of proteins S1 and S21, the translationally active ribosomes were devoid of the same set of r-proteins, which were found to be absent in the 61S particles (Figure S2D). For the reasons mentioned in the Discussion, we consider the presence of S1 and S21 in the polysome fraction to be a consequence of direct binding to ImRNA rather than originating from 61S particles.

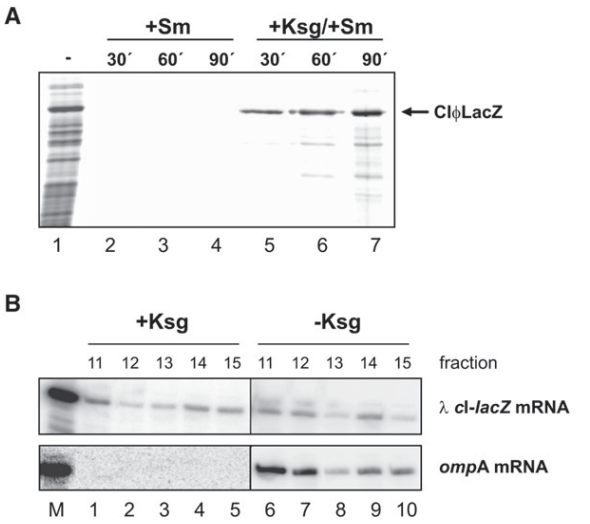



**Figure 3. 61S Particles Are Proficient in Translation of lmRNA In Vivo**

(A) *E. coli* strain MG1655(pRB381-1) was grown in M9 medium until the $OD_{600}$ reached 0.2. Then, the culture was divided into two flasks. Sm (100 μg/ml) was added to one culture, and Ksg (750 μg/ml) was added to the other. Aliquots of the Sm-treated culture were pulse labeled as described in the Experimental Procedures, 30 (lane 2), 60 (lane 3), and 90 min (lane 4) upon addition of Sm. The Ksg-treated culture was incubated for 60 min to allow for formation of the 61S particles. Then, Sm (100 μg/ml) was added to the culture. A total of 30 (lane 5), 60 (lane 6), and 90 min (lane 7) later, aliquots of the Ksg/Sm-treated culture were pulse labeled. Lane 1, pulse labeling of strain MG1655(pRB381-1) at an $OD_{600}$ of 0.5 in the absence of antibiotics. The position of the ClΦLacZ fusion protein in the autoradiograph is shown.

(B) The cI-*lacZ* lmRNA is present in polysome fractions upon addition of Ksg. Total RNA from polysome fractions (fractions 11–15) of the ribosome profiles shown in Figures 2A and 2C was purified. The presence of leaderless cI-*lacZ* mRNA and canonical *ompA* mRNA was determined by primer extension analysis with primers O8 and AvaII (see Table S1), respectively. Lanes 1–5, primer extension signals with total RNA prepared from polysomes of cells grown in the presence of Ksg. Lanes 6–10, extension signals with total RNA prepared from polysomes of cells grown without Ksg. The numbers of the respective fractions (see Figures 2A and 2C) are given on top of the autoradiograph. Extension signals obtained with in vitro-transcribed cI and *ompA* mRNAs are shown in lane M.

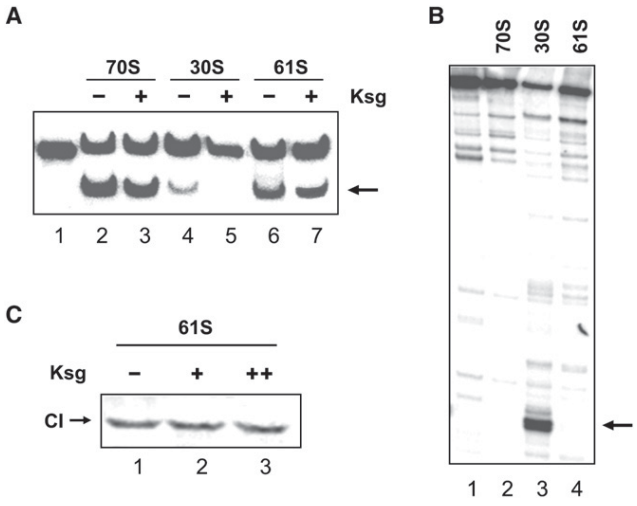



**Figure 4. Isolated 61S Particles Form Translation Initiation Complexes at 5′-Terminal Start Codons and Translate lmRNA**

(A and B) Translation initiation complex formation (toeprinting) in the presence of $tRNA_f^{Met}$ on (A) leaderless *cI* mRNA and (B) *ompA* mRNA. (A) Lane 1, primer extension on *cI* mRNA in the absence of ribosomes and $tRNA_f^{Met}$. Lanes 2, 4, and 6, toeprint analysis with 70S ribosomes, 30S subunits, and 61S particles, respectively, in the absence of Ksg. Lanes 3, 5, and 7, toeprint analysis with 70S ribosomes, 30S subunits, and 61S particles, respectively, in the presence of a 10,000-fold molar excess of Ksg. The position of the toeprint signal resulting from a translation initiation complex located at position +15 of the *cI* mRNA is indicated by an arrow. (B) Lane 1, primer extension on *ompA* mRNA in the absence of ribosomes and $tRNA_f^{Met}$. Lanes 2, 3, and 4, toeprint analysis with 70S ribosomes, 30S subunits, and 61S particles, respectively, in the absence of Ksg. The position of the toeprint signal is indicated by an arrow.

(C) In vitro translation of *cI* lmRNA with 61S particles in the absence of Ksg (lane 1) and in the presence of a 100-fold (+, lane 2) and a 10,000-fold (++, lane 3) molar excess of Ksg over ribosomes. The position the CI protein is indicated.

## Selective Translation of lmRNA by 61S Particles In Vitro

Using toeprinting, we next tested for the proficiency of the purified 61S particles to form translation initiation complexes at the 5′-terminal AUG of the leaderless λ cI mRNA. As expected from previous results (Moll et al., 2002a, 2004) and the experiments shown in Figure 3B, 70S and 61S particles, respectively, failed to form a translation initiation complex at the canonical ribosome-binding site of *ompA* mRNA (Figure 4B, lanes 2 and 4). In contrast, both the isolated 61S particles and 70S ribosomes formed a translation initiation complex at the 5′-terminal start codon of the λ *cI* mRNA in the absence (Figure 4A, lanes 2 and 6) and in the presence (Figure 4A, lanes 3 and 7) of Ksg.

In addition, in vitro translation assays were performed with an S100 extract containing purified 61S particles, which was programmed with the canonical *ompA* and the leaderless λ *cI* mRNA. As expected, the 61S particles were unable to translate the canonical *ompA* mRNA (data not shown). In contrast, the purified 61S particles were functional in translation of the leaderless cI mRNA (Figure 4C, lane 1), and the addition of increasing amounts of Ksg did not affect protein synthesis (Figure 4C, lanes 2 and 3), whereas increasing concentrations of Ksg inhibited in vitro translation in an S100 extract in the presence of added 30S/50S ribosomes (see Figure 1B). Taking the in vivo and in vitro data together, these experiments demonstrated that the 61S particles are functional in translation of lmRNA.

## 61S Particles Contain Modified 16S rRNA

Resistance to Ksg can result from the lack of the dimethylations at two adjacent adenosines at positions 1518 and 1519 in h45 at the 3′ terminus of the 16S rRNA (Figure 5A) (Helser et al., 1972; Van Buul et al., 1983). These nucleotides are modified during assembly by the methyltransferase KsgA. We therefore wondered whether the Ksg resistance of the 61S particles is caused by a lack of these methylations. Therefore, primer extension analyses were performed with primer S1525 (Table S1), which binds to the 3′ end of 16S rRNA. Due to the presence of the dimethylations at positions A1518 and A1519 in 16S rRNA isolated from MG1655(pRB381-1) 70S ribosomes and 30S subunits, respectively, a stop signal was observed at position 1520 (Figure 5B, lanes 1 and 2). In contrast to the 16S rRNA derived from 21S particles (Figure 5B, lane 4), the 16S rRNA

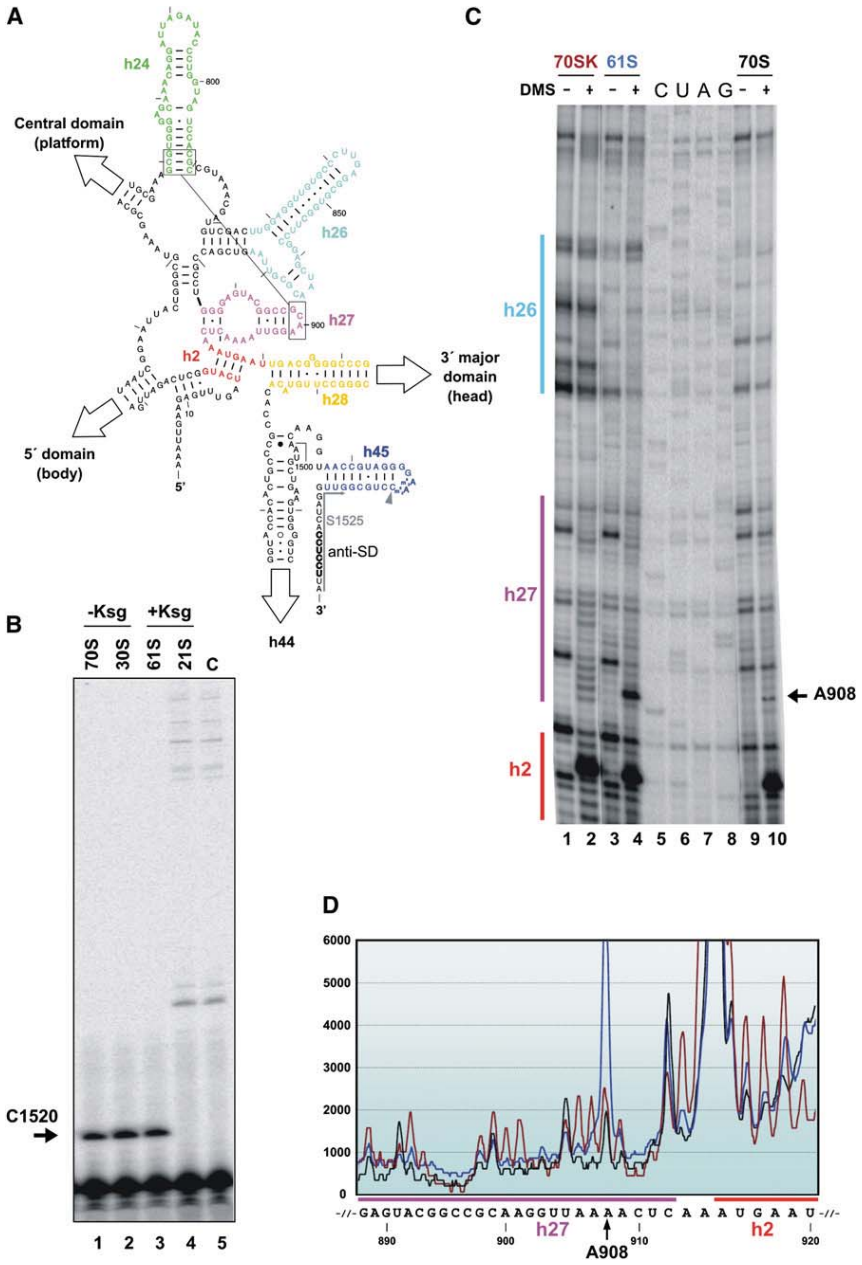



**Figure 5. Primer Extension Analyses of the 16S rRNA**

(A) The secondary structure of the central portion of the *E. coli* 16S rRNA, connecting the three major domains of the 30S subunit (head, body, and platform). Helices h2 (red), h24 (green), h26 (cyan), h27 (magenta), and h28 (yellow), which are affected by Ksg in vivo, as well as h45 (blue) are highlighted. The packing of the 900 loop of h27 toward the three boxed base pairs at the bottom of h24 is indicated (Belanger et al., 2004). The binding of primer S1525 (gray line, Table S1) as well as the stop signal obtained in the presence of the modification at A1518/1519 at position 1520 (gray arrowhead) are indicated. The figure was adapted from the Comparative RNA Web Site (http://www.rna.ccbb.utexas.edu).

(B) 16S rRNA purified from 70S ribosomes and 30S subunits isolated from strain MG1655 (pRB381-1) grown in the absence of Ksg (Figure 2A, 70S and 30S) as well as 16S rRNA purified from 61S and 21S particles of strain MG1655(pRB381-1) treated with Ksg (Figure 2C, 61S and 21S) was used for primer extension analysis with primer S1525 (Figure 5A; Table S1). The presence of the dimethylation at residues 1518/1519 in the 16S rRNA from 70S ribosomes (lane 1), 30S subunits (lane 2), and 61S particles (lane 3) stopped cDNA synthesis, as indicated by the signal (arrow) corresponding to nucleotide 1520 (also see Figure 5A, gray arrowhead). Primer extension of the 16S rRNA from 21S particles did not result in a stop signal, indicating the lack of modification (lane 4). In vitro-transcribed 16S rRNA served as an unmodified control (C, lane 5).

(C) 70S ribosomes isolated from strain MG1655(pRB381-1) (Figure 2A, 70S) as well as 70S ribosomes and 61S particles from strain MG1655(pRB381-1) treated with Ksg (Figure 2C, 70SK and 61S) were purified as specified in the Experimental Procedures. Upon DMS treatment, the rRNA was isolated and the sites of methylation were determined by primer extension analysis with primer V43, binding to positions 939–955 in 16S rRNA (Table S1). Primer extension analysis of 16S rRNA of 70SK ribosomes (lanes 1 and 2), 61S particles (lanes 3 and 4), and 70S ribosomes (lanes 9 and 10) in the absence of DMS (lanes 1, 3, and 9) or in the presence of DMS (lanes 2, 4, and 10). C, U, A, and G (lanes 5–8): sequencing reactions. The position of helices h2, h27, and h26 are indicated.

(D) PhosphorImager quantification of the primer extension analysis shown in (C) of the region spanning h27 (indicated in magenta) and h2 (indicated in red) of 16S rRNA of 70SK ribosomes (red, lane 2), 61S particles (blue, lane 4), and 70S ribosomes (black, lane 10) upon modification by DMS. The corresponding 16S rRNA sequence is shown below.

purified from the Ksg-induced 61S particles of strain MG1655(pRB381-1) was likewise modified at the respective positions in h45 (Figure 5B, lane 3). Hence, the resistance of the 61S particle against Ksg could not be attributed to the absence of the methylations at residues A1518/A1519.

## Ksg Affects the Central Structure of the 16S rRNA

To test whether the lack of the r-proteins in the 61S particles can be reconciled with structural changes in the 16S rRNA, we performed in vitro chemical probing experiments using the adenine-specific chemical probe dimethylsulfate (DMS). 70S ribosomes from strain MG1655(pRB381-1) grown in the absence of Ksg (Figure 2A) and 61S particles as well as 70S ribosomes accumulating in the strain treated with Ksg (70SK, Figure 2C) in vivo were purified. The sites of chemical modifications of nucleotides in the 16S rRNA were identified by primer extension analysis with several primers (Table S1). The most striking differences in the modification pattern were observed in the central

pseudoknot region, connecting the three major domains of the 16S rRNA (Poot et al., 1996) and h27 (Figure 5A). In contrast to 70S ribosomes, where the nucleotides located in h2 and h27 are protected from DMS modification by base pairing (Figure 5C, lane 10; Figure 5D, black line), we observed an enhanced reactivity of these nucleotides in 70SK ribosomes, indicating a disruption of these helices (Figure 5C, lane 2; Figure 5D, red line). The formation of h2 was shown to be essential for binding of proteins S1, S2, S6, S18, and S21 (Poot et al., 1996). It is striking that the same set of proteins is missing in the 61S particles. Nucleotide A908 in 61S particles shows a strong enhancement in DMS reactivity when compared with 70S and 70SK ribosomes (Figure 5C, lane 4; Figure 5D, blue line). This might be attributed to the lack of protein S12, the N-terminal tail of which lies in close proximity to this nucleotide (Lambert et al., 2005).

We observed several stop signals of reverse transcriptase (RT) in the absence of DMS, which apparently coincide with stable secondary and/or tertiary structures in the 16S rRNA (Figure 5C, lanes 1, 3, and 9). Additional stop signals seen in h26 within 70SK ribosomes as compared with 70S indicate a distortion of this helix (Figure 5C, lanes 1 and 9). As shown in Figure 7B, h26 is located on the solvent side of the 30S subunit, where it interacts with the heterodimer S6/S18 (Greuer et al., 1987; Powers and Noller, 1995) and directly contacts protein S1 (Golinska et al., 1981; Sengupta et al., 2001). Again, all three r-proteins are absent in the 61S particles.

