RsfS (YbeB) is an universally conserved ribosome silencing factor

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Zusammenfassung

Wenn sich Bakterien der stationären Phase nähern, reduziert sich ihre Teilungsrate, die metabolischen Aktivitäten werden zurück gefahren und die Translationsaktivität der Ribosomen nimmt rapide ab. Letzteres bedingt den wohlbekannten Effekt, daß Ribosomen, isoliert von Zellen aus der stationären Phase, nur eine geringe Aktivität *in vitro* haben. Wir haben nur geringe Kenntnis von den Ursachen der reduzierten ribosomalen Aktivität („silencing“) während der stationären Phase und unter Stressbedingungen. Faktoren wie RMF, HPF und sein Homolog PY wurden vorgeschlagen, über Dimerisation von 70S inaktive 100S Partikel zu bilden. Jedoch gibt es keine Übereinstimmung über die Rolle und das Auftauchen dieser Partikel. Ferner ist bekannt, daß die Entfernung des RMF Gens die Lebensfähigkeit von *E. coli* Zellen in der stationären Phase verschlechtert. Weiterhin wurden 100S Partikel auch in logarithmischer Phase beobachtet, was heißen mag, daß die 100S Partikel eine andere Rolle spielt oder weitere Funktionen besitzt.

Wir präsentieren hier eine Studie des kürzlich von uns und mit meiner Beteiligung beschriebenen „Ribosome Silencing Factor“ (RsfS, früherer Name YbeB), ein Protein das mit Ribosomen assoziiert ist. RsfS kommt fast in allen Bakterien, Mitochondrien und Chloroplasten vor und bindet an L14 der großen, ribosomalen Untereinheit, eines der am besten konservierten Proteine des Ribosoms. Die Wechselwirkung von RsfS mit L14 ist vom Bakterien bis zum Menschen konserviert.

Wir zeigen, daß RsfS wichtig für das Überleben ist, wenn immer die Wachstumsrate herunter gefahren werden muß, d.h. während des Übergangs von der logarithmischen zur stationären Phase oder vom reichen zum armen Medium. Im letzteren Fall ist das Wachstum blockiert, bis es nach etwa 15 h langsam wieder Fahrt aufnimmt. Entfernung des RsfS Gens erhöht die Translationsaktivität in der stationären aber nicht in der logarithmischen Phase. *In vitro* hemmt RsfS und sein mitochondriales Homolog die Translation über die Bindung an L14 in der großen ribosomalen Untereinheit und blockiert damit die 70S Bildung aus Untereinheiten oder dissoziiert leere 70S Ribosomen. Interessanterweise wird die Effizienz zur Dissoziation empfindlich gestört, wenn programmierte Ribosomen

tRNAs tragen. RsfS hemmt nicht die Translation von chemisch vernetzten 70S Ribosomen, woraus wir schließen, daß die Translationshemmung zum wesentliche Teil auf Dissoziation der 70S Ribosomen bzw. Hemmung der Assoziation der ribosomalen Untereinheiten zurückgeführt werden kann.

Wir haben auch RsfS mit den Faktoren RMF, PY und HPF genetisch und funktionell verglichen, um die jeweilige Bedeutung und eine mögliche Kooperation zu entdecken. Wir fanden, (i) daß 100S Bildung keine obligates Merkmal der stationären Phase ist, (ii) daß der schwere Phänotyp des *ΔrsfS* Stammes mit entsprechenden KO-Mutanten der drei anderen Faktoren nicht beobachtet wird, (iii) daß die Lebensfähigkeit von *ΔrsfS* Zellen und besonders der *Δrmf* Zellen, aber nicht der *Δhpf* or *Δpy* stark eingeschränkt ist, und daß schließlich *in vitro* RsfS die stärkste Translationshemmung sowohl bei natürlichen mRNAs als auch bei hoch definierten Elongationsexperimenten zeigt. Die Hemmung der anderen Faktoren ist additiv, nicht kooperativ. Zusammengefaßt zeigen unsere Daten, daß RsfS eine Schlüsselrolle für das ribosomale “silencing” hat, wobei es von den anderen Faktoren unterstützt wird.

Summary

Bacterial cells approaching stationary growth phase reduce division rates, cut back metabolic activities and thus decrease protein translation causing the well-known effect that ribosomes isolated from stationary growth phase show a low translational activity. We have a scarce knowledge about the mechanism of ribosome silencing during stationary growth phase or under stress condition. HPF, its homolog PY and RMF have been proposed to bring translation to a halt by dimerization of 70S ribosomes into 100S particles. However, there is no consensus about the function or occurrence of 100S particles. Deletion of RMF decreases the viability of *E. coli* in stationary growth phase. On the other hand, 100S particles have been observed also in logarithmic growth phase, suggesting that 100S particles have a different role than ribosome silencing. Here we present a study of the recently (with my participation) described Ribosome Silencing Factor S (former name YbeB), a protein associated with ribosomes. RsfS is present in almost all bacteria, mitochondria and chloroplasts and binds to protein L14, one of the most conserved proteins of the large ribosomal subunit. This interaction is conserved from bacteria to man.

We demonstrate that RsfS is important for cell survival, whenever the growth rate has to be decreased, *i.e.* during the transition from the logarithmic growth phase to the stationary growth phase and from rich to poor media. In the latter case Δ*rsfS* strain stops growing for about 15 h before growth is launched again. Deletion of RsfS gene increases the translation activity during stationary growth phase but not during logarithmic growth phase. *In vitro* RsfS and its mitochondrial homolog inhibits translation by binding to 50S protein L14 and thus i) inhibits 70S formation from subunits and ii) dissociates empty 70S ribosomes. Interestingly, the efficiency of 70S dissociation decreases, when 70S is programmed with tRNA at the P site. RsfS does not inhibit translation of non-dissociable 70S suggesting that ribosome silencing by RsfS is mediated predominantly *via* 70S dissociation or anti-association of the ribosomal subunits.

We also compared RsfS with RMF, PY and HPF to elucidate the importance and possible interplay between these factors in ribosome silencing. We found that i) 100S formation is not an obligatory feature of stationary-phase

E. coli cells; ii) the strong phenotype of *ΔrsfS* strain, *viz*. a block of growth for about 15 h after transfer from rich to poor media, is not seen with the knock-out of one of the other factors; (iii) viability at stationary phase is impaired in *ΔrsfS cells* and even stronger in *Δrmf* cells, but not in *Δhpf* or *Δpy* cells; (iv) RsfS is the only factor that impairs translation in stationary growth phase; (v) *in vitro* RsfS shows the strongest inhibition in both translation of natural mRNAs and in highly resolved elongation assays; the inhibition of the other factors is additive, not cooperative. Collectively, the data indicate that RsfS plays a key role in silencing the ribosomal activity under conditions characterized by a reduced growth rate, and that it is supported by the other silencing factors.