### In Vitro Formation of the 61S Particles

The presence of the dimethylations in 16S rRNA of 61S particles indicated that they originated from 70S ribosomes. To test this, we next examined whether the 61S particles can be formed in vitro from 70S ribosomes. The sucrose gradient analysis shown in Figure 6 revealed that incubation of 70S ribosomes with both Ksg and ImRNA for 90 min resulted in the formation of 61S particles (red line). In contrast, no 61S particles were formed in the presence of either Ksg (Figure 6, cyan line) or ImRNA (Figure 6, blue line). We also noted that these conditions led to a partial dissociation of the 70S ribosomes into subunits, which could indicate a destabilization of 70S ribosomes upon addition of either ligand. However, in the absence of Ksg or ImRNA, 70S ribosomes remained stable (Figure 6, black line).

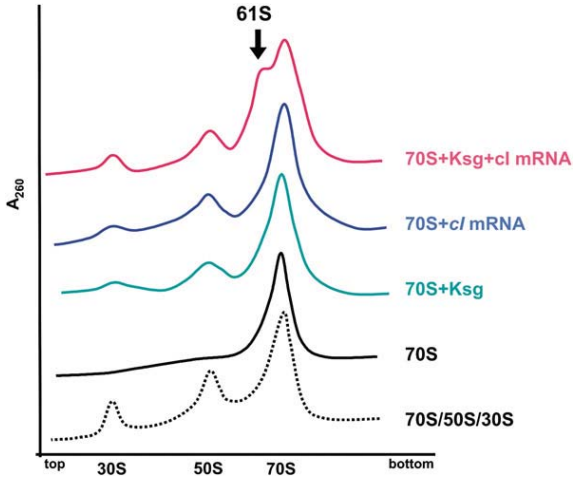



**Figure 6. In Vitro Formation of 61S Particles from 70S Ribosomes in the Presence of Ksg and ImRNA**

Sucrose gradient analysis of 70S ribosomes incubated for 90 min in the absence of *cI* mRNA and Ksg (black line), and in the presence of either Ksg (cyan line) or *cI* mRNA (blue line) as specified in the Experimental Procedures. The presence of both Ksg and *cI* mRNA resulted in the formation of 61S particles (red line). A mixture of equimolar amounts of 30S, 50S, and 70S served as a marker for the position of the subunits and ribosomes (dotted line).

## DISCUSSION

### Selective Translation of ImRNA by 61S Particles

Previous in vitro studies have shown that Ksg does not interfere with translation initiation complex formation on ImRNA by 70S ribosomes (Moll and Bläsi, 2002; Schluenzen et al., 2006). As we have shown that Ksg inhibits ImRNA translation by crosslinked 70S ribosomes in vitro (Figure 1C), the initiation inhibitor Ksg seems to affect translation at a postinitiation step, which remains to be elucidated. Surprisingly, translation of ImRNA is performed by 61S particles accumulating in the presence of Ksg in vivo (Figure 2C) and lacking more then six proteins (S1, S2, S6, S12, S18, and S21). Since the modern ribosome can be viewed as a protein-stabilized ribozyme (Steitz, 2008) and most r-proteins are essential (Wilson and Nierhaus, 2005), this observation is unprecedented.

Protein S1 appears to be essential for the translation initiation of canonical mRNAs in *E. coli*, since it facilitates mRNA binding (Boni et al., 1991; Tedin et al., 1997), whereas ribosomes devoid of both S1 and S2 are functional in ImRNA translation (Moll et al., 2002a). Moreover, protein S21, which stimulates the base-pairing potential of the SD-aSD interaction (Backendorf et al., 1981), is essential for translation of MS2 RNA, whereas the lack of protein S21 did not affect poly(U) translation (Van Duin and Wijnands, 1981). Poly(U) resembles ImRNA in that it is translated in vitro under conditions in which 70S monosomes are prevalent (Moll et al., 2004). Collectively, the absence of r-proteins S1, S2, and S21 from 61S particles (Figure 2D) can thus readily explain the strongly diminished translation (initiation) of bulk mRNA (Figure 1A), as well as the deficiency of the 61S particles to form translation initiation complexes on a canonical ribosome-binding site (Figure 4B). Likewise, S12 was shown to be dispensable for poly(U) translation (Nomura et al., 1969) and, more recently, for in vitro translocation (Cukras et al., 2003), again observations which are consistent with the competence of 61S particles to translate ImRNA (Figures 3A and 4C).

With the exception of S1 and S21, all r-proteins missing in 61S particles were also absent in polysomes translating ImRNA in the presence of Ksg (Figure S2D). Protein S1 has been reported to bind to single-stranded RNA stretches rich in pyrimidines (Draper and von Hippel, 1978), and gel-shift assays showed that free protein S1 binds to the leaderless *cI* mRNA (I.M., unpublished data). In addition, we have recently shown that binding of S1 to the ribosome depends on protein S2 (Moll et al., 2002a), which was absent in the 61S particles (Figure S2D). Likewise, the interaction of protein S21 with mRNA was shown by UV crosslinking (Schouten, 1985). Therefore, we consider the appearance of proteins S1 and S21 in polysomes translating

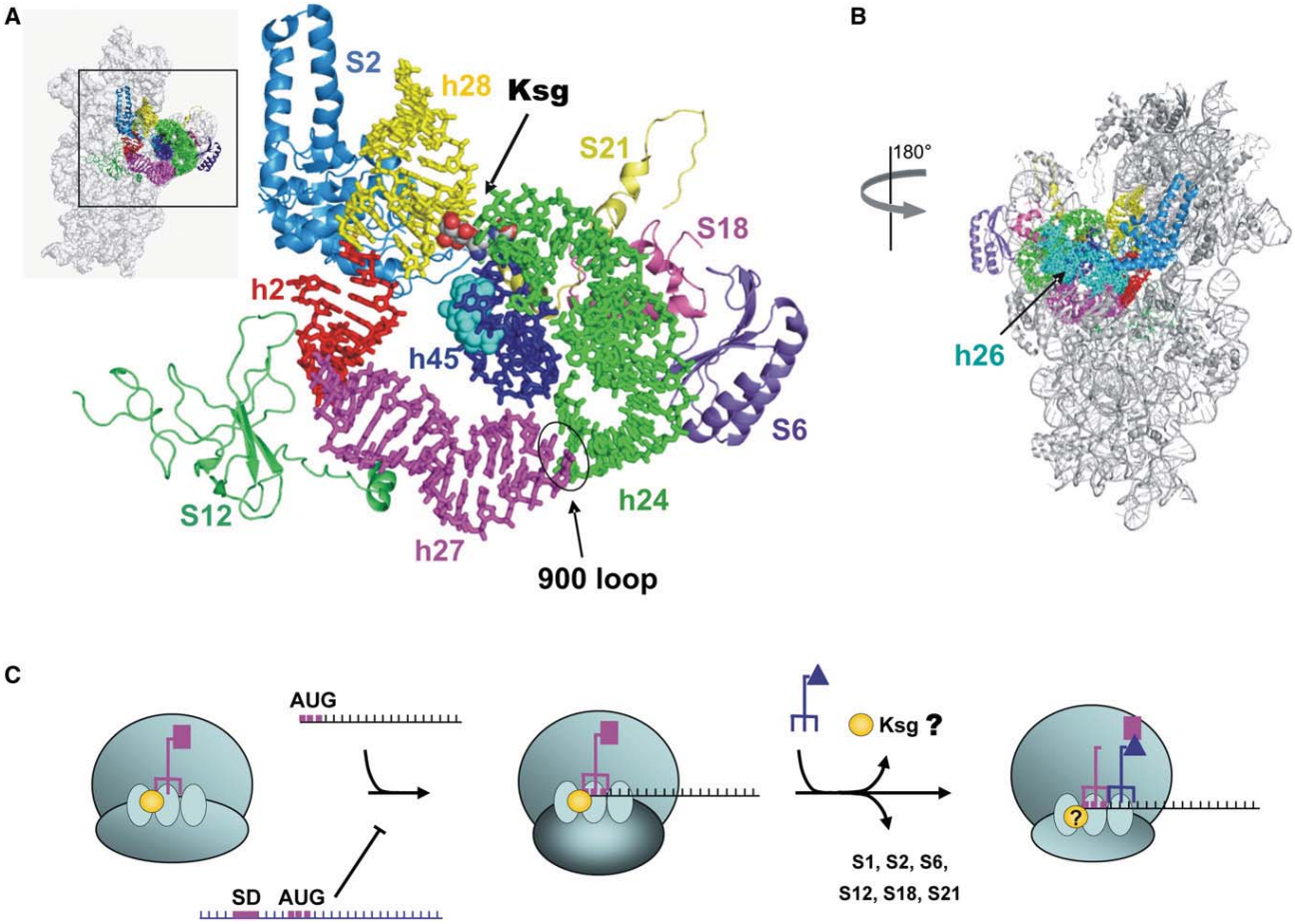



**Figure 7. Ksg Affects the Conformation of the 16S rRNA Central Pseudoknot**

(A) The 30S subunit as seen from the subunit interface and enlargement of the region affected by Ksg in vivo. The helices that are affected by Ksg are highlighted using the same color code as shown in Figure 5A. H26 has been omitted for clarity. Binding of Ksg (sphere model) between the G926 of h28 and the 790 loop of h24 is shown (Schluenzen et al., 2006; Schuwirth et al., 2006). The r-proteins S2 (blue), S6 (purple), S12 (green), S18 (pink), and S21 (yellow) are absent in 61S particles. Nucleotides A1518/A1519 in h45 are shown by blue spheres.

(B) The 30S subunit shown from the solvent side. H26 (cyan) is located on the platform interacting with proteins S18 (pink) and S1 (not shown). The structures were modeled by using PyMOL molecular system software (DeLano, 2002) and PDB file 1VS5 (Schuwirth et al., 2006).

(C) Working model for the formation of the 61S particle: 70SK ribosomes with a P site-bound fMet-tRNA$_f^{Met}$ (magenta) can form a translation initiation complex on lmRNA (black) containing a 5′-terminal start codon (AUG), but not on a canonical mRNA (blue) containing a Shine and Dalgarno sequence (SD), as the position of the 5′-UTR of the latter would clash with bound Ksg (yellow sphere) (Schluenzen et al., 2006; Schuwirth et al., 2006). At precisely which step Ksg induces the release of ribosomal proteins, i.e., upon formation of the initiation complex or at the beginning of the elongation phase, e.g., upon binding of the charged tRNA cognate to the second codon (blue), remains to be elucidated. At present, it is also unknown whether Ksg is released from or remains bound to the 61S particle during translation.

lmRNA in the presence of Ksg to result from direct binding of these proteins to lmRNA.

## Formation of Protein-Depleted Ribosomes in the Presence of Ksg

The nucleotides at positions A1518 and A1519 in the 16S rRNA of the 61S particles are dimethylated (Figure 5B). These methylations belong to the small group of universally conserved modifications seen in all kingdoms of life (O'Farrell et al., 2004). They are important for a stable folding of the 3′ end of 16S-type rRNA (Micura et al., 2001), suggesting that the conformation of this ultimate stem-loop is crucial for ribosome function (Rife and Moore, 1998; Micura et al., 2001). The pivotal role of the modifications in h45 is supported by the appearance of aberrant ribosomes sedimenting between 70S and 50S as determined by ribosome profile analysis of an *E. coli* strain deficient for the KsgA methylase, which is responsible for the modifications (data not shown). However, the lack of an active enzyme confers resistance against Ksg (Helser et al., 1972; Van Buul et al., 1983), which might be explained by the direct interaction between h45 and h24, the latter involved in binding of the antibiotic (Schluenzen et al., 2006) (Figure 7).

For methylation activity, KsgA requires a minimal particle consisting of the 16S rRNA and eight r-proteins (Thammana and

Held, 1974; Desai and Rife, 2006). Half of these eight proteins were either absent (S6 and S18) or only present in significantly reduced amounts (S16 and S17) (Figure 2D) in the 61S particles. The presence of the methylations in the 61S particles is therefore difficult to reconcile with the assumption that Ksg exerts its effect on an assembly intermediate before binding of the proteins S6, S18, S16, and S17.

Moreover, the structural analysis of the 16S rRNA of 70S ribosomes obtained in the presence of Ksg in vivo (70SK) revealed a disruption of h2 and h27 (Figure 5C, lane 2). These helices together with h24 and h28 form a loop structure (Figure 7A), which entraps the primary binding site of Ksg at the position of the mRNA start codon in the P-site located between G926 of h28 and A792 and A794 of h24 (Woodcock et al., 1991; Schluenzen et al., 2006). Since (1) the presence of a lmRNA is required to stimulate the formation of the 61S particles (Figures 2C and 6), (2) the formation of a 70S initiation complex on lmRNAs is not inhibited in the presence of Ksg (Figure 4A) (Moll and Bläsi, 2002; Schluenzen et al., 2006), and (3) 61S particles can be formed by incubation of 70S ribosomes with Ksg and lmRNA in vitro (Figure 6), we suggest the following model for in vivo formation of these particles (Figure 7C): (1) 70S ribosomes form an initiation complex exclusively with lmRNA in the presence of Ksg (Figures 4A and 4B); (2) Ksg changes the 70S conformation, in particular that of the loop-forming helices h2, h27, h24, h28, and h26 (Figures 5C and 7A); (3) disruption of some of these helices (h2, h26, and h27) (Figure 5C) triggers the release of r-proteins directly or indirectly attached to this loop.

## 61S Particles: A Look into the Past?

Since ribosomes of organisms of all three domains can translate lmRNA (Grill et al., 2000; Moll et al., 2002b), they may be viewed as remnants of ancestral mRNAs before domain separation. The 61S particles proficient in lmRNA translation contain a reduced number of 30S proteins. With the exception of S2 and S12, which are conserved in all three evolutionary domains, most of the proteins reduced or absent in 61S particles were most likely added later in evolution, after separation of the kingdoms (Mears et al., 2002). It is therefore tempting to speculate that the 61S particles mirror an ancient form of the small subunit present in proto-ribosomes before domain separation, approximately three billion years ago.

Crosslinked 70S ribosomes can translate lmRNA (Figure 1C) (Moll et al., 2004), and 61S particles are more stable at low $Mg^{2+}$ concentrations and in the presence of an S100 extract than 70S ribosomes (A.C.K. and I.M., unpublished data). It is therefore possible that the 61S particle acts as a stable monosome, and thus resembles the proposed one-subunit proto-ribosome that catalyzed peptide bond formation initiating at the 5′ terminus of single-stranded polynucleotides (Moore and Steitz, 2002). Furthermore, the 16S rRNA of 61S particles is methylated at the two residues A1518 and 1519 (Figure 5A), which is an important feature for stable folding of the 3′ end of 16S-type rRNA (Micura et al., 2001) comprising the decoding center. Since adenine methylation occurs at this functional hot spot in ribosomes of all three domains, it has probably been acquired at or even before the existence of the bacterial proto-ribosome and thus also before domain separation. We find it a total surprise that under distinct stress conditions ribosome particles are formed in vivo in present bacteria, which might reflect ancient bacterial proto-ribosomes.

## EXPERIMENTAL PROCEDURES

### Bacterial Strains

*E. coli* strain MG1655 (Blattner et al., 1997) has been described. Unless otherwise indicated, bacterial cultures were grown in LB broth (Miller, 1972) at 37°C in the presence of 100 μg/ml ampicillin to maintain selection of plasmids pRB381 (Brückner, 1992) and pRB381-1. Growth was monitored by measuring the optical density at 600 nm ($OD_{600}$). Plasmid pRB381-1 confers ampicillin resistance and harbors a translational fusion in which the first 63 codons of the leaderless λ cI gene are abutted on the eighth codon of the *lacZ* gene (Moll et al., 2001).

### De Novo cI-*lacZ* Synthesis in the Presence of Ksg and Streptomycin

*E. coli* strain MG1655 (pRB381-1) was grown in M9 minimal medium. At an $OD_{600}$ of 0.3 either no antibiotic, Ksg (1 mg/ml), or streptomycin (Sm, 100 μg/ml) was added. A total of 30, 60, and 90 min after addition of antibiotics, 150 μl aliquots were withdrawn from all three cultures. After 60 min of incubation with Ksg, Sm was added (100 μg/ml) to half of the culture. A total of 30, 60, and 90 min after addition of Sm, aliquots were withdrawn from both cultures (with and without Sm). At each time pulse, labeling was carried out by addition of 1.5 μl L-[U-$^{14}$C]-aa mix (50 μCi/ml), and by further incubation for 5 min at 37°C. The reactions were stopped by the addition of an equal volume of cold 10% TCA, followed by incubation on ice for 15 min and subsequent centrifugation for 15 min at 15,000 rpm at 4°C. The cell pellets were washed once with 90% acetone, dried under vacuum for 5 min, resuspended in SDS-protein sample buffer, and boiled for 5 min prior to loading onto a 12% SDS-polyacrylamide gel. For the different $OD_{600}$ values, the same amounts of total cellular protein were subjected to electrophoresis. The gels were dried and exposed to a Molecular Dynamics PhosphoImager for visualization.

### In Vitro Translation Assays

Full-length λ cI and *ompA* mRNA (4 pmol) were translated in vitro with *E. coli* S100 extracts and 30S/50S ribosome, crosslinked 70S ribosomes, or 61S particles as described before (Moll et al., 2004). Briefly, 15 μl Mix A (16.6 mM MgOAc, 80 mM NH4Cl, 30 mM Tris-HCl [pH 7.7], 3.3 mM DTT, 1.6 mg/ml *E. coli* tRNA, 0.2 mM citrovorum, 16.6 mM KCl, 0.33 mM amino acids (-lys), 66.6 μM [14C]-lys, 3.3 mM ATP, 0.66 mM GTP, 16.6 mM phosphoenol-pyruvate, and 0.04 mg/ml of pyruvate kinase) was mixed with 5 μl S100 extract and 5 pmol of either 30S/50S ribosomes, crosslinked 70S ribosomes, or 61S particles. The reactions were started by the addition of 5 pmol mRNA. After incubation of the reactions for 30 min at 37°C either in the absence or in the presence of 1.6 μM, 16 μM, 160 μM, or 1.6 mM Ksg, translation was stopped by the addition of 4 volumes of 90% acetone. The samples were resuspended in SDS-protein sample buffer and loaded onto a 12% SDS-polyacrylamide gel. Gels were dried and exposed to a Molecular Dynamics PhosphoImager for visualization and quantification.

### Chemical Probing and Primer Extension Analysis

DMS-modification reactions of the rRNA of purified 70S and 70SK ribosomes and 61S ribosomal particles were performed as described (Stern et al., 1988). Briefly, 2 pmoles ribosomes or particles were modified in a reaction volume of 45 μl in buffer containing 10 mM Tris-HCl (pH7.3), 60 mM $NH_4Cl$, 10 mM MgOAc, and 6 mM β-mercaptoethanol by the addition of DMS (5 μl in a 1/12 dilution in ethanol), followed by incubation on ice for 30 min. Control reactions were performed by adding ethanol without DMS. Reactions were stopped by ethanol precipitation. After phenol/$CHCl_3$ extractions, the modified rRNA was resuspended in 24 μl 1× RT-buffer (50 mM Tris-Cl [pH 8.3], 60 mM NaCl, 6 mM $MgCl_2$, 10 mM DTT). For primer extension analysis, 2.4 μl (0.2 pmol) of the respective rRNAs were annealed to the 5′-end-labeled primers (Table S1) (2.4 μl, 0.6 pmol) in 1× RT-buffer, heated to 80°C for 3 min, snap frozen in liquid nitrogen, and slowly thawed on ice. Primer extension reactions were performed in RT-buffer by using the AMV reverse transcriptase (Promega) by

incubation at 42°C for 15 min essentially as described previously (Moll et al., 2004). The samples were separated on an 8% PAA-8M urea gel, and the extension signals were visualized by using a Molecular Dynamics PhosphoImager. Quantification was performed by using the ImageQuant program. To analyze the in vitro DMS modification, we used DMS-nonspecific stops as internal standards (G888, U891, G898, C899) to normalize the amounts of the different 16S rRNA.

### In Vitro Formation of 61S Subunits

A total of 250 pmol 70S ribosomes prepared under low-salt conditions (60 mM $NH_4Cl$) was incubated in the absence or presence of an equimolar amount of full-length cI mRNA in a reaction volume of 100 μl in a buffer containing 10 mM Tris-HCl (pH 7.3), 60 mM $NH_4Cl$, 6 mM MgOAc, and 6 mM β-mercaptoethanol either in the presence or absence of 25 mM Ksg. After incubation at 37°C for 90 min, the reactions were layered on 7%–47% sucrose gradients made up in the reaction buffer, and centrifugation was performed as described above.

## SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures (including information on the preparation of ribosomes, ribosome profile analysis, determination of the protein composition of 61S particles by immunoblotting, detection of mRNAs [cI-*lacZ* and *ompA*] in polysomes, and toeprinting analysis), two figures, and one table and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(08)00857-5.

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## 6.2. Ksg-induced formation and translation of leaderless *infB* mRNA variants in *Escherichia coli*

## SUMMARY

The aminoglycoside antibiotic kasugamycin (Ksg) inhibits translation initiation on canonical mRNAs by indirectly inhibiting P-site tRNA binding through perturbation of the mRNA-tRNA codon-anticodon interaction. In contrast, Ksg does not inhibit translation initiation on leaderless mRNAs (lmRNAs) *in vivo*, which lack ribosomal recruitment signals other than the 5'-terminal start codon. Previous studies have shown that Ksg induces formation of protein-depleted *Escherichia coli* ribosomes (61S particles) *in vivo* and *in vitro*, which are devoid of more than six ribosomal proteins of the small subunit, including the functionally important proteins S1 and S2. Biochemical analysis revealed that 61S particles are proficient in translation of lmRNAs in the presence of Ksg. lmRNAs can alternatively be translated by 70S ribosomes formed upon association of the 30S and 50S ribosomal subunits and their association is stimulated by the initiation factor 2 (IF2 encoded by the *infB* gene). I studied the potential effect of IF2 on the formation of heterogeneous, i.e. protein deficient, ribosomes.

The *in vivo* experiments presented here revealed that overexpression of IF2 stimulates the formation of 61S particles in the presence of Ksg. Moreover, western blot analyses employing anti-IF2 antibodies revealed that Ksg treatment additionally leads to accumulation of truncated IF2 polypeptides. Their formation was attributed to translation of leaderless *infB* mRNA variants lacking the 5'-regions of this transcript. Primer extension analysis revealed that accumulation of some leaderless *infB* mRNA variants occurs in the wild-type *E. coli* strain upon Ksg treatment. Futher studies indicate that the formation of the leaderless *infB* mRNA variants can be attributed to the endoribonuclease MazF. This interferase represents the toxin component of the MazEF toxin-antitoxin (TA) system. Although this TA system is silent under normal conditions, due to inhibition of the MazF activity by the antitoxin component MazE, it can be activated in response to some environmental stresses (e.g. antibiotic treatment). Therefore, our data suggest that truncated forms of the *infB* transcript are likely generated by means of *infB* mRNA processing by MazF during Ksg treatment. Moreover, our data suggest that similar mechanisms are likely involved in formation of other leaderless mRNAs that are still translated in the presence of Ksg. Taken together; these observations suggest a novel adaptation mechanism leading to formation of leaderless mRNAs selectively translated by modified ribosomes accumulating upon stress.

# INTRODUCTION

The aminoglycoside antibiotic kasugamycin (Ksg) inhibits protein synthesis by interfering with the initiation of translation (Tanaka et al., 1971). In contrast, Ksg does not inhibit translation of leaderless mRNAs (lmRNAs) *in vivo*, which, besides the 5'-terminal start codon, have no other ribosome recruitment signals (Chin et al., 1993; Moll et al., 2002b). Recent structural studies have shown that Ksg binds to the mRNA track of the ribosome thereby preventing the formation of the translation initiation complex on canonical mRNAs (Schluenzen et al., 2006; Schuwirth et al., 2006). In contrast, Ksg binding to the ribosome has no affect on translation of lmRNAs, which continues unabated in the presence of this antibiotic *in vivo* (Chin et al., 1993; Moll et al., 2002b).

Our recent studies revealed that upon expression of the leaderless *cI*-mRNAs, Ksg can induce the formation of protein depleted ribosomal particles (61S particles), which are stable and proficient in selective translation of lmRNAs *in vivo* and *in vitro* (Kaberdina et al., 2009). These 61S particles lack several ribosomal proteins (r-proteins) in the 30S subunit including the functionally important r-proteins S1 and S2. Comparative structural analysis suggests that the r-protein S2 is positioned in the universal stand-by site on the 30S platform, which is involved in binding regulatory 5' mRNA elements (Marzi et al., 2007). Likewise, S1 is also proposed to be located in the vicinity of the platform binding site. As S1 has a helicase activity (Rajkowitsch and Schroeder, 2007), it may also play a role in binding of structured mRNA during translation initiation. Apparently, due to its assisting role in binding of canonical mRNAs to ribosomes (Sengupta et al., 2001), S1 is essential for translation of bulk mRNA in *E. coli* (Sorensen et al., 1998; Tedin et al., 1997). Thus, these findings provide an additional support for the notion that the absence of r-protein S1 and S2 in 61S particles blocks the translation of bulk mRNA (Moll et al., 2002a).

Previous studies have shown that initiation factor 2 (IF2) has a stimulatory effect on lmRNA translation (Grill et al., 2000) and could stimulate formation of 61S by increasing the pool of 70S ribosomes (Antoun et al., 2003). Furthermore, GTP hydrolysis on IF2 induces structural rearrangements in the 30S subunit (Marshall et al., 2009), which might result in or induce the loss of the respective proteins to form 61S particles.

In this study, we further analyzed effects of IF2 on formation of 61S ribosomes and found that IF2 promotes their formation during Ksg treatment. Moreover, we also observed translation of shorter IF2 variants lacking the N-terminal part of IF2. We were able to attribute the appearance of the truncated IF2 variants to the accumulation of their cognate leaderless *infB* transcripts lacking the corresponding regions at their 5'-ends. Moreover, further analysis implicated the toxin MazF in the formation of truncated *infB* transcripts during Ksg treatment. These results suggest a new role for MazF, the toxin component of the MazEF toxin-antitoxin

system, in generation of alternative lmRNAs under stress. Taken together, our data suggest that the observed formation and translation of leaderless mRNAs might be a general mechanism that enables translation of specific mRNAs during adaptation to stress.

# MATERIALS AND METHODS

### *Bacterial strains and plasmids*

The *E. coli* strains MG1655 (Blattner et al., 1997), MC4100 (*relA*$^+$) (Hazan et al., 2001), and MC4100/ (*relA*$^+$ Δ*mazEF)* (Engelberg-Kulka et al., 1998) have been described previously. Plasmid pSA1 is a derivative of pQE30 (Qiagen, Hilden, Germany), which carries *mazF* under control of the *lac* operator. It also carries the *lacI*$^q$ and Amp resistance genes (Amitai et al., 2009). Plasmid pLBYC8-*infB* carries the *E. coli infB* gene under control of the $P_{tac}$ promoter (Bremaud et al., 1997). Plasmid pRB381-1 confers ampicillin resistance and harbors a translational fusion, in which the first 63 codons of the lambda phage *cI* gene are abutted on the eighth codon of the lacZ gene (Moll et al., 2001).

### *Media and Growth Conditions*

Cells were routinely grown in liquid Luria-Bertani (LB) medium containing antibiotics to maintain the used plasmids as described previously (Miller and Ordal, 1972).

### *Preparation of the* E. coli *S30 fractions and analysis of their ribosomal profiles*

Strain *E. coli* MG1655 harboring pLBYC8-*infB,* which carries the *E. coli infB* gene under the $P_{tac}$ promoter was grown in LB medium supplemented with 100 µg/ml Amp at 37$^o$C to early-logarithmic phase ($OD_{600}$~0.3). The culture was split into two parts. One portion was used to induce *infB* expression by adding 0.5 mM IPTG and incubation for 30, 60 and 90 min at 37$^o$C. The other portion was induced for 30 min with IPTG followed by addition Ksg to a final concentration of 1 mg/ml and was further incubated for additional 60 min. The cells from each culture (100 ml) were collected by centrifugation (6,000 g, 5 min, 4 $^o$C) and the cell pellets were further suspended in 1 ml of VD buffer (10 mM Tris-HCl, 10 mM $Mg(OAc)_2$, 60 mM $NH_4Cl$, 3 mM β-mercaptoethanol, 100 mM PMSF, 100 mM benzamidine, 100 mM DTT, pH 7.5) containing 10 units/ml DNase I and 0.2 mg/ml lysozyme. Then the cells were disrupted by repeated freezing/thawing steps. To remove cell debris, the cell lysates were cleared by centrifugation (12,000 g, 10 min, and 4$^o$C). The supernatant was withdrawn and re-centrifuged (30,000 g, 10 min, 4 $^o$C). Likewise, the S30 extract from cells that were not induced with IPTG was prepared. Aliquots of the resulting S30 extracts (supernatants) were analyzed by electrophoresis in 12% gels followed by Coomassie blue staining to monitor the amount of IF2. Then the supernatants were separately loaded onto sucrose gradients (7%-47%) prepared in VD buffer. After centrifugation (16 h, 30,000 rpm, 4 $^o$C) in SW40Ti BECKMAN rotor, the presence of ribosomal subunits and 70S ribosome or 61S ribosomal particles in the gradient fractions was examined by recording their absorbance at 260 nm using an ISCO UV/VIS detector (ISCO, US).

### *Preparation of total* E. coli *RNA*

*E. coli* strain MG1655(pRB381-1), MC4100 and MC4100/ΔmazF strains were grown in LB medium at 37°C to an early-logarithmic phase ($OD_{600}$~0.3), then the cultures were divided and further incubated for 90 min at 37°C in the presence or absence of Ksg (1 or 0.05 mg/ml, respectively). In addition *E. coli* MC4100 cells were freshly transformed with pSA1. The cells were grown in LB medium supplemented with Amp (100 µg/ml) at 37°C to mid-logarithmic phase ($OD_{600}$~0.5). The culture was divided, and one part was treated with 1mM IPTG and incubated for 15 min without shaking to induce *mazF* expression. The uninduced cells were used to prepare a negative control.

To isolate total RNA, 10 ml aliquots were withdrawn from each culture and mixed with 1.25 ml of stop solution (5 % water-saturated phenol (pH~7.0) in ethanol). The cells were harvested by centrifugation (6,000 g, 2 min and 4°C. The supernatant was discarded and the pellets were re-suspended in 800 µl of preheated (1-2 min, 64°C) TE buffer (pH 8.0) containing 0.5 mg/ml lysozyme, 1% SDS. The resulting mixtures were further incubated at 64°C for 1-2 min to complete cell lysis. After addition of NaOAc (pH 5.2) to a final concentration of 0.1 M, the samples were extracted twice with hot phenol (pH~7.0, 64°C), phenol/chloroform (1:1) and RNA was precipitated with ethanol. The resulting pellets were dissolved in 200 µl of DNase I reaction buffer (10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT) (Fermentas) containing 10 u RNase-free DNase I , RNase inhibitor (20 U) and incubated at 37 °C for 30 min. The samples were again extracted with phenol, phenol/chloroform to remove DNase I, and ethanol precipitated. The pellets were washed with 1 ml 80% ethanol, dried at room temperature, and suspended in 50 µl RNase-free $H_2O$ (Fermentas). The concentration of total RNAs was determined by using a micro-volume spectrophotometer (NanoDrop2000).

### *Primer extension analysis*

For primer annealing, 0.5 pmol of 5'-[$^{32}$P]-labelled primers were individually mixed with 20 µg of *E. coli* total RNA, heated at 80°C for 3 minutes and snap frozen in liquid nitrogen. The primers used in this study are listed in Table 1. After thawing, each sample was mixed with 4.1 µl extension mix (1 u of AMV, 6 mmol dNTPs) that was preheated at 42°C (or 50°C for IF2 primers) for 3 min and the resulting mixtures were further incubated for 15 min at 42°C (or 50°C for IF2 primers). The reactions were stopped by adding 10 µl of 2X TBE containing 8M urea, 0.025% xylene cyanol FF and 0.025% bromophenol blue. To generate the DNA ladder, *Hinf*I digested ΦX174 DNA (Fermentas) was 5'-end-labelled with $^{32}$P. Before loading onto an 8% polyacrylamide sequencing gel, the samples were preheated at 95°C for 3

minutes. After electrophoresis, the extension signals were detected by using the Molecular Dynamics Phosphoimager.

## Western blot analysis

Aliquots (0.05 u $OD_{260}$) of S30 fractions as well as equivalent amounts of fractions obtained after sucrose gradients were separated in 12% SDS polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-IF2, anti-L2 and anti-S2 antibodies. The signals corresponding to IF2, L2 and S2 polypeptides were visualized using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) as chromogenic substrates.