Abbreviations

β-ME	β-mercaptoethanol
aa	amino acid
A	Ampere
Å	Ångström
aaRS	aminoacyl – tRNA synthetase
aa-tRNA	aminoacyl-tRNA
AcPhe	acetyl phenylalanine
APS	ammonium peroxodisulfate
Da	Dalton
DNA	desoxyribonucleic acid
DMS	dimethyl-suberimidate
E. coli	*Escherichia coli*
EF-G	elongation factor G
EF-Ts	elongation factor thermo-stable
EF-Tu	elongation factor thermo-unstable
EM	electron microscopy
f.c.	final concentration
fMet tRNA	formyl methionyl tRNA
GAC	GTPase associated center
GDP	guanosine diphosphate
GTP	guanosine triphosphate
H95	helix 95 of large ribosomal subunit
h44	helix 44 of small ribosomal subunit
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethan-sulfonic acid
HPF	Hibernation Promoting Factor
IF	initiation factor
IPTG	isopropyl- thio-β-D-galactosidose
kb	kilobase, 1,000 base pairs
LB	lysogen broth
Lys	lysine
mRNA	messenger RNA

nt(s)	nucleotide(s)
PAGE	polyacrylamide gel electrophoresis
PY	Protein Y
PTC	peptidyl transferase center
RF	release factor
RMF	Ribosome Modulation Factor
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RsfS	Ribosome Silencing Factor S
S	Svedberg unit (sedimentation coefficient)
SDS	sodium dodecylsulphate
TCA	trichloracedic acid
TEMED	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
V	volt
v/v	volume/volume
w/v	weight/volume

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1 Introduction

1.1 Structure and function of translation apparatus

According to the central dogma of biology all living organisms contain genetic information encoded in DNA, which is transcribed to mRNA, a template for protein translation mediated by the ribosome. The ribosome catalyses the polymerization of amino acids (10-20 amino acids per second; (Dennis and Bremer 1973; Wilson and Nierhaus 2003)) and ensures a proper accuracy of aminoacyl tRNA decoding (overall one mistake per 3,000 incorporations (Bouadloun, Donner et al. 1983)). Furthermore, it protects about 30 last incorporated amino acids in the ribosomal tunnel. The ribosome consists of rRNA and proteins. Two thirds of the mass of the bacterial ribosome is build of rRNA and 1/3 of proteins. These proportions are inverted in the mitochondrial ribosome.

We separate all organisms into three domains: the Eukarya, Archaea and Bacteria. One classifies the ribosome according to their sedimentation constant, *viz*. the 80S and 70S ribosomes (Eukarya and Archaea plus Bacteria, respectively). The two crucial tasks, decoding and peptide bond formation performed by the small subunit 30S (40S eukaryotes) and the large 50S (60S eukaryotes), respectively, are applying to every cell on earth implicating the existence and importance of ribosome in early evolution stages at the beginning of life.

1.1.1 Structure

The bacterial ribosome (*Escherichia coli*) has a mass of 2.4 Mega Daltons (MDa), a diameter around 250 Å and contains 3 rRNAs and 54 ribosomal proteins. A single molecule of 16S rRNA is a part of 30S subunit, whereas 2 molecules, 5S rRNA and 23S rRNAs, belong to 50S subunit. The crystal structure of ribosome revealed two novel RNA structures called A-minor motif and K turn (Nissen, Ippolito et al. 2001). The A-minor motif is an interaction between an adenine nucleotide with the minor groove of a Watson-Crick base pair, where H-bonds form with the 2’ - OH groups of a base pair. A-minor motif is a most prominent

RNA structure in the ribosome and plays a crucial role in decoding of translation. K turn is a kink in the axis of helical RNA, with an angle around 60° (Klein, Schmeing et al. 2001); (Nierhaus 2009).

The large subunit of the bacterial ribosome (50S) contains 33 proteins whereas small subunit (30S) contains 21 proteins. Contrary to other cellular macromolecular complexes most of ribosomal proteins, present at the surface, have globular shape with long extensions protruding into the centre of the ribosome. Such unique feature might play an important role for assembly and stability of ribosome.

Bacterial ribosomes have a globular shape with distinct landmarks (**Figure 1**). 30S is docking station of tRNAs, where the mRNA and tRNAs form codon-anticodon interactions. On the other hand, structure of the big subunit resembles a hemisphere containing three protuberances: the L1 stalk, the central protuberance and the L7/L12 stalk (Frank 1989; Stark, Mueller et al. 1995).

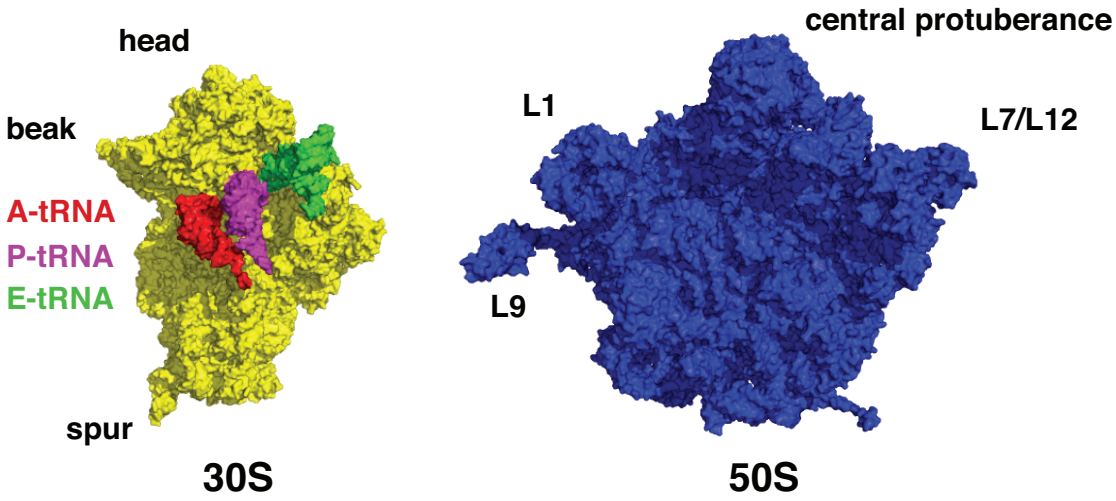

Figure 1. Crystal structures of the 50S the 30S ribosomal subunits. A) A view at the interface region of 30S subunit with three tRNAs. **B**) A view at the interface region of 50S; PDB files: 30S: 2X9T; 50S: 2X9S.

The final product of translation – an amino-acid polymer or protein - is an outcome of incorporation of single amino acids into a peptide chain. Prior to engagement into the process of translation, the amino acid has to be attached to transfer RNA (tRNA) by an enzyme called aminoacyl – synthetase (aaRS, aminoacyl tRNA synthetase). Each one of 20 aminoacids is charged by one

specific aminoacyl tRNA synthetase. A tRNA in this complex serves as a carrier placing the correct (cognate) amino acid on the ribosome. Functional studies performed in the last century have shown that the ribosome displays three distinct sites for tRNA binding: the A site, the P site and the E site. Each of them executes unique function during translation process. The A site is a place of delivery of aminoacyl-tRNA (together with GTP and EF-Tu), a substrate for peptide synthesis, whereas the P site is occupied by a tRNA holding a product – the nascent peptide chain before peptide-bond formation.