**Table 1: The sequence of the primers used in the primer extension assays.**

| ***mRNAs*** | ***Sequence*** | ***Coordinates of the complementary region within the infB coding sequence*** |
|---|---|---|
| ***infB*****(IF2-β/γ)** | **CGTGCTTCTTCTTCGAG** | **583-599** |
| ***infB*****(IF2-C terminus)** | **GCCAGAACCGATGATC** | **2157-2172** |

# RESULTS

## *IF2 overexpression stimulates the formation of 61S particles in the presence of kasugamycin*

Translation initiation on lmRNAs is known to involve 70S ribosome particles assembled from 50S and 30S ribosomal subunits prior to their binding to leaderless transcripts (Moll et al., 2002b; Moll et al., 2004). We have recently shown that assembly of 70S monosomes is impaired in *E. coli* cells treated with the aminoglycoside antibiotic Ksg. Instead, exposure to this antibiotic results in formation of 61S ribosome particles that functionally resemble 70S monosomes (i.e. they are able to initiate translation of lmRNAs). However, in contrast to 70S subunits, these ribosomal particles lack more than 6 r-proteins including r-protein S1 and S2 (Kaberdina et al., 2009). Since IF2 actively stimulates the association of the 30S and 50S subunits (Antoun et al., 2003), thus increasing the pool of 70S monosomes, it was tempting to speculate, whether IF2 could stimulate the formation of 61S particles. Moreover, IF2-dependent GTP hydrolysis induces structural rearrangements in the 30S subunit (Marshall et al., 2009), which might result in or induce the loss of the respective proteins to form 61S particles. Therefore, we were interested in investigating whether this translation initiation factor is also able to facilitate the formation of 61S ribosomal particles during Ksg treatment. To test this hypothesis, we analyzed ribosomal profiles of cells grown in the presence and absence of Ksg upon overepression of *infB* using the *E. coli* strain MRE600 carrying the *infB* gene on plasmid pLBYC8-infB.

Analysis of ribosome profiles employing sucrose gradients revealed that in accordance with previous data, overexpression of *infB* resulted in increased levels of 70S ribosomes compared to free 30S and 50S subunits (Figure 1A, B and C). However, upon overexpression of *infB* in the presence of Ksg, we observed the stimulated formation of 61S particles (Figure 1D) when compared to the ribosome profiles in the absence of the antibiotic (Figure 1C). These data indicate that IF2 can stimulate the Ksg-dependent formation of 61S ribosomes *in vivo* even in the absence of lmRNAs. This result is consistent with the previously reported IF2 function to selectively stimulate lmRNAs translation (Grill et al., 2000) by increasing the concentration of 70S ribosomes in the cell which can initiate translation selectively on leaderless mRNAs (Moll et al., 2004). However, addition of Ksg resulted in the formation of a large peak corresponding to 61S particles. When compared to ribosome profile analysis performed in the absence of *infB* overexpression the presence of lmRNA and Ksg shifts the ratio of 70S *vs.* 61S particles upon overexpression of *infB (Figure 1E, (Kaberdina et al., 2009))*.

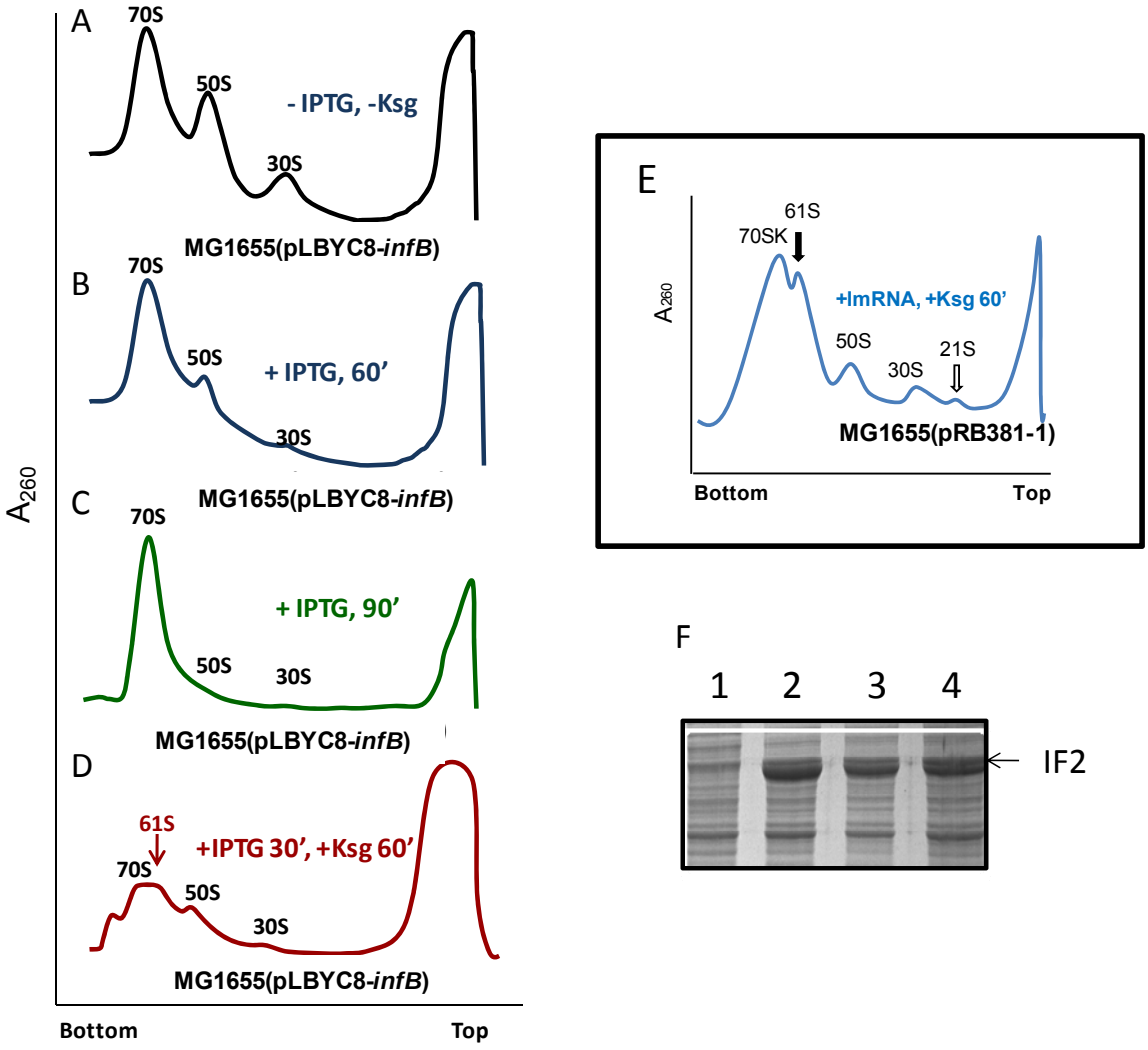


***Figure 1. IF2 stimulates the formation of 61S ribosomes.*** Ribosome sedimentation profiles of the strain MG1655 (pLBYC8-infB) obtained without IF2 overexpression and Ksg treatment (panel A) or upon IF2 overexpression induced by 0.5 mM IPTG for 60 min, 90 min (panels B and C respectively) or for 30 min followed by Ksg treatment (1 mg/ml final concentration) for additional 60 min (panel D). The peak corresponding to 61S particles formed in the presence of Ksg and overexpressed IF2 (panel D) is indicated by a red arrow. A ribosome sedimentation profile of the strain MG1655 (pRB381-1) obtained in the presence of lmRNA and upon Ksg treatment (1 mg/ml final concentration) for 60 min is shown in panel E). The peaks representing 30S and 50S subunits and 70S ribosomes are indicated. The peaks corresponding to 21S and 61S particles formed in the presence of Ksg and lmRNA (panel E) are indicated by open and closed arrows, respectively. Electrophoretic analysis of cell lysates, which were used to obtain ribosome sedimentation profiles shown in panels A to D, is presented in panel F (lanes 1-4, respectively). The position of IF2 is indicated by an arrow.

### *Formation of truncated IF2 polypeptides upon Ksg treatment*

Next, we tested for the binding of IF2 to the ribosome upon Ksg treatment. During the course of these experiments, we observed the appearance of short variants of protein IF2 (Figure 2A, lane 1, 3, 4 and 6) upon Ksg treatment. However, it has been shown that the *E.*

*coli infB gene* encodes three isoforms of IF2, termed IF2-α (97.3 kDa), IF2-β (79.7 kDa) and IF2-γ (78.8 kDa) (Laursen et al., 2002; Nyengaard et al., 1991). Moreover, translation of smaller isoforms (IF2-β and IF2-γ) starts from independent (but in-frame) alternative initiation codons that are just upstream of the sequence coding for domain II (Laursen et al., 2002). In contrast, in phase translation of shorter IF2 variants (85, 70, 30 and 24 kDa, Figure 2A, lane 6) has never been reported previously and no other canonical ribosome binding site is present within the *infB* gene.

Next we used western blot analysis to check for the presence of IF2 in the ribosomal subunits upon Ksg treatment (Figure 2B & 2C). This analysis revealed that the amount of the individual ribosome-bound variants of IF2 was differentially affected by Ksg treatment. As shown in Figure 2C, upon Ksg treatment, the 70 kDa variant of IF2 was present in polysome fraction (Figure 2C, lane 1), 70S ribosomes (Figure 2C, lane 2) and ribosome free top fraction (Figure 2C, lane 5), but not in 50S subunit (Figure 2C, lane 3), which is similar to the results obtained with Ksg untreated cell (Figure 2B, lane 3). Upon Ksg treatment, the amount of the 30 kDa variant of IF2 protein was decreased in 70S subunit but was increased in top (ribosome-free) fractions. In contrast, the amount of the ribosome-bound 85 kDa IF2 polypeptide remained nearly the same after Ksg treatment. In addition, we could not detect the 24 kDa IF2 fragment, and this result could likely be attributed to proteolysis occurred in this experiment.

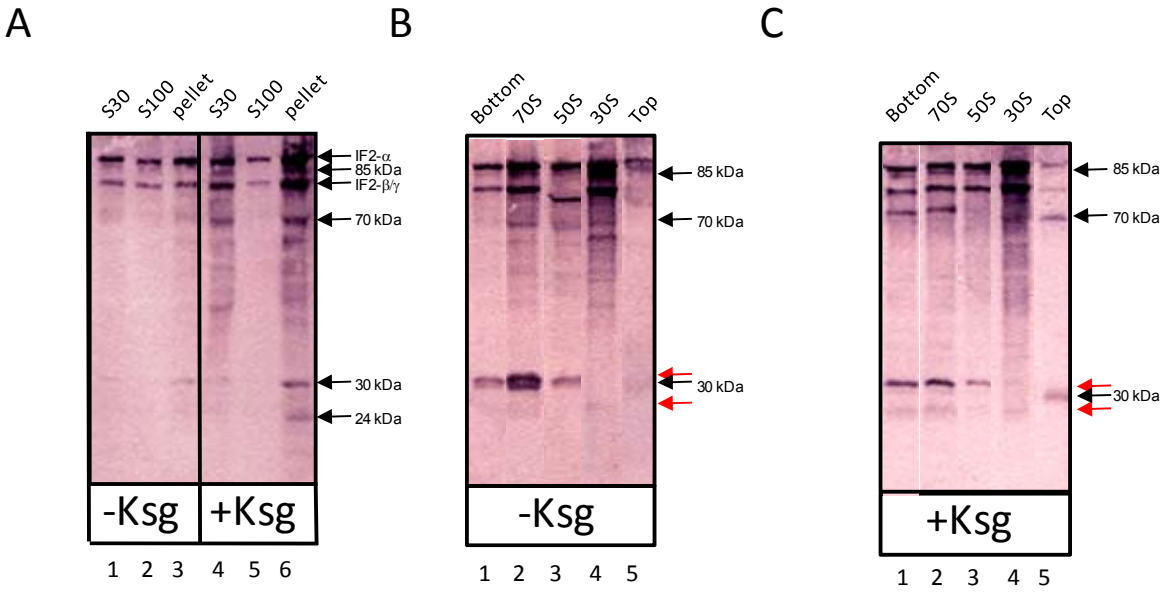


***Figure 2. Effect of Ksg treatment on the level of IF2 truncated polypeptides bound to the ribosomal subunits.*** S30 prepared from E. coli MG1655 (pLBYC8-infB) in the absence (-Ksg) or presence (+Ksg) of Ksg added to a final concentration of 1 mg/ml (see Materials & Methods) were subjected to ultra-centrifugation to separate ribosomes (pellet) and S100 supernatant (A) or alternatively fractionated in analytical sucrose gradients (B and C). Equal protein amounts ($OD_{260}$ 0.05u) of each fraction were analyzed by western blotting using antibodies directed against IF2.

### *Synthesis of* E. coli *IF2 is affected by kasugamycin*

Experiments performed in our laboratory revealed that after prolonged treatment with Ksg (up to 120 min), translation of about 5% of *E. coli* proteins was resumed compared to their inefficient synthesis during the first 60 minutes of Ksg treatment, which can be attributed to the formation of conditional leaderless mRNAs by the RNA interferase MazF upon Ksg treatment (Vesper *et al*, manuscript in prep.). Our finding that IF2 facilitates formation of 61S particles upon Ksg treatment suggests that IF2 might belong to the group of proteins that are still translated in the presence of this antibiotic *in vivo*. To test this possibility, *E. coli* strain MC4100 was grown to an $OD_{600}$ of 0.3 and then treated with Ksg (1 mg/ml). Western blot analysis of total cell lysates using antibody directed against IF2 has shown that Ksg treatment inhibited IF2 translation causing its degradation during the first 30 min of Ksg treatment. However, the level of IF2 isoforms and truncated variants occurred after 120 min of Ksg treatment (i.e. after the number of the translationally active 61S particles has achieved a critical level) (Figures 3A).

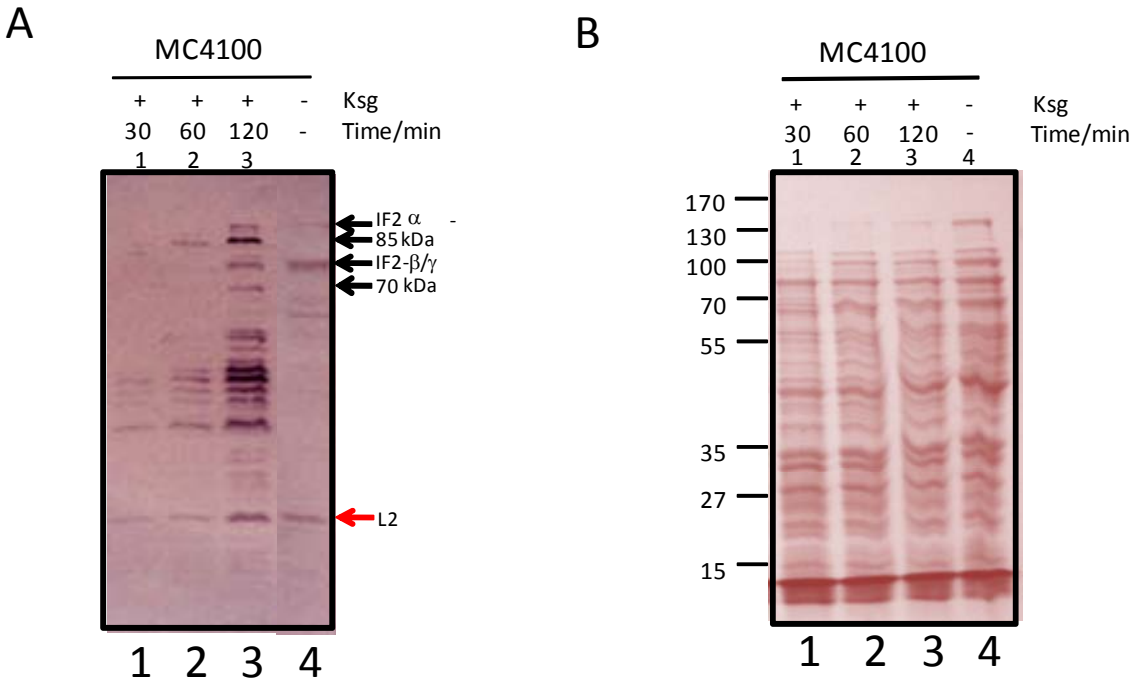


***Figure. 3. Selective IF2 Protein Synthesis in the Presence of the Translation Initiation Inhibitor Ksg* in vivo.** *E. coli* MC4100 cells grown to an $OD_{600}$ of 0.3 and then incubated without Ksg for 120 min (lane 4) or treated with this antibiotic (1 mg/ml) for 30, 60 and 120 min (lanes 1, 2 and 3, respectively), were used to prepare total cell lysates as described in Materials & Methods. Aliquots of each lysate were fractionated in 12% SDS gels and further analyzed by western blotting using antibodies specific for IF2 and L2 (A) or by Coomassie blue staining (B).

In addition, these experiments revealed that Ksg treatment also affects the ratio between the full-length IF2 (IF2-α) and the in-phase translated IF2 variants (IF2-β and IF2-γ) (Figure 3A, compare lane 3 versus lane 4). This finding is consistent with the previous observation that the ratio between the full-length IF2 and IF2-β/IF2-γ is affected by different growth rates (Giuliodori et al., 2004; Howe and Hershey, 1983; Sacerdot et al., 1992).