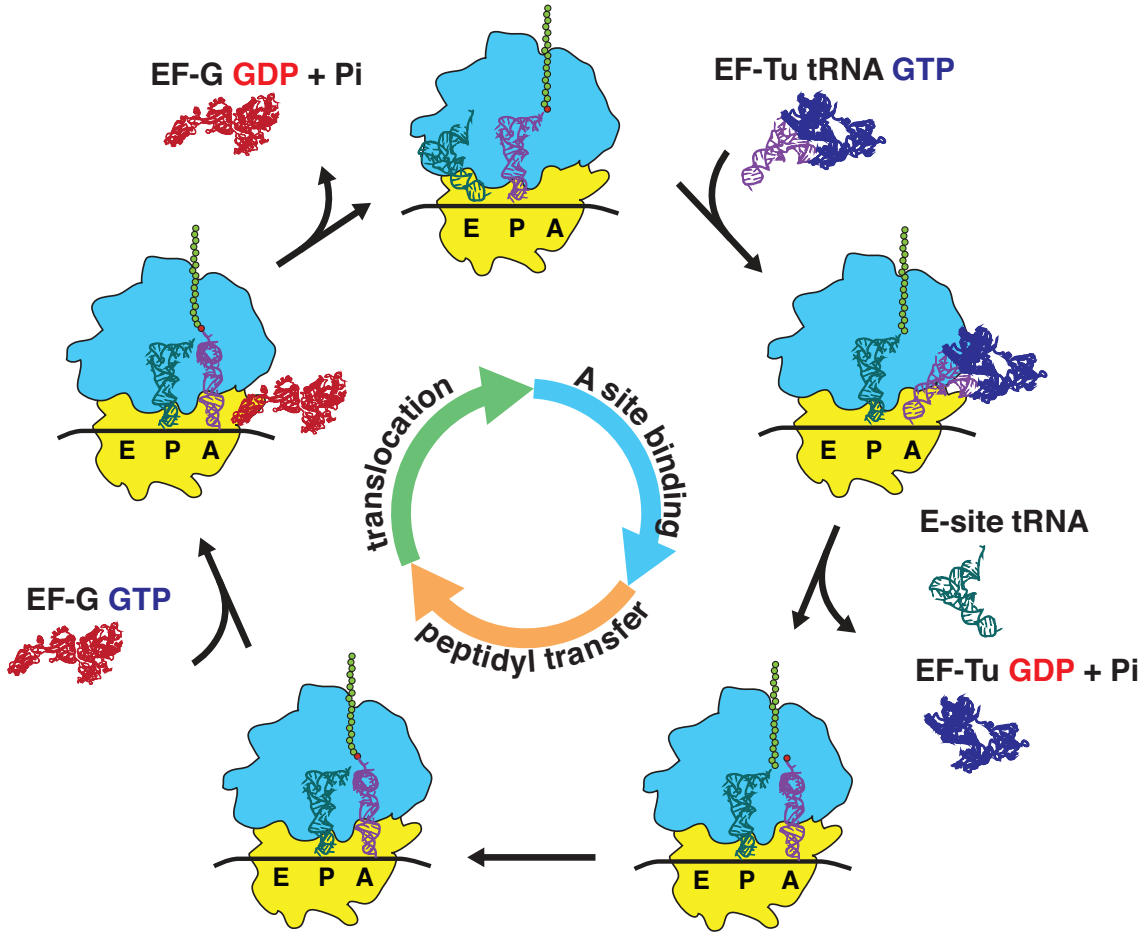

Figure 2. Elongation step of bacterial translation cycle. During decoding step a ternary complex consisting of aminoacyl-tRNA, EF-Tu and GTP binds to the ribosomal A site. After successful decoding peptide bond is formed and peptidyl-tRNA is present at the A site. In the translocation step EF-G facilitates the movement of A and P site tRNA together with mRNA to P and E site, respectively. Once EF-G is released, another ternary complex can bind to the A site.

The ribosomal E site is involved in control of translation fidelity. It binds deacylated tRNA and releases it upon A-site occupation. (Rheinberger, Sternbach et al. 1981). The process of translation is divided into four stages: initiation, elongation,

termination and recycling of the ribosome to a new initiation event. The functional phases of translation are presented schematically in **Figure 2**.

1.1.2 Initiation

In bacteria translation initiation depends primarily on 30S ribosomal subunit, which together with fMet-tRNA and initiation factors IF1, IF2 and IF3 governs the formation of 30S initiation complex (30SIC). In this classical type of initiation 30S subunit recognizes Shine-Dalgarno (SD) sequence, which is a part of the initiation region of the mRNA upstream of the start codon (AUG). An interaction between anti SD sequence at the 3'-end of 16S rRNA in the 30S subunit and SD sequence on the mRNA ensures the identification of the initiation codon AUG (Shine and Dalgarno 1974). The subsequent binding of the initiator fMet-tRNA, where the tRNA anticodon loop is complementary to the start codon AUG at the P site, occurs within the IF2●GTP complex. (Gualerzi, Severini et al. 1991; Myasnikov, Marzi et al. 2005; Simonetti, Marzi et al. 2008). Binding of fMet-tRNA●IF2●GTP complex is stimulated by Initiation factor 1 (IF1).

IF1 is universal, essential for viability (Cummings and Hershey 1994). It is a smallest in *E. coli*, consisting of only 72 amino acids. IF1 binds to the 30S subunit (Celano, Pawlik et al. 1988), near the A site and interacts with proteins S1 and S12 and with Initiation Factor 2 (IF2). Upon binding IF1 induces rearrangements in the nucleotides the helix 44, namely adenines 1492 and 1493 (A1492/1493). These two nucleotides play important roles in the decoding process. Furthermore, IF1 interferes with binding of aa-tRNA●EF-Tu●GTP-ternary complex. Another factor, Initiation factor 3 (IF3) binds to the 30S ribosome, overlapping with the E site region and is thought to block subunit reassociation (Kaempfer 1972). Subsequent association of the 50S subunit with the 30SIC is thought to induce the release of IF3 and IF1. The 50S subunit contains the GTPase associated center that triggers GTP hydrolysis and leads to release of IF2●GDP from the ribosome and full accommodation of tRNA at the P site (Nierhaus and Wilson 2004). The ribosome programmed in Pi state (the P site occupied) is ready to enter the second phase of translation, namely the elongation phase.

Recently another type of translation initiation has been described, called 70S scanning (Yamamoto et al.; in press). In 70S scanning after termination, 70S

does not dissociate into ribosomal subunits, but instead scans mRNA upstream and downstream until AUG codon is found. IF3 is essential for 70S scanning whereas IF1 plays important role to block elongation-ternary complex from binding to 70S initiation complex. 70S scanning requires no energy source and is observed in mRNA containing only single gene (monocistronic) as well as in mRNA with multiple genes (polycistronic mRNA). This novel type of initiation explains why ribosomal from a single polycistronic mRNA are synthesized in precise stoichiometric proportions.

1.1.3 Translation elongation

1.1.3.1 Decoding and accommodation of A site tRNA

The aminoacyl-tRNA is delivered to the ribosomal A site as a ternary complex aa-tRNA•EF-Tu•GTP. Upon binding of correct aminoacyl-tRNA the interaction between codon of mRNA and anticodon loop of tRNA is tested by the 16S rRNA of the 30S subunit. Three key nucleotides are involved in testing Watson - Crick pairing in the decoding centre. Binding of correct aminoacyl-tRNA induces conformational changes of three conserved bases of 16S rRNA: A1492, A1493 and G530. The A1493 base facilitates formation of type I A–minor interaction with the first base-pair (bp) of the codon - anticodon interaction. Determination of correct pairing involves the formation of three hydrogen bonds between A1493 and residues at the minor groove of the first bp, which occurs as a consequence of correct Watson Crick pairing (Ogle, Brodersen et al. 2001). The arrangement of the second pair is stabilized by two bases: A1493 and G530 resulting in formation of type II A-minor interaction. Verification of a correct third base is less specific and allows Wobble pairing (non-Watson-Crick pairing (Ogle, Brodersen et al. 2001)). Apart from the nucleic acid interaction, the protein S12 (Ser 50) contacts the decoding center, supporting the correctness of middle position pairing. Intermolecular interactions in decoding center are depicted in **Figure 3**.

A recent crystal structure of ribosome with non-cognate tRNA bound to the A site shows that non-cognate tRNA induces similar rearrangement of A1493, A1493 and G530 as cognate tRNA (Demeshkina, Jenner et al. 2012). Therefore,

the mechanism of discrimination between cognate and near cognate tRNA, proposed by Venkatraman Ramakrishan in 2001 has to be extended.

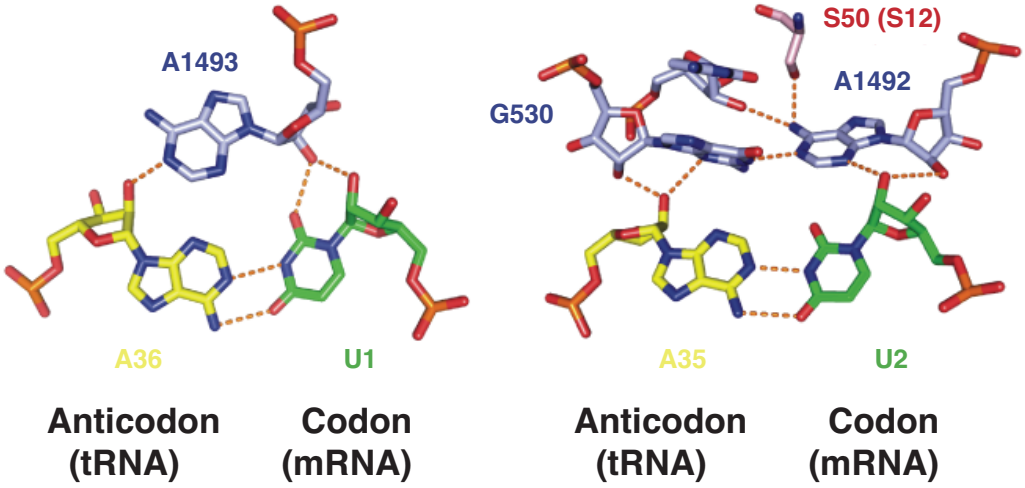

Figure 3. Codon anticodon interactions at the first and second nucleotide. Three nucleotides of 16S rRNA (A1492, A1493 and G530) involved in verification of Watson – Crick base pairing during the decoding step (A – first pair, B - second pair). According to (Ogle, Brodersen et al. 2001)

Once decoding step is complete, the GTPase center of EF-Tu is triggered by the GTPase Associated Center (GAC) of the 50S subunit, which contains the L7/L12 stalk, L11 with its binding site on helices H43/44 and the sarcin-ricin stem-loop on H95. The hydrolysis of GTP occurs as a consequence of opening a hydrophobic gate, which is a part of EF-Tu (**Figure 4A**).

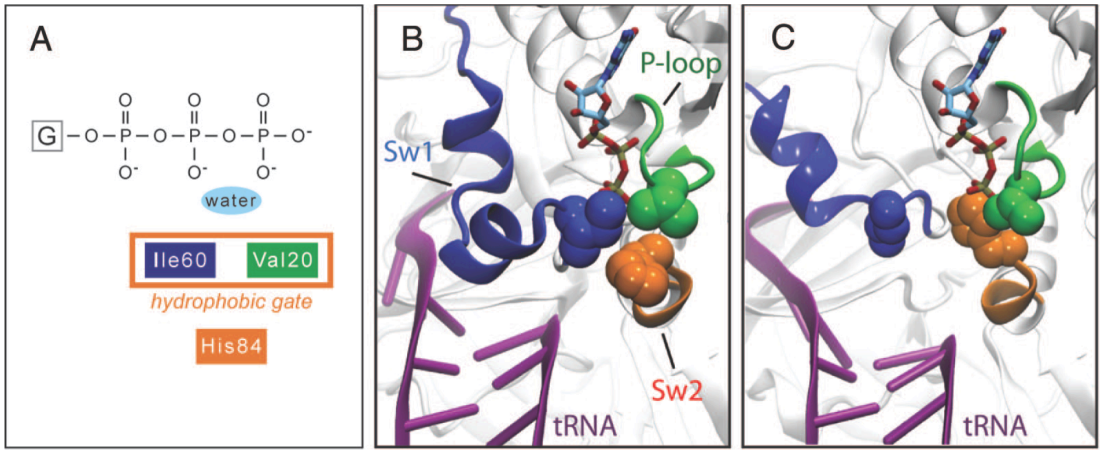

Figure 4. GTP cleavage induced after correct codon-anticodon interaction. A) Schematic representation of hydrophobic gate. **B**) Closed gate in the crystal structure of ternary complex bound to the ribosome, stalled with kirromycin. **C**) Open gate as a consequence of a successful decoding step.

Sw1 – switch one of EF-Tu, Sw2 – switch two of EF-Tu (Villa, Sengupta et al. 2009).

It consists of two residues: isoleucine 60 (Ile 60) and valine 20 (Val 20), forming a steric barrier for histidine 84 (His 84). If access of His 84 is allowed to the bound GTP, it organizes the access of H_2O, which then cleaves the β-γ-phospho-anhydrid bond of GTP (Sprinzl, Brock et al. 2000; Villa, Sengupta et al. 2009). The accommodation of tRNA induced by GTP cleavage and subsequent EF-Tu•GDP release is followed by formation of peptide bond between aminoacyl-tRNA and peptidyl - tRNA. This step is mediated by the 50S enzymatic region called Peptidyl Tranferase Center (PTC).