### *Truncated* infB *transcripts variants accumulated in the presence of Ksg*

The results shown in Figure 3 revealed that, similar to other 5% of *E. coli* proteins (Moll *et al.*, unpublished data), translation of three IF2 isoforms and their truncated variants (85 kDa, 70 kDa and several shorter polypeptides) resumed after Ksg treatment for 120 min. Since Ksg inhibits translation of regular mRNAs with canonical translation initiation signals, the resumed protein translation upon prolonged Ksg treatment was previously attributed to translation of leaderless mRNAs that were formed upon Ksg treatment (Vesper et al, manuscript in prep.). If the same mechanism is accounted for the translation of truncated IF2 variants, than their cognate leaderless mRNAs should be generated *in vivo* by a Ksg-dependent processing of the full-length *infB* mRNA.

To test for the possible formation of *infB* lmRNAs upon Ksg treatment, we used primer extension to map the 5'-end of the putative *infB* leaderless RNAs *in vivo.* In agreement with the anticipated processing of *infB* mRNA, we detected several stops upstream of the translation starting sites of IF2-*β* (79.7 kDa), IF2-$\gamma$ (78.8 kDa) and IF2-C-terminal (23.6 kDa, initiator tRNA binding domain) polypeptides (Fig. 4A and 4B). The predicted sizes of the detected leaderless *infB* mRNA variants indicate that their products of translation should still retain the central GTP binding domain and the C-terminal domain. Two transcriptional stops (positions 476A and 479A) are located exactly in frame with the initiation AUG codon of IF2-$\gamma$, and are both detectable using RNA from S100 fraction (Figure 4A, Lane 8). In addition, one cleavage site (indicated by a red arrow) is predominantly detectable using RNA from the ribosome fraction and is located after the same AUG codon (Figure 4A, Lane 7; Figure 4D). The cleavage at this site may regulate the ratio of the three isoforms of IF2 (Nyengaard et al., 1991) in response to the growth rate or environment stresses (Giuliodori et al., 2004; Howe and Hershey, 1983; Sacerdot et al., 1992).

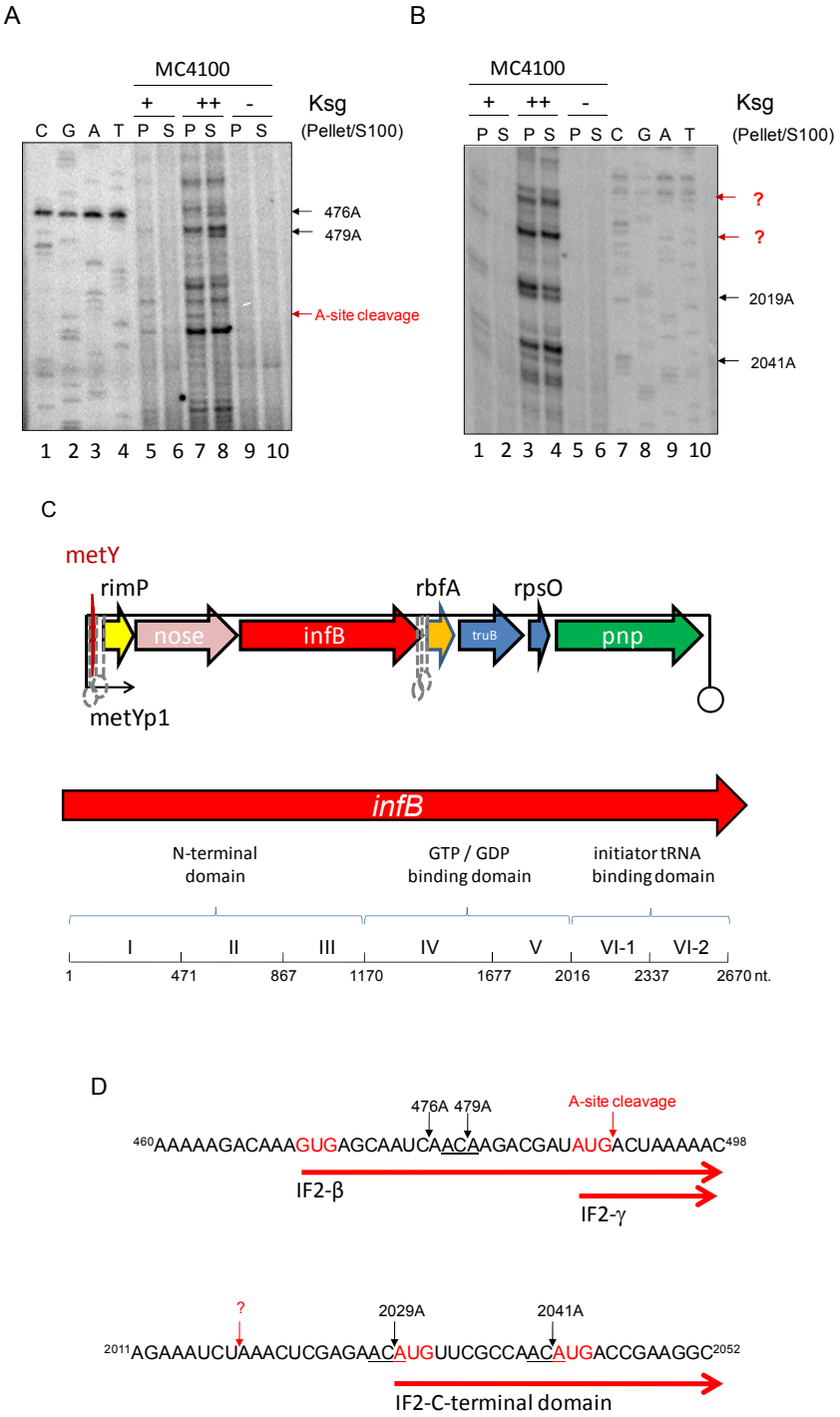


***Figure 4. Mapping the Ksg-dependent cleavage sites in* E. coli infB *mRNA*.** Primer extension analysis with primers complementary to a region located downstream of the internal start codon of IF2-β/γ (A) or region encoding the C-terminal portion of IF2 (B) were performed with total RNA isolated from ribosomal fraction (P: pellet) or S100 extracts (S: S100) prepared from the *E. coli* MRE600(pRBcI-*lacZ)* strain. Cells were grown at 37°C to an $OD_{600}$ of 0.3, and then further incubated in the absence (-) or presence (+) of Ksg for 120 min. The transcriptional stops that correspond to the putative ribonuclease cleavage sites are indicated by arrows. The sequencing ladder (lanes C, G, A, and T) was generated using a primer specific for 23S rRNA. (C) Organization of the *E. coli metY-rimP-nusA-infB-rbfA-truB-rpsO-pnp* operon. The domain structure of IF2 (encoded by the *infB* gene) (Sands et al., 1988) is also shown The nucleotides flanking each functional domain of IF2 are numbered. The portions of the *infB* mRNA comprising the AUG codon of IF2-β/γ (D, top) and part of the C-terminal domain (D, bottom) are indicated by horizontal red arrows within the corresponding sequences of the transcript. The putative MazF cleavage sites and others are indicated by vertical black and red arrows, respectively.

### *MazF overexpression leads to formation of leaderless infB mRNA variants*

Primer extension analysis of *infB* mRNA (Figure 4) as well as other unpublished data from our lab (Vesper et al, manuscript in prep.) revealed that Ksg treatment of *E. coli* cells can lead to formation of conditional leaderless mRNAs *in vivo* and these RNAs are likely generated by endonucleolytic cleavages of their cognate primary transcripts. We also found that some of the Ksg-dependent cleavages within the *E. coli infB transcript* occur in close proximity to ACA triplets known to be recognized by the RNA interferase MazF a toxin component of the mazEF toxin-antitoxin system (Zhang et al., 2005a; Zhang et al., 2003; Zhang et al., 2005b). Due to the presence of the putative MazF cleavage sites at positions 463A, 476A and 479A in the translation initiation region of IF2-$\beta\gamma$ and positions 2029A and 2041A in the putative translation initiation region preceeding the sequence coding for the C-terminal domain of IF2, we proposed that *infB* mRNA might represent a potential target for MazF known to be activated upon inhibition of translation.

To investigate whether MazF is involved in *infB* mRNA processing upon Ksg treatment, we performed primer extension analysis of this transcript using total RNA purified from *E. coli* MC4100 (pSA1-mazF) cells grown without or upon MazF overexpression. Without MazF overexpression, two stop signals corresponded to the 5'-end of the full-length *infB* mRNA and its product of cleavage at position 193A (indicated by red arrows in Figure 5A lane 1). Translation of these RNAs results in the full length IF2 (97.3 kDa) and shorter IF2 polypeptide of 85 kDa initially detected by western blotting *in vivo* (Figure 2 and 3A).

As anticipated, we found that MazF overexpression leads to the formation of the conditional leaderless *infB* mRNAs that lack the 5'-proximal fragments and their 5' ends are positioned at 479A, 2029A and 2041A (Figure 5A, lane 2 and 5C). As shown in Figure 5C, all three transcriptional stops overlap with potential MazF cleavage sites (see Figure 5C) usually located within ACA triplets. One of these stops (479A) is located between the alternative ribosome binding sites of IF2-$\beta$ and IF2-$\gamma$. The putative MazF cleavage at this site would generate a leaderless mRNA encoding IF2-$\gamma$ thereby leading to an increase in the relative level of this isoform upon MazF up regulation *in vivo*.

Two other transcriptional stops (positions 2029A and 2041) were found exactly in front of the two in frame AUG codons (Figure 5B and C). MazF cleavages at these sites will produce leaderless mRNAs encoding the C-terminal domain of IF2 (polypeptides 209 and 213 a.a. in length) with the predicted molecular weights of 23.2 and 23.6 kDa, respectively. The size of these IF2 polypeptides is similar to the size of one of the truncated IF2 fragments (24 kDa) appeared upon Ksg treatment (Figure 2A, lane 6). These data suggest the putative role of MazF in formation of leaderless *infB* mRNA variants that are presumably translated by 61S ribosome particles to produce N-terminally truncated IF2 polypeptides.

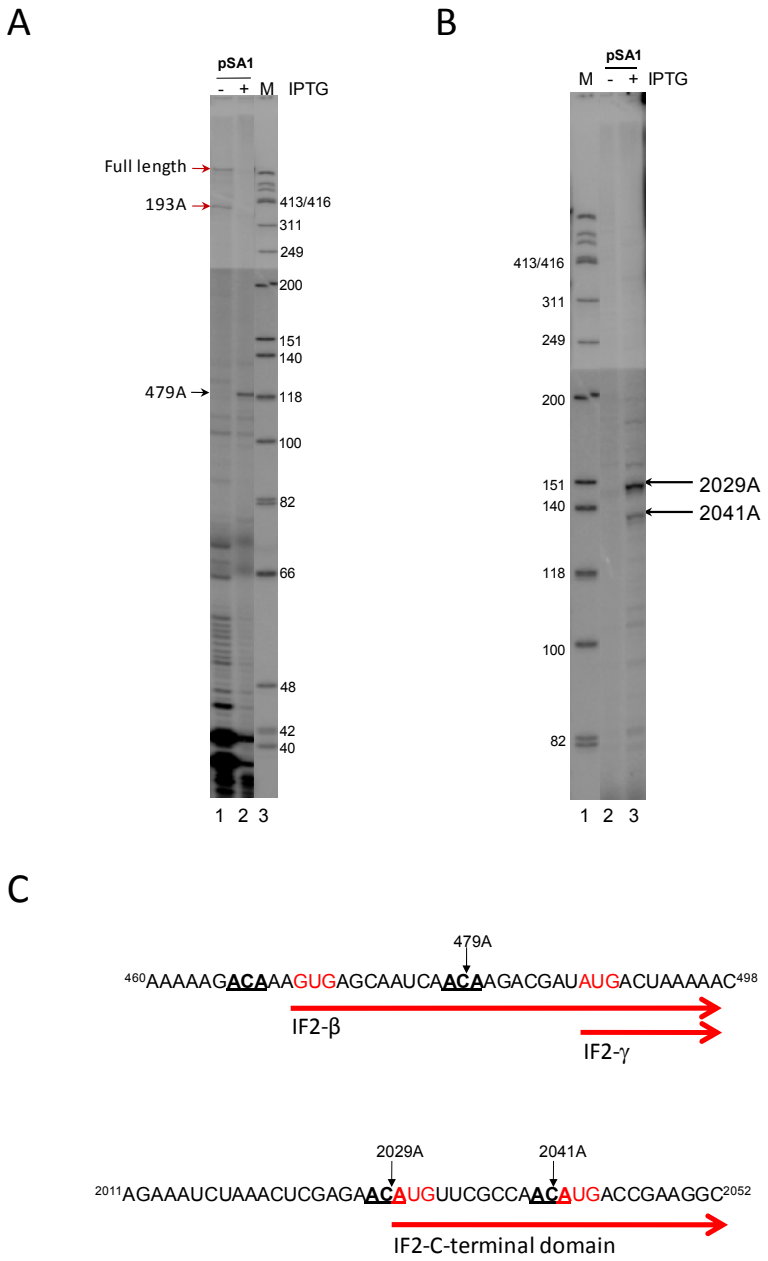


***Figure 5. Mapping the 5'-ends of the putative E. coli infB lmRNA accumulating upon MazF overexpression.*** Primer extension analysis with primers complementary to a region located downstream of the internal start codon of IF2-β/γ (A) or region encoding the C-terminal portion of IF2 (B) was performed with total RNA isolated from *E. coli* MC4100 carrying pSA1 plasmid encoding MazF. MazF overexpression (+) was induced by IPTG addition (1 mM) and incubation for 15 min. The positions of the transcriptional stops that overlap with the putative MazF cleavage sites (in bold) are indicated by black arrows. Lane M: $^{32}$P-labelled *Hinf*I-digest of ΦX174 DNA included as a size marker. (C) Parts of the *infB* coding region comprising the GUG and AUG start codons (highlighted in red) of IF2-β/γ (indicated by red arrows at the top) and part of the C-terminal domain (indicated by a red arrow at the bottom). The transcriptional stops that overlap with putative MazF cleavage sites (in bold) are indicated by vertical black arrows.

# DISCUSSION

The aminoglycoside antibiotic Ksg inhibits protein synthesis at the initial stage of translation (Tanaka et al., 1971). We have recently shown that Ksg specifically inhibits translation initiation on canonical mRNAs by affecting 70S ribosome structure and leading to formation of new ribosome particles (61S ribosomes) that lack several ribosomal proteins and can selectively translate lmRNAs (Kaberdina et al., 2009).

Since previous studies have revealed that the initiation factor IF2 promotes selective translation of leaderless mRNAs (Grill et al., 2000), increases the pool of 70S ribosomes (Antoun et al., 2003) and induces structural rearrangements (Marshall et al., 2009) potentially facilitating the loss of several r-protein to form 61S particles, we were interested to investigate whether IF2 can facilitate formation of the protein-depleted ribosomes upon inhibition of translation with Ksg. Our analysis of ribosome profiles obtained in the absence and presence of Ksg treatment revealed that IF2 overexpression could stimulate formation of 61S ribosomal particles after prolonged incubation with Ksg.

Moreover, we found by western blot analysis that the stimulatory effect of IF2 was paralleled by a decrease in the level of the full-length IF2 and led to accumulation of N-terminally truncated IF2 variants (IF2 polypeptides of 85, 70 and 30 kDa), and these N-terminally truncated IF2 variants retained the capacity to bind to ribosomes. This finding is consistent with the previous observation that the N-terminal domain of IF2 is a non-essential for ribosome binding (Moreno et al., 1999). The C-terminus of IF2 contains the initiator tRNA binding domain and together with the GTP-binding domain stimulates ribosome binding of the initiator tRNA and its accommodation in the P site (Gualerzi et al., 2001). We proposed that some of these N-terminally truncated IF2 polypeptides could be produced *in vivo* by translation of their cognate leaderless mRNAs (*infB* lmRNAs) accumulating upon Ksg treatment. Consistent with this idea, we were able to detect a number of leaderless *infB* transcripts by primer extension.

Accumulation of leaderless *infB* mRNAs can be attributed to endoribonucleolytic cleavage of their primary transcript (i.e. *infB* mRNA) by an endoribonuclease activated by Ksg treatment. We found that the 5' end of several *infB* lmRNAs were in close proximity to ACA triplets known to be recognized by the toxin endoribonuclease MazF, a component of the MazEF toxin-antitoxin system. The endoribonuclease MazF is activated under several stress conditions including inhibition of translation (e.g. during antibiotic treatment), amino acid starvation or in response to heat and oxidative stresses. Under these conditions, most of the *E. coli* mRNAs are known to be degraded by MazF cleavage at ACA sites present in single-strand regions of most mRNAs (Zhang et al., 2003). We therefore hypothesized that some *infB* lmRNAs are generated *in vivo* by MazF-dependent cleavages. In agreement with this idea, we found that the level of *infB* lmRNAs was significantly increased upon

overexpression of MazF and therefore this endoribonuclease likely plays an important role in generation of leaderless *infB* RNAs encoding N-terminally truncated IF2 polypeptides presumably important for cell survival under stress.