1.1.3.2 Peptide bond formation

The main enzymatic activities of the ribosome are peptide-bond formation during elongation and peptidyl-tRNA hydrolysis during termination. Crystal structure analysis of the archeal 50S subunit identified conserved nucleotides of 23S rRNA at the PTC that might be involved in peptide bond formation (Nissen, Hansen et al. 2000). Interestingly, in the radius of 18 Å from PTC there was no protein which prompted the famous statement: ”ribosome is ribozyme” (Cech 2000). The mechanism of peptide bond formation is the following: ribosomal A site and P site are occupied by aminoacyl - tRNA and peptidyl - tRNA, respectively. In both cases acyl residues are attached to adenine (A76) 3’ of tRNAs *via* ester bonds. The peptidyl residue of peptidyl-tRNA is transferred to the amino groups of the aminoacyl-tRNA. The crucial step is a nucleophilic attack of the α-amino group of A-tRNA on the carbonyl carbon group of the peptidyl-tRNA resulting in a peptide bond between the peptidyl residue and aminoacyl group at the A site. Although there is no doubt about the ground state (substrate and product) of peptide formation, the detailed mechanism including intermediate states as well as the nature of this reaction is still under discussion (Pech and Nierhaus 2012). The ribosome is increasing the rate of uncatalyzed peptide bond formation by a factor of 3×10^5 (Nierhaus, Schulze et al. 1980). Such an enhancement would rather stem from the physical concept (proper alignment of substrate, enhancements by a factor of up to 10^8 are possible) rather then chemical reaction, which increases the rate only up to 10^3 fold. The crystal structure of Nissen et al. suggested that N3

of A2451 is an element of acid base catalysis of peptide bond formation (Nissen, Hansen et al. 2000). However, under *in-vivo* conditions pK of the N3 is too low (pK = 1) to donate a proton. Furthermore, mutagenesis of this base has proven not to be essential for peptide bond formation (Amort, Wotzel et al. 2007). On the other hand, atomic mutagenesis of 2'OH group of A2451 showed that 2' OH group is important for peptidyl transferase activity (Clementi, Chirkova et al. 2010). Later, 2'OH of A76 has been proposed to be important for peptide bond formation, because mutation to desoxyadenine led to a decrease of the reaction rate by 10^3 up to 10^6 fold (Zaher, Shaw et al. 2011). Based on biochemical studies and molecular dynamics simulation six and eight membered model of proton shuttle during peptide bond formation was proposed (**Figure 5A**). In a six membered model, during nuleophilic attack of α-amino group of aminoacyl tRNA a proton moves from the α-amino group of A - tRNA to the 2'-O group of the peptidyl-tRNA at the P site. This causes a transfer of its own proton to the 3'–O of the P-RNA and results in cleavage of the ester bond. In an eight-membered-proton shuttle a water molecule is involved (**Figure 5B**). However, this model was not in agreement with experiments performed by the Sprinzl group (Koch, Huang et al. 2008; Huang and Sprinzl 2011), who showed that 2'OH is important but only until 10-12 aminoacid are incorporated into nascent peptide chain. A recent paper of Hiller et al gave us hints, whether peptide-bond formation is based on a chemical reaction or only substrate positioning (Hiller, Singh et al. 2011). The study is based on the assumption that the ribosomal rate enhancement of peptide-bond formation is limited to defined substrate positioning. If so then the mechanism should be equivalent to the uncatalysed one. The uncatalysed reaction comprises three steps with two intermediates T+/- and T-. Nucleophilic attack of the α-amino group leads to the zwitterionic T- intermediate with a negatively charged oxygen and a positively charged nitrogen, and deprotonation of the attacking amine to the T- intermediate, which decomposes to products. However, the results showed that in contrast to the uncatalysed reaction, formation of the tetrahedral intermediate and proton transfer from the nucleophilic nitrogen both occurred in the rate-limiting step. The detailed reaction mechanism is not fully concerted, therefore in addition to substrate positioning, the ribosome is contributing to chemical catalysis by changing the rate-limiting transition state.

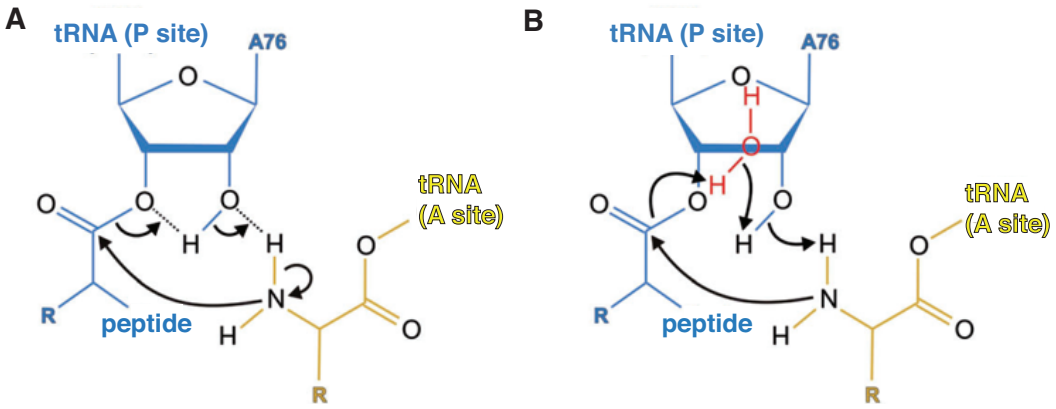

Figure 5. Principles of peptide bond formation. A) Six membered proton shuttle. B) Eight membered proton shuttle. According to (Pech and Nierhaus 2012).

1.1.3.3 Translocation

Following the transfer of peptidyl group from the P site to the A site, another amino acid has to be incorporated into the nascent peptide chain. However, the binding of new aminoacyl-tRNA cannot occur, because the A site remains still occupied by the newly formed peptidyl-tRNA. The EF-G acts as a catalyst promoting the transfer of two tRNAs present in A and P sites to P and E sites respectively, together with the mRNA. This process called translocation is strongly dependent on successful decoding and accommodation of aminoacyl-tRNA in the A site. The ribosome with the A and the P sites occupied is called PRE state (*i.e.* pre translocation state), whereas tRNAs presence at the P and the E sites defines the ribosomal POST state (*i.e.* post translocation state). The translocation mediated by EFG involves a third step called hybrid state (Moazed and Noller 1989). Recent advances in single-molecule ribosome studies showed that ribosome is highly dynamic during translocation (Munro, Altman et al. 2007; Ratje, Loerke et al. 2010). First, EF-G in the GTP state binds to the ribosomal A site of a PRE state ribosome, which induces a conformational change on the ribosome, called ratcheting. This is a 7° anti-clockwise rotation of the small subunit provided that the 50S subunit is fixed and one looks on top of the cytoplasmic site of the 70S ribosome. In addition, the head of 30S moves by 6° towards the E site, a rotation called the swivelling.

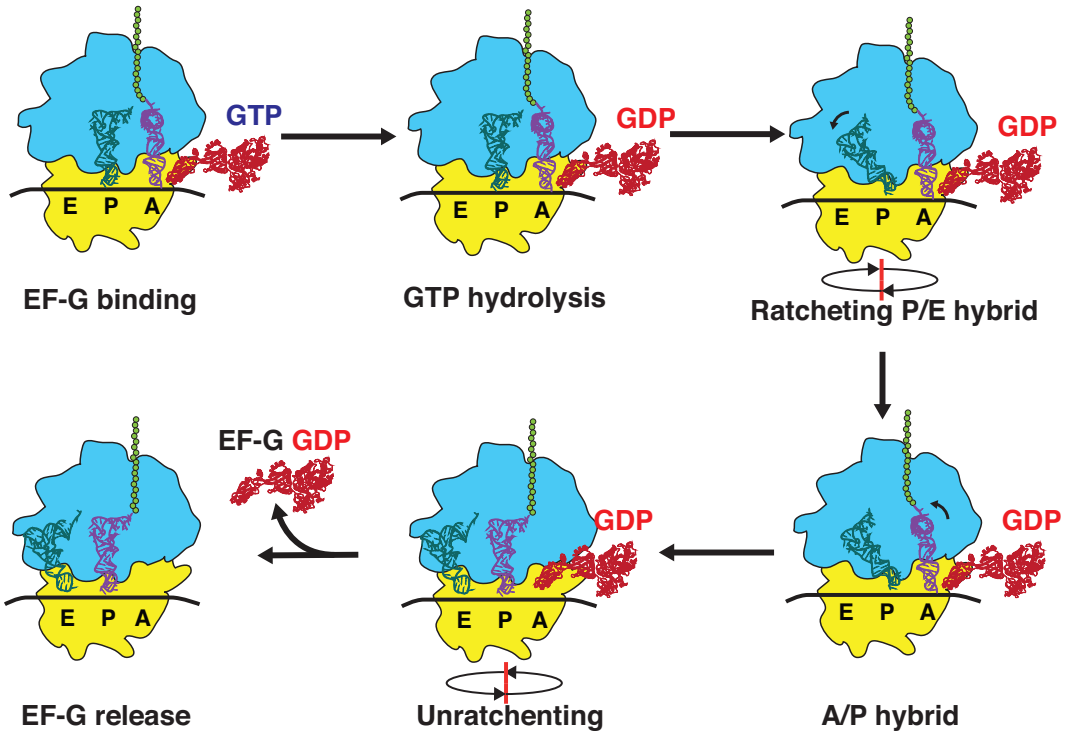

Figure 6. The mechanism of translocation facilitated by EF-G. EF-G binds to the ribosome with pre translocation state (PRE). The binding of EF-G induces conformational change on the ribosome (ratcheting), which leads to translocation of tRNA. Note that it is not the EF-G dependent GTPase activity, which drives the translocation, but the EF-G binding, because EF-G•GDPNP can also perform a translocation reaction. For details see text. After translocation is finished EF-G leaves the ribosome in the post translational state (POST).

Furthermore, the L1 stalk of 50S subunits moves towards the ribosomal E site, termed L1 closed conformation. As a consequence of ratcheting the 50S part of P-site tRNA moves by 15 Å towards the E site, thereby forming the hybrid 2 state (P/E). Subsequently, the 50S part of A-site tRNA forms the hybrid 1 state (A/P), which involves a 3 Å motion towards P site. (Munro, Sanbonmatsu et al. 2009). The ratcheting-unratcheting of the ribosome accompanied by moving the tRNAs between classical and hybrid sites occurs also in the absence of EF-G, at least in a fraction of the ribosomes. In contrast, the rotation of the head is seen only in the presence of EF-G, the forward (counterclockwise) rotation is accompanied by mRNA movement from A and P sites to P and E sites, respectively, on the 30S subunit (Guo and Noller 2012). Interestingly, the EF-G dependent GTP hydrolysis is not strictly coupled to the translocation reaction, which also can occur with the non-cleavable GDPNP analog, whereas the Pi release is the essential step after translocation. Pi release triggers a

conformational change of EF-G, upon which this factor leaves the ribosome accompanied by unratcheting, involving movement of A and P site tRNA to P an E site respectively, resulting in formation of classic P/P and E/E state. L1 stalk forms open conformation and EF-G leaves the ribosome. The translocation reaction is schematically depicted in **Figure 6**. Once the A site is vacant, a new aminoacyl-tRNA can be delivered in the form of a ternary complex, which causes the release the of E-tRNA (Triana-Alonso, Chakraburtty et al. 1995). The release of the deacylated tRNA from the E site is triggered after decoding and before accommodation of A site tRNA (Dinos, Kalpaxis et al. 2005). There are two implications of this mechanism. The ribosome shows an allosteric coupling between A and E sites in a sense of a negative cooperativity: the consequence is that in the PRE state A and P sites are occupied and in the POST state E and P sites (Rheinberger, Geigenmüller et al. 1990). Additionally, the E site plays important role in translation fidelity. The occupation of E site by deacylated tRNA restricts the interaction of aminoacyl-tRNA with A site only to the decoding centre, resulting in accommodation of cognate and near-cognate tRNAs exclusively (Rheinberger, Sternbach et al. 1986; Di Giacco, Marquez et al. 2008). As important as accurate incorporation of a single amino acid during the elongation cycle is the termination of protein synthesis, without which the nascent peptide chain cannot leave the ribosome.

1.1.4 Termination

Protein synthesis enters the termination phase as soon as one of the stop codons UAG, UGA or UAA appears at the A site.The recognition of termination codons relies on proteins termed class I release factors (RF), (Capecchi 1967). We distinguish two types of RFs specific for the stop codons: RF1 recognizes UAG and UAA, and RF2 recognizes UAA and UGA (Caskey, Redfield et al. 1967; Wilson, Ito et al. 2000). Domain two of the RFs contains a conserved amino acid motif interacting with stop codons: P(A/V)T for RF1 and SPF for RF2 (Ito, Uno et al. 2000). A correct recognition of stop codon by release factor induces the signal, which rearranges the PTC of the 50S subunit and leads to peptidyl-tRNA hydrolysis. The universally conserved tripeptide GGQ (glycine, glycine, glutamine) of domain 3 of both RF1 and RF2 facilitates the hydrolysis of the ester bond linking

tRNA and peptide chain and thereby induces release of nascent polypeptide (Frolova, Tsivkovskii et al. 1999).