Taken together, our data suggest a potential stress adaptation mechanism under adverse conditions. We have shown here that Ksg treatment induces the MazF-dependent cleavage of *infB* mRNA within its coding region and in close proximity to the in frame AUG codons, thereby leading to formation of leaderless transcripts. Translation of these lmRNAs by protein deficient ribosomes results in production of truncated IF2 polypeptides that possess the C-terminal domain of this protein, which can interact with the initiator tRNA (Gualerzi et al., 2001) thereby leading to predominant translation of lmRNAs. It could be envisaged that synthesis of truncated IF2 variants affects the specificity of the translational machinery under stress conditions. The formation of alternative ribosome particles that selectively translate specific mRNAs under stress conditions is consistent with the ribosome filter hypothesis proposed by Mauro *et al*. (Mauro and Edelman, 2002). In their work, Mauro *et al.* refer to a mechanism, which regulates cell differentiation in eukaryotic cells by employing ribosome particles composed of different sets of r-proteins (Ramagopal and Ennis, 1981). They proposed that ribosomal proteins that are present on the ribosome in duplicate have additional roles in translation by affecting localization of particular mRNAs (Amikura et al., 2001; Komili et al., 2007). Another example of similar regulation is described for ribosomes deficient in P-proteins (eukaryotic L7/L12 orthologs), which, unlike ribosomes that have P-proteins, selectively translate a subset of mRNAs present in the stationary phase (Remacha et al., 1995; Saenz-Robles et al., 1990).

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## 6. 3. Direct interaction between ribosomal proteins S1 and S2 in *Escherichia coli*

## *SUMMARY*

Ribosomal protein S1 is essential for translation in *Escherichia coli* and apparently in many pathogenic species belonging to gamma subdivision of proteobacteria. Unlike many other ribosomal proteins, protein S1 is not evolutionarily conserved and no functional homologues exist in eukaryotic cells and Gram-positive bacteria which possess genomes with low G/C-content. Previous studies proposed that binding of protein S1 to the ribosome was assisted by a protein-protein interaction mediated by ribosomal protein S2. Moreover, it has been shown that the N-terminal domains of ribosomal protein S1 are essential for the binding to the ribosome. Therefore, we further characterized the S1/S2 interaction using C-terminally truncated variants of S1 ($S1_{106}$, residues 1-106; and $S1_{194}$, residues 1-194) by (i) analyzing the effects of their overproduction on ribosome functions *in vivo*, (ii) by Far-western blot analysis, and (iii) NMR probing with reporter ligands and affinity tags. The results presented here indicate that S1 and S2 can directly interact with each other. Furthermore, the truncated variants of S1, $S1_{106}$ and $S1_{194}$, bind S2 in the same manner as the full-length S1 protein. Moreover, we show that overproduction of $S1_{1\text{-}106}$ inhibits bacterial growth. This effect could be attributed to the ability of this polypeptide to substitute endogenous S1, thus yielding translational inactive ribosomes. Collectively, our data indicate that S1 and S2 interact directly and suggest that their binding interface can be considered as a perspective target for novel antimicrobial compounds selectively acting against Gram-negative pathogens.

## *INTRODUCTION*

In the past six decades, antibiotics have been indispensable to treat bacterial infections. However, their extensive use led to the appearance and rapid spread of drug-resistant pathogens, causing a major problem in public health care (Diekema et al., 2004; Wisplinghoff et al., 2004). Due to its pivotal role in gene expression and its complex structure, the ribosome carries many sites for functional interference, and is therefore a major target for most of the clinically important antibiotics (Brodersen et al., 2000; Carter et al., 2000; Steitz, 2005).

Previous studies revealed that many of these antibiotics predominantly target ribosomal RNAs, thus affecting their functional sites, which are highly conserved across all kingdoms of life (Mankin, 2000; Sutcliffe, 2005). This high conservation of functionally important sites on the ribosome restrains the clinical use of several antibiotics due to their toxic side effects. Moreover, despite the theoretically large number potential drug target sites, there are only few sites where antibiotics interfere with ribosome function (Mankin, 2006; Yonath, 2005). As a result, an acquired resistance to one antibiotic can often confer resistance to other antibiotics.

The ribosomal protein (r-protein) S1 is essential for translation of canonical mRNA in *E. coli* (Sorensen et al., 1998) and other Gram-negative bacteria (Szer et al., 1975) . It binds to a pyrimidin-rich region in the 5' untranslated region (5'-UTR) upstream of the Shine-Dalgarno (SD) sequence of canonical ribosome binding site (Sengupta et al., 2001). In contrast, protein S1 or its functional homologues are absent in ribosomes that perform protein synthesis in eukaryotic cells and organelles. This fact implies that compounds that interfere with the function of protein S1 or with its binding to the ribosome may have less or no side effect on eukaryotic cells. In addition, such drugs could be selective against a number of Gram-negative pathogens without affecting the majority of the beneficial Gram-positive flora, as many of these species do not possess homologues of S1.

It has been shown that S1 is the only r-protein that binds to the 30S subunit *via* protein-protein interaction (Boni et al., 1982). Despite significant progress in analyzing the ribosome structure, the structure of protein S1 and its binding site on the 30S subunit has not been analyzed due to its intrinsic flexibility. Nevertheless, biochemical and genetic studies were able to provide some insights in the nature of the interaction between 30S particles and S1, which suggested that this interaction could be mediated by the r-protein S2 (Moll et al., 2002). This notion was initially based on results of cross-linking experiments indicating a direct interaction between these proteins (Laughrea and Moore, 1978). Moreover, analysis of a temperature sensitive *E. coli* mutant that carried a conditionally lethal mutation affecting the *rpsB* gene coding for

ribosomal protein S2 (Bollen et al., 1979) indirectly affected S1 expression. These data are in agreement with the recent observation that preparations of the *E. coli* ribosome, which lack ribosomal protein S2, are likewise devoid of S1 (Moll et al., 2002). Collectively, these findings and recent analysis of the assembly landscape of the 30S subunit (Sykes and Williamson, 2009) suggest that S2 is required for the binding of protein S1 to the ribosome.

Since the putative S1/S2-interaction appears to be essential for S1 binding to the ribosome, we have used biochemical and biophysical approaches for further characterization. Our analysis confirmed the interaction between S1 and S2 *in vivo* and *in vitro*. Therefore, these results support the notion that this interaction surface could serve as a potential target for the design of novel antimicrobial compounds acting semi-selectively against Gram-negative pathogens.

# *MATERIALS AND METHODS*

## *Bacterial strains, plasmids and growth conditions*

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultivated in Luria-Bertani medium (Miller and Ordal, 1972) at 37°C. When appropriate, kanamycin (20 µg/ml) or ampicillin (100 µg/ml), was added. Where indicated, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM or 1 mM.

**Table1. Strains and plasmids used in this study.**

| ***Strains and Plasmids*** | ***Description*** | ***Reference*** |
|---|---|---|
| *E.coli strains* | | |
| Rosetta(DE3)pLysS | $F^-$ ompT $hsdS_B$ (*$rB^-$ $mB^-$)* gal dcm λ(DE3 [*lacI lacUV5*-T7 *gene 1 ind1 sam7 nin5*]) pLysSRARE (*$Cam^R$*) | Novagen |
| Tuner | $F^-$ ompT $hsdS_B$ (*$rB^-$ $mB^-$)* gal dcm lacY1 | Novagen |
| DH5α | $F^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17($r_K^-$ $m_K^+$), λ– | *(Meselson and Yuan, 1968)* |
| TOP10F' | F'[lacI$^q$ Tn10(tet$^R$)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str$^R$) endA1 λ$^-$ | *(Durfee et al., 2008)* |
| Plasmids | | |
| pJS418 | encodes N-terminal 116 amino acid residues of r-protein S1 of *E.coli* K12 under ptac promoter, Amp$^R$ | (Schnier et al., 1986) |
| pET22b (+) | 5.4 kb *E.coli* expression vector His-tag at C-terminal, T7 promoter, Amp$^r$ , IPTG induction | Novagen |
| pET22b-S1-106 | pET22b:: 318 bp PCR product containing $S1_{-106}$ gene. | This work |
| pET21b-S1-194 | pET21b:: 582 bp PCR product containing $S1_{-194}$ gene. | (McGinness and Sauer, 2004) |
| pET42b-SH2 | pET42b:: 321 bp PCR product containing SH2 gene. | (Ludwiczek et al., 2004) |
| pET22-rpsB | pET22b:: 723 bp PCR product containing S2 gene. | This work |
| pET42-SH2-S2 | pET42b-SH2:: 723 bp PCR product containing S2 gene. | This work |
| pET42-SH2-S1-194 | pET42b-SH2:: 582 bp PCR product containing $S1_{-194}$ gene. | This work |
| pBAD | *ara*BAD promoter (PBAD), Provides tight, dose-dependent regulation of heterologous gene expression | (Guzman et al., 1995) |
| pBAD-S2 | pBAD:: 723 bp PCR product containing S2 gene. | This work |

### *Construction of plasmids*

To obtain plasmid pET22-$S1_{106}$, the DNA fragment encoding the N-terminal 106 amino acid residues of the r-protein S1 was amplified using plasmid pJS418 (Schnier et al., 1986) as a template and primers R29-fw-rpsA 5´- TATACATATGACTGAATCTTT TGCTCAACTCTTTGAAG-3' and S29-rev-$rpsA_{116}$ 5´-TATACTCGAGTTCAGCATCTT CGTAAGCTTTTTCCAGC-3´. The amplified fragment was digested with NdeI/XhoI and ligated into a *Nde*I/*Xho*I-treated pET22b (+) vector (Novagen), thus creating the plasmid pET22-$S1_{106}$. The transcription of the $S1_{106}$ protein gene in pET22b-$S1_{106}$ is controlled by a T7-promoter and the His-6 tag is fused to the C-terminus of protein. The resulting plasmid was transformed into *E. coli* Rosetta (DE3)/pLysS cells (Novagen).

To generate a plasmid for the synthesis of the SH2-S2 fusion protein, a DNA fragment containing the full-length S2 protein with flanking *Nco*I and *Bam*HI restriction sites was generated by PCR using pET-rpsB as a template and primers G29-fw 5'-TATACCATGGCAACTGTTTCCATGCGCGAC -3' and H29-*Bam*HI 5' -TATGGATCC TTACTCAGCTTCTACGAAGCTTTCTTCCGCC-3'. The resulting PCR product was treated with *Nco*I and *Bam*HI and ligated into *Nco*I / *Bam*HI -treated pET42-SH2 (Ludwiczek et al., 2004). The resulting plasmid was transformed into *E. coli* Tuner cells (Novagen).

To generate a plasmid for the synthesis of the SH2-$S1_{194}$ fusion protein the DNA fragment coding for the N-terminal 194 amino acid residues of S1 and flanking *Nco*I and *Bam*HI restriction sites was generated by PCR using pET21b-$S1_{194}$ as a template (McGinness and Sauer, 2004) and primers N33-SH2-S1his-fw 5'-TATACCATGACTGAATCTTTTGCTCAACTCTTTGAAGAGTCC-3' and T7 terminator primer (Novagen). The PCR product was digested with *Nco*I and *Bam*HI and further ligated into *Nco*I / *Bam*HI-treated pET42-SH2 (Ludwiczek et al., 2004). The resulting plasmid was transformed into *E. coli* Tuner cells (Novagen).

To generate the plasmid for the synthesis the 6His-tagged S2 protein, a DNA fragment coding for the full length S2 protein with flanking *Xho*I and *Hind*III restriction sites was generated by PCR using pET22-rpsB as a template and primers N39 and O39. The PCR product was further digested with *Xho*I and *Hind*III and ligated into *Xho*I and *Hind*III-treated plasmid pBAD (Guzman et al., 1995). The resulting plasmid was transformed into *E. coli* TOP10 strain.

### *Expression and purification of S1 variants and $S2_{6His}$ fusion protein*

5 ml overnight culture of *E. coli* Rosetta pLysS cells harbouring pET22b-$S1_{106}$ and pET21b-$S1_{194}$ plasmids were used to inoculate 5 l LB medium supplemented with ampicillin (100 µg/ml). Cells were grown with shaking at 37°C until the cultures reached

an $OD_{600}$ ~ 0.6 to 0.8, and IPTG was added to a final concentration of 1 mM. The cells were further incubated with shaking for 2 hours at 37°C and harvested by centrifugation for 10 min at 6,000 g. For purification of $S2_{6His,}$ 5 ml overnight culture of *E. coli* Top10F' cells harbouring pBAD-$S2_{6His}$ was used to inoculate 5 l LB medium supplemented with ampicillin (100 µg/ml). Cells were grown with shaking at 37°C until the cultures reached an $OD_{600}$ ~ 0.6 to 0.8, and L-arabinose was added to a final concentration of 0.04%. The cells were further incubated with shaking for 4 hours at 37°C and harvested by centrifugation for 10 min at 6,000 g.

The supernatant was removed and the cell pellet was resuspended in 50 ml of lysis buffer (10% glycerol, 50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 100 mg/ml lysozyme, 24 mg/ml RNase A, pH 8.0). The cells were lysed by sonication with ten 1 min sonic bursts with intervening periods of 30 sec (Heat Systems Ultrasonics Cell Disruptor W375; settings: 50% amplitude, intensity 4; pulsed). The cell lysates were centrifuged for 15 min, 4°C at 16,400 g. The supernatant (cytoplasm fraction) was collected and filtered through a 0.45-µm-pore-size-filter (Millipore). The filtrate was mixed with 2 ml of Ni-NTA agarose (Qiagen, US), which was initially pre-equilibrated with binding buffer (10% glycerol, 50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0) at 4°C for 1 hour. The column was washed with buffer (1.5 M NaCl, 50 mM $NaH_2PO_4$, 0.1 % Triton-X 100, 20 mM imidazole, pH 8.0), and the individual polypeptides were eluted with the elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions containing the respective protein were collected and dialyzed twice against 1 L NMR-binding buffer (10 % glycerol, 20 mM $NaH_2PO_4$, 100 mM NaCl, 1 mM DTT pH 7.0) or 1x VD buffer (10 mM Tris-Cl, 10 mM $Mg(OAc)_2$, 60 mM $NH_4Cl$, 3 mM β-mercaptoethanol, 100 mM PMSF, pH 7.5) for 12 hour at 4°C.

### *Expression and purification of the SH2-S2 fusion proteins*

5 ml overnight culture of *E. coli* Tuner (DE3) carrying pET42-SH2-S2 plasmids were used to inoculate 5 l LB medium supplemented with kanamycin (50 µg/ml). Cells were grown shaking at 37°C until they reached an $OD_{600}$ ~ 0.3, and IPTG was added to a final concentration of 0.5 mM. Cultures were further incubated for 12 to 14 hours at 28°C with shaking and the cells were collected by centrifugation for 10 min at 6,000 g. The cell pellet was resuspended in 50 ml of the lyses buffer (10% glycerol, 20 mM $NaH_2PO_4$, 100 mM NaCl, 1 mM DTT pH 7.0), the cells were lysed by sonication, after centrifugation for 15 min, 4°C at 16,400 g, the supernatant containing the overexpressed polypeptide was filtered through a 0.45-µm-pore-size filter (*Millipore). The filtrate was mixed with 12 ml cation exchange cellulose resin (Sigma)* which was initially equilibrated with binding buffer (10% glycerol, 20 mM $NaH_2PO_4$, 150 mM NaCl,

1 mM DTT pH 7.0) at 4°C for 1 hour. The gel slurry was loaded on a column; the column was further washed with 60 ml binding buffer, and then SH2-S2 fusion proteins were eluted with the elution buffer (10% glycerol, 20 mM $NaH_2PO_4$, 2 M NaCl, 1 mM DTT pH 7.0). The fractions containing SH2-S2 fusion protein were combined and run through a gel-filtration column to get rid of additional impurities and to replace the elution buffer with the buffer (10% glycerol, 20 mM $NaH_2PO_4$, 0.1 M NaCl, 1 mM DTT pH 7.0) for NMR analysis.