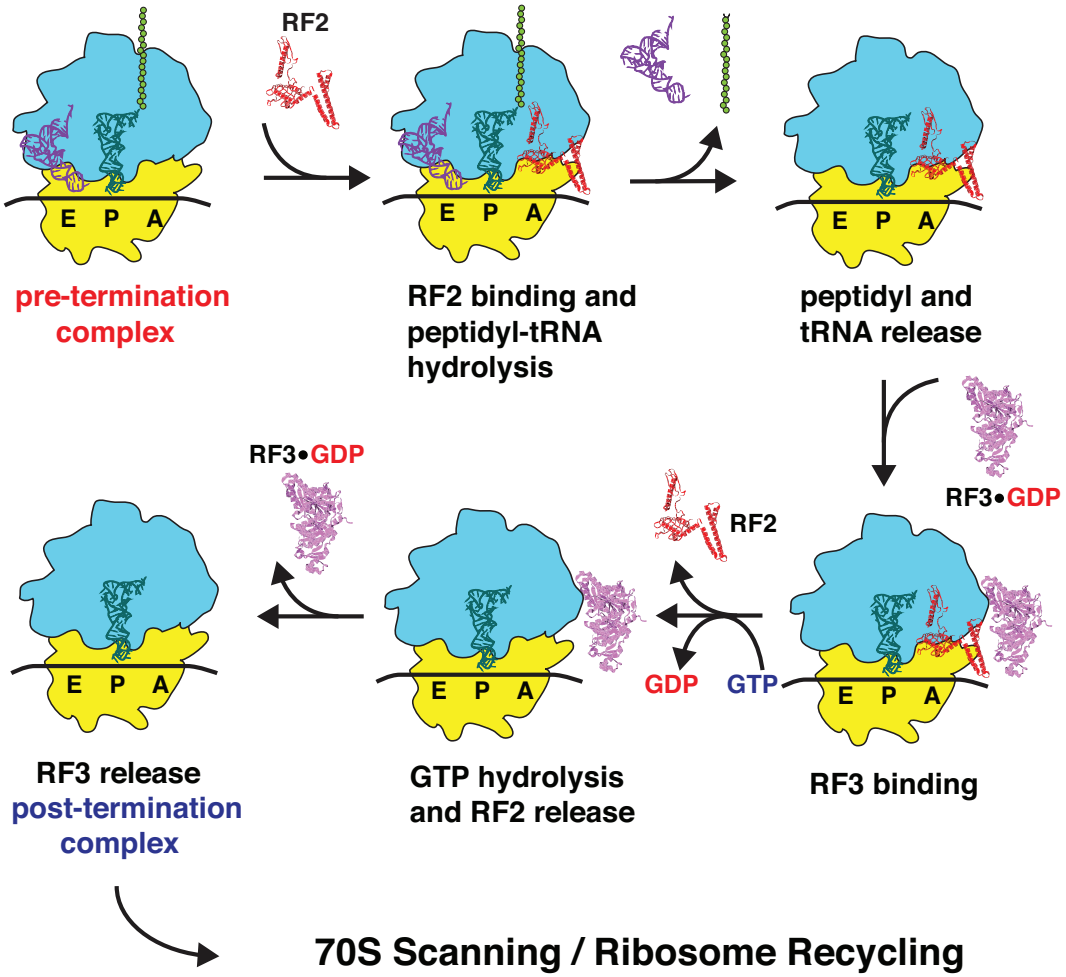

Figure 7. Schematic representation of translation termination. RF2 binds to the ribosomal A site (if an UAG or UAA stop-codon is present in the decoding center of the A site) and promotes the hydrolysis of peptidyl-tRNA. Subsequently, E-site tRNA and peptidyl residue leaves the ribosome. RF3•GDP complex binds to the ribosome. Upon binding RF3 exchanges GDP to GTP, induces GTP hydrolysis and promotes dissociation of P site tRNA. When ribosome is free, it can be either recycled or scans the mRNA for next initiation codon.

A side chain of conserved glutamine contacting the A76 nucleotide is buried in pocket formed by rRNA, RF and tRNA. The mechanism of accommodation of a water molecule to the peptidyl-ester bond remains unclear. Following the peptide chain release, RF1/RF2 is removed by release factor class II (RF3) accompanied by GTP cleavage. RF3 binds to the ribosome in a GDP form. On the ribosome GDP is exchanges to GTP. Finally, the GTP hydrolysis triggers the release of RF3 (Freistroffer, Pavlov et al. 1997; Zavialov, Buckingham et al. 2001). When

ribosome is factor free, the ribosomal recycling factor (RRF) promotes dissociation into subunits in concert with EF-G and IF3 (Karimi, Pavlov et al. 1999; Hirokawa, Iwakura et al. 2008). Alternatively, if the subsequent translation initiation occurs *via* 70S scanning, the ribosome does not dissociate into the ribosomal subunits, but instead scans the mRNA upstream and downstream for AUG codon (Knud Nierhaus, personal communication). Figure 7 shows schematically the termination of translation.

1.2 Regulation of translation during stress

Any kind of stress on the bacterial cell has an immediate influence of transcription and translation. For instance, amino acid starvation increases the pool of deacylated tRNA. Consequently those tRNAs bind codon-dependent to the ribosomal A site and stall the ribosome. Bacteria have evolved numerous systems to deal with stalled ribosome as well as other stress. Some of them, like RelBE, RelA and tmRNA are essential for surviving amino acid starvation and resolve ribosome stalling. Others like EF4 (LepA) prevent misincorporation by backtranslocating the POST to the PRE state. Below we describe the various regulation patterns of protein synthesis under stress.

1.2.1 Translation under high ionic strength facilitated by EF4 (LepA)

EF4 is one of the most conserved bacterial proteins ranging from 55 % to 68 % amino-acid identity (Margus, Remm et al. 2007). Deletion of the *lepA* gene has little effect on viability (Dibb and Wolfe 1986; Kiser and Weinert 1995), however at low pH and high concentrations of K^+ and Mg^{2+} EF4 becomes essential for growth (Bijlsma, Lie et al. 2000; Pech, Karim et al. 2011). EF4 is a G protein sharing high homology to EF-G, although lacking domain IV and the G' domain within domain I. EF4 has a low intrinsic GTPase activity that is stimulated by 70S ribosomes (Qin, Polacek et al. 2006). Overexpression of EF4 *in vivo* is toxic to the cell, and consistently addition of increasing amounts of EF4 protein progressively inhibits *in vitro* translation (Pech, Karim et al. 2011). On the other hand, addition of low amounts of EF4 increases the fidelity of translation *in vitro* counteracting the translation misreading induced by increased Mg^{2+} ion

concentration (Qin, Polacek et al. 2006). EF4 recognizes a POST state ribosome, induces a back-translocation, moving the P and E site tRNAs back into the A and P sites, respectively (Qin, Polacek et al. 2006). Therefore, EF4 is considered as a stress response elongation factor working under conditions of low pH (≤6.5) or high ionic strength (K^+ or $Mg2^+$), a condition usually leading to misincorporation of amino acids. In such a case, EF4 detects these defective POST ribosomes and induces a back-translocation, thus providing EF-G another chance to translocate the ribosome correctly. Recently, it has been demonstrated that EF4 recognized also stalled ribosomal PRE states, which might be even the preferential substrate for EF4 (Liu, Chen et al. 2011).

1.2.2 RelA, SpoT and the stringent response

A stringent response is an adaptation response in bacteria under nutrient starvation. In bacteria there is a tight coupling of protein and RNA synthesis: if protein synthesis stops, the synthesis of rRNA is stopped within a fraction of a minute. There are two effects of stringent response. Genes involved in translation are repressed at the transcriptional level (Lazzarini and Dahlberg 1971; Dennis and Nomura 1974). On the other hand, genes encoding metabolic enzymes, especially those involved in amino acid synthesis are upregulated (Cashel, Gentry et al. 1996; Zhou and Jin 1998). Stringent response is activated upon the shortage of one or more amino acids, which eventually increases the pool of uncharged tRNA (deacylated tRNA) for the corresponding amino acid(s). Under optimal conditions the fraction of deacylated tRNA rarely exceeds 15 %, whereas amino acid starvation elevates the pool of deacylated tRNA up to 85 % (Yegian, Stent et al. 1966). The shortage of aminoacyl-tRNA allows deacylated tRNA to bind to the ribosomal A site, given that it is a cognate tRNA (Jenvert and Schiavone 2005). A deacylated-tRNA bound to the ribosomal A site triggers RelA-dependent synthesis of guanosine 5’- triphosphate 3’ diphosphate (pppGpp) and guanosine 3’, 5’ bisphosphate (ppGpp) from ATP and GTP or GDP, respectively (Haseltine, Block et al. 1972; Haseltine and Block 1973; Sy and Lipmann 1973). The products, collectively referred to as (p)ppGpp exert a regulatory effect on transcription *via* an interaction with the β-subunit of the transcriptase (Travers 1976; van Ooyen, Gruber et al. 1976; Chatterji, Fujita et al. 1998). Binding of RelA to 70S ribosomes