### *Purification of crude ribosomes upon overexpression of the S1 variants, $S2_{6His}$ and SH2-S2 fusion protein*

Overexpression of recombinant polypeptides has been induced essentially as described in the previous section. The cells were collected by centrifugation at 6,000 g and the cell pellet was further resuspended in 1 ml of VD buffer (10 mM Tris-Cl, 10 mM $Mg(OAc)_2$, 60 mM $NH_4Cl$, 3 mM β-Mercapto-EtOH, 100 mM PMSF, 100 mM Benzamidine, 100 mM DTT, pH 7.5) containing 10 units/ml DNase I and 0.2 mg/ml lysozyme. Then the cells were disrupted by repeated freezing/thawing steps. To remove cell debris, the cell lysates were cleared by centrifugation at 12000 x g for 10 min. The supernatant was withdrawn and re-centrifuged at 15000 x g for 30 min. 0.5 ml of the supernatant was layered onto the same volume of the 1.1 M sucrose in VD buffer. After centrifugation at 100,000 x g for 4 hours in Beckman TLS-100 rotor, the supernatant was discarded and the remaining pellet containing crude ribosomes was resuspended in 1 ml VD buffer and stored at -80°C. To control the quality of the purified proteins, equal amounts of total protein from cell lysate, supernatant and pellet fractions, respectively were loaded on two 12% SDS polyacrylamide gels. One gel was stained with Coomassie blue, and the other was used to transfer the separated proteins to a nitrocellulose membrane (Whatman, Germany), which was used for Western blotting to detect S1 variants fragments and S2 fusion protein with anti-S1, anti-S2 or anti-His antibodies.

### **In vitro** *reconstitution of 30S(-S1) ribosomes with truncated protein S1 variants*

The 30S ribosomal subunits were prepared essentially as described by Spedding (Spedding, 1990) and depleted protein S1 by affinity chromatography (Subramanian, 1983) on poly (U)-Sepharose 4B (Pharmacia).

The protein S1 depleted 30S subunits and purified protein S1 variants were incubated with in a 1:2 ratio at 37°C for 20 min followed by loading onto a sucrose gradient (10%-30%) prepared in VD buffer. Upon centrifugation for 5 h at 45000 x g,

the gradient fractions were examined using an ISCO UV/VIS detector (ISCO, US). The fractions containing reconstituted 30S ribosomes were treated with 5% TCA at 4°C and precipitated proteins were separated on a 12% SDS-PAGE as described by Laemmli (1970). The protein S1 variants bound to the 30S ribosomes were detected by Coomassie blue staining.

### *Western blot analysis of proteins co-purifying with S1 and its truncated variants*

Proteins co-purifying with protein S1 variants were separated in polyacrylamide SDS gels, transferred to a nitrocellulose membrane, and further determined by goat polyclonal antibodies specific for ribosomal proteins.

### *Far-western blot analysis*

To determine protein-protein interactions using Far-western blotting, 2.5 µg total cell extracts of *E. coli* cells overexpressing the SH2-S2 fusion protein were separated in a 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane, and the membrane was washed with 1XPBS to remove SDS. Then proteins bound to the membrane were refolded by incubation in 6M and then 3M Guanidine-HCl in AC buffer (10% glycerol, 100mM NaCl, 20 mM Tris, 0.5 mM EDTA, 1mM DTT and 0.1% Tween-20) supplemented with 2% milk powder for 30 min at room temperature. Then the blot was washed with 1M (and then 0.1M) Guanidine-HCl in AC buffer supplemented with 2% milk powder for 30 min at 4ºC. The membrane was quickly rinsed in AC buffer and then was blocked in AC buffer containing 2% skim milk at 4ºC for overnight. After incubation the blocked membrane with 300, 30, 3 µg/ml or without bait proteins (protein S1 or $S1_{106}$) at 4ºC for 2-3 hours, the membrane was washed twice wash with AC buffer for 10 min at room temperature and further incubated with anti-S1 antibody in AC buffer with 2% milk. After two times washing with AC buffer for 10 min at room temperature, it was incubated for 30 min with the secondary goat antibody conjugated with alkaline phosphatase and further visualized using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrayolium (NBT) as chromogenic substrates.

### *NMR probing of protein-protein interaction using reporter ligands and affinity tags*

The SH2 domain from PLCγ1 (Manning et al., 2003), which binds with high specificity to polypeptides containing phosphotyrosine residues, was used as a ligand binding domain, which was in turn fused to the protein S2 to yield SH2-S2 respectively. The phenyl phosphate was chosen as a reporter ligand to ensure fast exchange rate.

The dissociation constant ($K_D$) of the complex formed by the ligand and SH2-S2 with potential binding partners ($S1_{106}$ and $S1_{194}$) were studied *in vitro* at 25ºC in the 500 µl binding buffer (10% glycerol, 20 mM $NaH_2PO_4$, 150 mM NaCl, pH= 7.0). The selective inversion and excitation were measured using IBURP (22 ms, γB1= 250 Hz) and EBURP (20 ms, γB1= 215 Hz) pulses. The interaction was monitored by selective T1 measurements of the aromatic proton in *meta* position to the phosphate group of the ligand every 200 ms after polarizing their nuclear spin by a pulse (time 0). T1 indicate time points of the spin's ''relaxation'', i.e. returning back to the state when their spins are parallel in the static magnetic field. The relaxation rates are defined as R1=1/T1. The concentration of the ligand, phenyl phosphate was 250 µM, and the binding protein partner (protein SH2-S2, protein S1 variants, and protein $S2_{6His}$) was 50 µM. The Varian Unity Inova 800MHz spectrometer was used to obtain the inversion recovery spectra by characterizing 64 transitions with a relaxation delay of 5 s (Ludwiczek et al., 2004).

# *RESULTS*

## Overexpression of $S1_{106}$ affects growth of *E. coli*

It has been shown that in *E. coli* the N-terminal domain of protein S1 comprising the first two domains (residues 1-194) binds to the ribosome and displaces endogenous protein S1, which leads to inhibition of protein synthesis and cessation of cell growth (McGinness and Sauer, 2004). To determine whether the very N-terminal domain of protein S1 consisting of 106 amino acids from the N-terminus inhibits cell growth (Figure 1B and 2A), the *E. coli* strain Tuner harboring plasmid pET22-$S1_{106}$, which codes for the truncated protein S1 was grown in LB and overexpression of variant $S1_{106}$ was induced by addition of IPTG. The results shown in Fig. 2A demonstrate that overproduction of $S1_{106}$ significantly inhibits *E. coli* growth. Nearly the same effect was observed upon overexpression of $S1_{194}$. In contrast, overexpression of the full-length S1 resulted only in a slight reduction of growth rate.

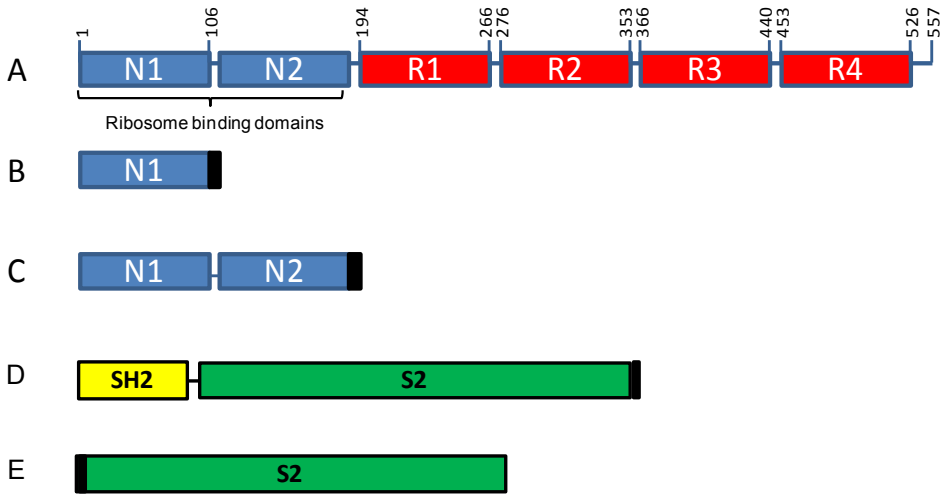


***Figure 1. Schematic representation of S1, variants of S1 and SH2-tagged S2 used in this study.***
(A) The domain structure of the *E. coli* ribosomal protein S1. The N-terminal domains that are required for binding to the ribosome (Giorginis and Subramanian, 1980) and the C-terminal RNA-binding domains (Bycroft et al., 1997) are indicated by blue and in red boxes, respectively. The numbers correspond to the first and the last amino acid of each domain. The C-terminally truncated variants of S1 comprising the first 106 (B) and 194 (C) amino acids as well as the full-length S2 harbouring an N-terminal 6His-tagged (indicated by black boxes) (E), and the SH2 tagged-S2 fusion protein (D).

## *The binding of $S1_{106}$ and $S1_{194}$ to the ribosome* in vivo

In order to confirm the binding of proteins $S1_{106}$ and $S1_{194}$ to the ribosome *in vivo*, ribosomes were isolated by ultra centrifugation from *E. coli* cells individually overexpressing these polypeptides. The proteins of the ribosome-free supernatant

fractions and cognate ribosome-containing pellets were individually analyzed by electrophoresis on a 12% SDS polyacrylamide gels and by Western blot analysis (Fig. 2C and D). The presence of the $S1_{106}$ and $S1_{194}$ proteins in the ribosome fractions indicates that proteins $S1_{106}$ and $S1_{194}$ are able to associate with ribosome. Therefore, the toxicity of the synthesis of $S1_{106}$ and $S1_{194}$ proteins could be attributed to the accommodation of the truncated protein S1 within the binding pocket of full length protein S1 on the 30S subunit. Consequently, this binding would inhibit assembly of endogenous protein S1 to the ribosome. Taken together, our data suggest that the N-terminal domain of protein S1 ($S1_{106}$) is sufficient for the interaction with the ribosome.

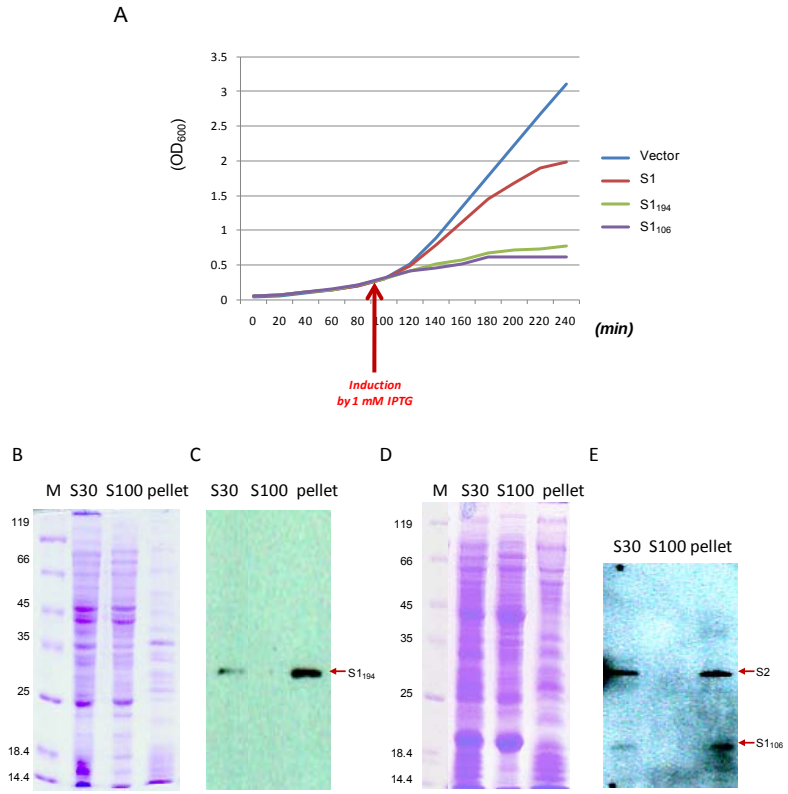


***Figure 2. The synthesis of $S1_{106}$ and $S1_{194}$ causes cell death by binding to the ribosome.***

(A) Growth of *E. coli* cells synthesizing $S1_{106}$. The cells carrying the corresponding plasmids (pS1, pET22b- $S1_{106}$, and pET21b- $S1_{194}$) harboring genes encoding for proteins S1, $S1_{106}$ or $S1_{194}$, respectively, were grown in LB. At $OD_{600}$ ~0.3 synthesis of the corresponding proteins was induced with 1 mM IPTG. Upon induction of the gene encoding $S1_{106}$ and $S1_{194}$, cessation of growth was observation. Overexpression of the *rpsA* gene encoding for the full length S1 protein only slightly affected cell growth. Ribosomes were isolated by ultra centrifugation from cultures expressing $S1_{106}$ or $S1_{194}$. The same amount of total protein from the S30 extract, the pellet and the S100 supernatant fractions, respectively, was separated on a 12% SDS gel and protein S1 and S1 variants were detect by Western blot analysis with anti-His (C) or with additional anti-ribosomal protein S2 (E) antibodies, or by staining with Coomassie blue (B) and (D). The position of $S1_{106}$, $S1_{194}$ and S2 are indicated by arrowheads.

### *The interaction between protein S1 variants and S2* in vivo

Previous work (Sengupta *et al*., 2001; Moll et al., 2004) and our biochemical data strongly suggest the existence of direct contacts between ribosomal proteins S1 and S2. Moreover, our experimental data also show that interaction between these proteins can take place when the full-length S1 is substituted by its C-terminally truncated variants, $S1_{106}$, and $S1_{194}$. To further characterize this interaction, we have employed the previously developed technique that enables to probe protein-protein interactions by NMR using SH2-tagged polypeptides (Ludwiczek et al., 2004). The S1 variants and the SH2-S2 proteins were purified as described in Materials and Methods. Howerver, during the purification procedure, several proteins were found to be co-purified with the overexpressed S1 variants (Fig. 3). These copurified proteins were found even upon increasing the stringency of the procedure. In addition, the cell lysates were treated with micrococcal nuclease prior to loading onto Ni-NTA columns. This step prevents contamination with RNA and excludes the interaction of proteins indirectly *via* RNA. Using Western blot analysis, we determined some of the co-purifying polypeptides to be ribosomal proteins. As shown in Fig. 3 ribosomal proteins S2, and S15 were found to co-purify with $S1_{106}$ and $S1_{194.}$ These results further support the interaction between proteins S1 and S2. However, experiments performed in our laboratory revealed that in solution the $S1_{106}$ is dimerized, and this may attributed to the results that compare with $S1_{194,}$ more ribosomal proteins (S5, S9, S10, S11 and S12 ) were found to co-purify with $S1_{106}$.

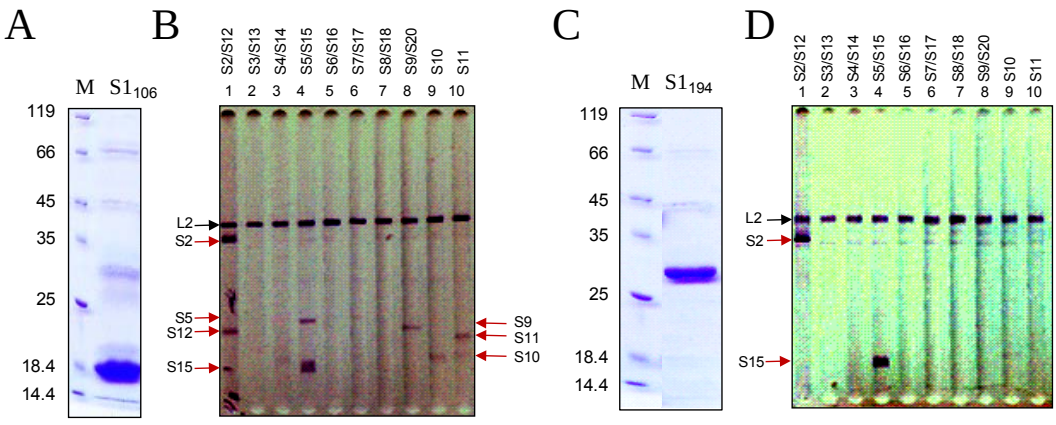


***Figure 3. Ribosomal proteins co-purifying with $S1_{106}$ and $S1_{194}$.***

S1 variants were purified by IMAC and analyzed by electrophoresis in SDS polyacrylamide gels followed by staining with Coomassie blue (panels A and C, respectively). The same preparations of $S1_{106}$ and $S1_{194}$ polypeptides were also analyzed by western blotting using antibodies specific for ribosomal proteins (panels B and D, respectively). (B) $S1_{106}$ and (D) $S1_{194}$ protein. The anti-r-protein antibodies are indicated. Ribosomal protein L2 containing multiple histidine residues which bind to the Ni-NTA resin (Graslund et al., 2008) was use as a loading control.

### *$S1_{106}$ and $S1_{194}$ bind to the 30S ribosomal subunit depleted for protein S1* in vitro

To further support our *in vivo* results, the binding of the purified proteins $S1_{106}$ and $S1_{194}$ to 30S(-S1) has been tested *in vitro.* Protein S1 depleted 30S subunits were incubated with a 10-fold excess of protein S1 variants for 30 min on 37ºC and subsequently separated on 10-30% sucrose gradients. Upon centrifugation the fractions were analyzed as described in Materials and Methods. The fractions containing the 30S subunits were tested for the presence of the protein $S1_{106}$ or $S1_{194}$ by SDS-PAGE. To ensure that the proteins were not precipitated during this procedure and to show the amount of free S1 variants proteins, the bottom and the top fractions were examined as well. The results of the sucrose density gradient centrifugation experiments (Fig. 4) revealed that both purified protein $S1_{106}$ and $S1_{194}$ can bind to 30S subunits *in vitro*.

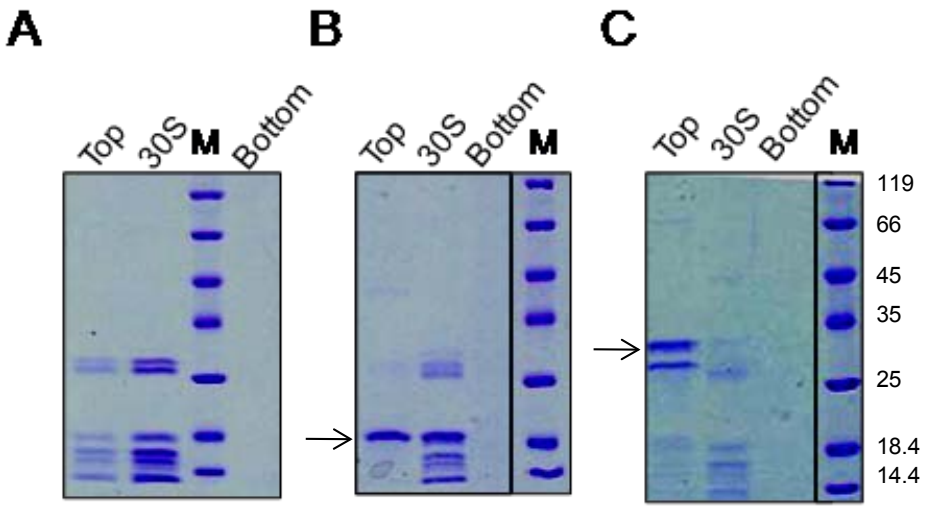


***Figure 4. Reconstitution of 30S subunits containing $S1_{106}$ and $S1_{194}$.***

The ribosome binding properties of $S1_{106}$ and $S1_{194}$ were examined in ribosome reconstitution experiments. The S1-depleted 30S ribosomes (5 pmol) were incubated without (A) or with 50 pmol of protein $S1_{106}$ (B) or $S1_{194}$ (C) for 30 min at 37ºC. The resulting mixtures were layered on 10% to 30% sucrose gradients. After centrifugation the reconstituted 30S ribosome fractions (indicated as 30S) and the top fractions which contained free proteins (indicated as Top) were collected and analysis by SDS-PAGE followed by Coomassie blue staining. The location of each S1 varients is indicated by arrowheads.

### *The 6His-tagged and SH2-tagged S2 proteins are able to associate with the ribosome* in vivo

The interaction between protein $S1_{106}$ and protein S2 will be studied *in vitro* employing 6His-tagged or SH2-tagged variants of protein S2. In order to test for their

functionality *in vivo*, we tested whether purified 6His-tagged and SH2-tagged proteins S2 could be assembled into the ribosome *in vivo*. Upon overexpression of these proteins individually, ribosomes were isolated by ultra centrifugation. The proteins of the ribosome-free supernatant fractions and cognate ribosome-containing pellets were individually analyzed by SDS PAGE and by Western blotting (Fig. 5). The presence of the 6His-tagged and SH2-tagged proteins S2, respectively, in the ribosome fractions, indicates that the 6His-tagged S2 and SH2-S2 protein are able to associate with the ribosome and therefore biochemically resemble untagged protein S2.

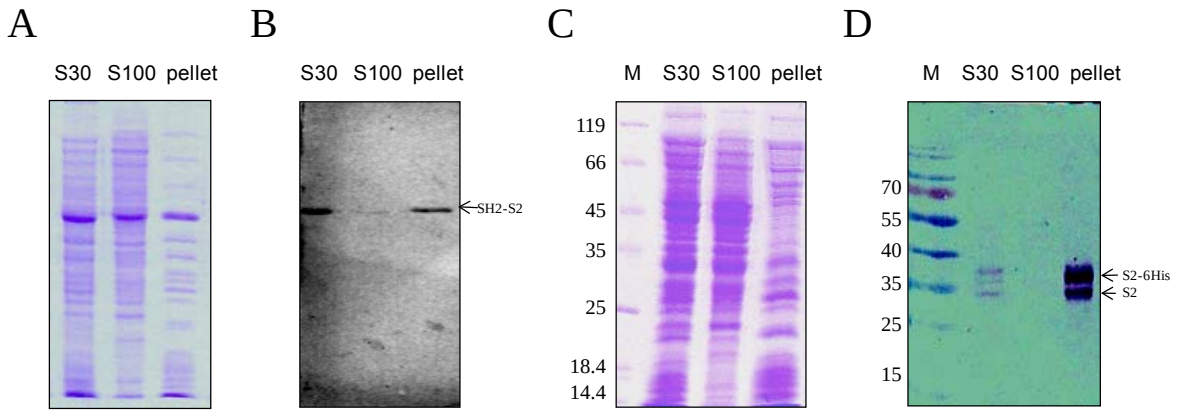


***Figure 5. SH2-S2 and S2-6His fusion proteins bind to the ribosome* in vivo.**

Ribosomes were isolated by ultra centrifugation from cultures expressing protein SH2-S2 or S2-6His. The same amount of total protein from the S30 extract, the pellet and the S100 supernatant fractions, respectively, was run on 12% SDS gel and detect by Western blot analysis with anti-His (B) or anti-ribosomal protein S2 (D) antibodies, or by staining with Coomassie blue (A) and (C). The position of protein SH2-S2, S2 and S2-6His are indicated by arrowheads.

## *Far-western blot analysis revealed interaction of $S1_{106}$, and $S1_{194}$ with SH2-S2*

To further support the interaction between the variants of protein S1 and protein S2, we used a Far western blot analysis. This method is similar to a Western blot analysis and is used to detect the specific interaction between the prey and bait proteins with anti-bait-protein antibody (Blackwood and Eisenman, 1991). Here, an extract of *E. coli* cells overexpressing protein SH2-S2 was separated on a SDS-PAGE. Upon blotting, the proteins were re-natured on the nitrocellulose membrane and incubated with the bait proteins S1 and $S1_{106}$, respectively. The interaction between prey and bait proteins was detected by an anti-S1 antibody. The results shown in Fig. 6 demonstrate that protein SH2-S2 and native protein S2 were successfully refolded on a membrane and interact with proteins S1 and $S1_{106}$.

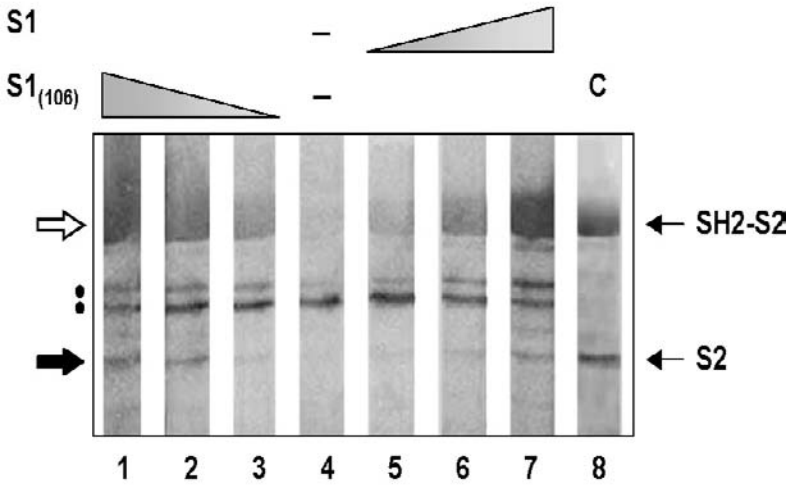


***Figure 6. The interaction between protein SH2-S2 and proteins S1 or $S1_{106}$ was detected by employing Far-western blotting.***

*2.5 µg of total extract of cells over expressing the SH2-S2 fusion protein were separated in a 12% SDS polyacrylamide gel and transferred onto nitrocellulose membranes. After renaturation, the membranes were individually incubated with different concentrations (lanes 1 and 7: 300 µg/ml, lanes 2 and 6: 30 µg/ml, lanes 3 and 5: 3 µg/ml) of purified* ***$S1_{106}$*** *(lanes 1-3) a****nd S1*** *(lanes 5-7), respectively. Lane 4: no bait protein was added. The blots were probed with anti-S1 antibody.* ***$S1_{106}$ and S1*** *bound to SH2-S2 fusion protein and S2 are indicated by an open and a closed arrow, respectively. The positions of the bands corresponding to SH2-S2 and native S2 protein were verified by probing the same membranes with anti-S2 antibody (lane C). Two signals that were also present in the absence of the bait proteins are likely detected due to non-specific binding of anti-S1-antibody to other polypeptides or to proteolysis forms of endogenous protein S1(marked with closed circles).*

## *Probing the protein-protein interaction between proteins S1 and S2 by NMR*

Next, we characterized the S1-S2 protein-protein interaction by NMR using SH2-tagged polypeptides. Since the SH2 tag can bind the low molecular weight ligand, phenyl phosphate, and its relaxation rate is strongly dependent on the molecular weight of the complex, this assay allows the determination of protein-protein interaction. The binding of the ligand to the SH2 domain on the SH2-S2 fusion protein in the absence and presence of protein S1 variants was monitored by selective T1 measurements of the aromatic proton in Meta position to the phosphate group. The results of these experiments are presented in Fig. 7. These data point to the direct interaction between protein S1 variants and protein SH2-S2 in solution. The full-length 6His-tagged protein S2 was added as a competitor for protein SH2-S2 and its effect on relaxation rates was recorded as well. As seen in Fig. 7D, addition of 6His-tagged protein S2 resulted in an

increase in the relaxation rate, thus confirming the substitution of protein SH2-S2 in the S1/SH2-S2 complex by 6His-tagged protein S2. Our data indicate that binding of protein S1 to the 30S subunit indeed requires the direct interaction of its N-terminal domain with protein S2.

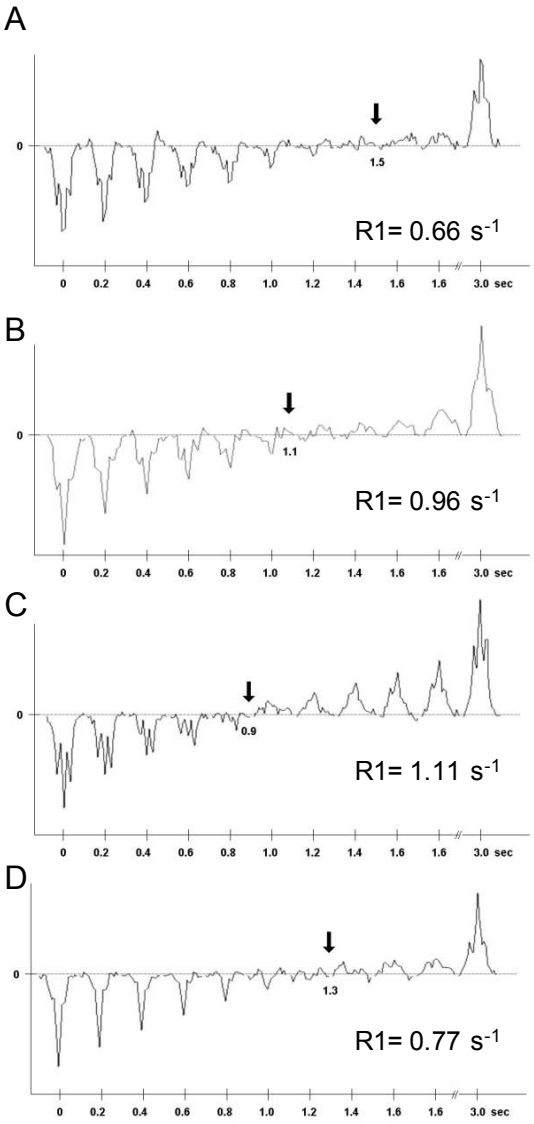


***Figure 7. Probing the S1/S2 interaction employing an NMR-based reporter system.***

*The protein-protein interaction was monitored by selective T1 measurements of the aromatic proton in Meta position to the phosphate group of the ligand. At time point 0, the ligand's nuclear spin was polarized by a pulse, and then every 200 ms the T1 values were selectively measured till time point 3.0 s. The experimentally determined selective relaxation rates R1=1/T1 are indicated. Selective T1 measurements of the ligand bound to the SH2-S2 fusion protein (A), to the SH2-S2 fusion protein in complex with $S1_{106}$ (B), to the SH2-S2 fusion protein in complex with $S1_{194}$ (C) and upon addition of native protein S2 to SH2-S2/ $S1_{106}$ complex (D) are shown. All measurements were carried out with 50 µM amounts of proteins and 250 µM amount of ligand (phenyl phosphate) in 0.5 ml at 25ºC.*

## *DISCUSSION*

Although antibiotics have widely been used for controlling bacterial infections for many years, the significant increase in the number of multidrug resistant pathogenic bacteria stimulated the search for new antimicrobials including synthetic compounds that can efficiently interfere with ribosome functions. Here we focused on the interaction surface of protein S1 on the *E. coli* ribosome, a possible target to interfere with assembly of protein S1. This ribosomal protein is known to play pivotal roles in translation initiation, elongation and trans-translation (Tzareva et al., 1994) (Potapov and Subramanian, 1992). Taken together these properties of protein S1 suggest that compounds, which are able to interfere with S1 binding to the ribosome can efficiently affect its ribosomal functions and thereby inhibit bacterial growth. Here, we have shown that overexpression of the first N-terminal domain of protein S1 ($S1_{106}$), which is not active in translation but still retains the ability to interact with the ribosome, inhibits cell growth. Since we were able to confirm $S1_{106}$ binding to ribosomes, the toxicity induced by overexpression of $S1_{106}$ could be attributed to the accumulation of non-functional 30S ribosomal subunits that accommodated $S1_{106}$ instead of endogenous S1 and therefore are not active in translation.

Several studies indicate that ribosomal protein S1 is located in close proximity to protein S2 in assembled 30S particles (Sengupta et al., 2001). Consistently, recent work in our lab (Moll et al., 2002) also suggested that protein S2 facilitates protein S1 recruiting to the ribosome via protein-protein interactions. Here, we tested this hypothesis by employing a number of experimental techniques including *in vitro* reconstitution of 30S particles with truncated variants of protein S1, Far western blot analysis and NMR probing of protein-protein interaction using reporter ligands and affinity tags. To simplify this analysis, we studied the S1/S2 interaction using truncated variants of S1, $S1_{106}$ and $S1_{194}$. We have shown in pilot experiments employing Far western blot analysis that both full- length and truncated protein S1 variant are able to interact with protein S2, thus indicating that the N-terminal domain of S1 ($S1_{106}$, residues 1-106) is sufficient for efficient binding to protein S2. This conclusion was further supported by the results of *in vitro* reconstitution of 30S particles with $S1_{106}$. To further characterize the S1/S2 interaction, we used a recently developed NMR-based technique (Ludwiczek et al., 2004). The data obtained have confirmed that protein $S1_{106}$ binds to protein S2 and indicated that this approach could further be applied in NMR drug discovery screening to develop ligands, which can specifically inhibit the S1/S2 interaction and therefore prevent protein S1 binding to the ribosome. The use of NMR in drug discovery offers additional benefits such as the possibility to (1) study the binding mode of a ligand in solution at high resolution, (2) carry out screening by

testing different cofactors, different protein activation states and environmental conditions, (3) identify low affinity ligands not detectable by other techniques (Villar et al., 2004) (Homans, 2004) (Meyer and Peters, 2003).

Finally, the possibility to inhibit binding of protein S1 to the ribosome by interfering with its binding to protein S2 appears to be used by bacteria under certain stress conditions. Previous work revealed that protein S2 is efficiently degraded by the Lon protease, which is activated during the stringent response (Kuroda et al., 2001) (Nishii et al., 2005). This could indicate that bacteria can potentially disrupt the S1/S2 interaction by reducing the concentration of free protein S2 under some stress condition, which, in turn, prevents protein S1 binding to 30S particles and inhibits the initiation of translation under stress.

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The last but not the least, I want to dedicate this thesis to the glory of the Lord who has given me the faith and spiritual strength to face and overcome all the difficulties during my study.

# *8. Curriculum vitae*


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## PUBLICATIONS


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