is essential for the production of (p)ppGpp synthesis (Ramagopal and Davis 1974; Richter, Nowak et al. 1975; Richter 1976). Furthermore, the synthesis of (p)ppGpp has been shown to be dependent on a deacylated-tRNA at the A site (Haseltine and Block 1973) and inhibited *in vivo* when L11 is absent (Friesen, Fiil et al. 1974). The binding of RelA to the ribosome is predominantly influenced by mRNA and not by deacylated-tRNA or L11 (Wendrich, Blaha et al. 2002). In contrast, RelA-catalyzed (p)ppGpp synthesis is strictly dependent on L11 and is coupled to RelA release from the ribosome. Furthermore, it is the release of RelA, not deacylated-tRNA, from the ribosome that is concomitant with (p)ppGpp synthesis. This explains how approximately 200 copies of RelA in a bacterial cell can control the fraction of stalled ribosome within a huge ribosome population of up to 70,000 per cell (hopping model of the RelA action (Wendrich, Blaha et al. 2002). The hopping model of RelA action was recently extended by the observation that the released RelA after the synthesis of a (p)ppGpp molecule continues the (p)ppGpp synthesis outside the ribosome (English, Hauryliuk et al. 2011).

1.2.3 RelBE – a toxin-antitoxin system

The stringent response is linked to another system of stress response called RelBE. RelBE is a suicide system in bacteria, consisting of a stable toxin and unstable antitoxin, which binds to toxin, thereby neutralizing it. The RelE toxin inhibits translation by cleaving after the second position of the codon of the mRNA located at the ribosomal A. RelE preferentially cleaves stop codons and some sense codons. When a deacylated tRNA is at the P site, then cleavage at A and E sites is observed with comparable efficiency, without ribosomes the mRNA is not cleaved (Pedersen, Zavialov et al. 2003). Notably, RelE has no resemblance to known RNases, having instead a globular structure. In fact, the toxin mimics, in overall dimension and folding topology, domain IV of the EF-G that interacts with the decoding center at the ribosomal A site (Pedersen, Zavialov et al. 2003). On the other hand, the antitoxin RelB has no hydrophobic core and therefore it is unstable in free state; it has a defined structure only in complex with RelE. This explains why RelB, which is unstructured in the free state, has a short half-life. Crystal structure of RelBE complex revealed that RelB wraps around RelE, thereby making the toxin to big to enter the ribosomal A site (Takagi, Kakuta et al.

2005). Both toxin and antitoxin are encoded by the same operon with toxin downstream of the antitoxin gene. Under stress conditions ribosomes are stalled with deacylated tRNA. This leads to decrease in translation of antitoxin RelB and degradation of RelB by proteases (Christensen, Mikkelsen et al. 2001). As a consequence RelE cleaves mRNA on stalled ribosome, which is eventually a target for another protein called tmRNA, described in the next chapter.

1.2.4 Rescue of ribosome by tmRNA and SmpB

When ribosome stalls on mRNA lacking stop codon, a tmRNA (also called SsrA or 10S RNA) comes to rescue. tmRNA is highly structured molecule, containing a tRNA like domain (TLD) and an mRNA like domain (MLD) (Keiler, Waller et al. 1996). TLD domain can be aminoacylated with alanine by alanyl-tRNA-synthetase. Then aminoacylated tmRNA is delivered to the A site by EF-Tu supported by the Small protein B (SmpB). TLD domain of tmRNA together with SmpB mimics the anticodon stem of a tRNA molecule (Gutmann, Haebel et al. 2003; Shpanchenko, Zvereva et al. 2005). However, mutations in conserved residues of the decoding center on 30S (A1492, A1493 and G530) do not affect peptidyl transfer rate or EF-Tu mediated GTP hydrolysis of the tmRNA•EF-Tu•GTP complex (Miller, Liu et al. 2011). Furthermore, mutations in the C-terminal region of SmpB only slow down peptide bond formation rather than GTP hydrolysis (Miller, Liu et al. 2011). On the other hand, truncation of C-terminal tail of SmpB abolishes trans translation reaction (Konno, Kurita et al. 2007). Therefore, although SmpB mimics a tRNA anticodon loop and enhances peptide bond formation, the decoding step is not required for subsequent peptidyl transfer. Once tmRNA together with SmpB is accommodated on the A site, a peptide bond is formed between stalled peptide and alanyl-tRNA. tmRNA is then translocated by EF-G to the P site. As a consequence the MLD domain enters the mRNA tunnel, moves to the A site and is translated (Ramrath, Yamamoto et al. 2012). MLD encodes a degradation signal for proteases followed by a stop codon. The consequence is that the truncated peptidyl moiety obtains the MLD encoded tag, is released form the ribosome and degraded by cellular proteases.

1.3 Silencing of protein synthesis

1.3.1 100S formation by RMF, HPF and PY

We distinguish three main stages of bacterial growth: lag, logarithmic and stationary phase. During the lag-growth phase bacterial cells adapt to new media conditions. During logarithmic growth phase cells double in an exponential manner, whereas in the stationary phase the cell division is ceased. In stationary growth phase, in *E. coli* we observe a 70S ribosome dimer called 100S particle. Three factors are involved in the regulation of 100S formation: The Ribosome Modulation Factor (RMF) and Hibernation Promoting Factor (HPF) induce dimerization of ribosome called 100S particle, whereas the protein Y (PY) blocks dimerization (Wada, Yamazaki et al. 1990)). Transition of 70S to 100S occurs in a stepwise fashion. First RMF stimulates the formation of 90S particle, which is a target for HPF promoting a maturation of 90S to 100S (Wada, Igarashi et al. 1995; Ueta, Yoshida et al. 2005). **Figure 8** shows the recent crystal structure of RMF, HPF and PY on the 30S ribosomal subunit.

RMF is a small basic protein (pI 11.3, 6.5 kDa) expressed in stationary phase (Wada, Yamazaki et al. 1990). Deletion of RMF abolishes 100S formation and decreases viability during stationary phase and at low pH, *e.g.* pH 3.5 (Ueta, Yoshida et al. 2005). Incubation of 70S with RMF is sufficient for dimerization of 70S to 90S particle (Wada, Igarashi et al. 1995). Furthermore, RMF inhibits poly(U) and MS2 dependent translation as well as binding of fMet-tRNA and Phe-tRNA to ribosomes (Yamagishi, Matsushima et al. 1993). Interestingly, RMF preferentially binds to stationary-phase ribosomes rather than to those from logarithmic phase (Yoshida, Ueta et al. 2009). Furthermore, a presence of the stringent alarmone (p)ppGpp has a positive effect on expression of RMF (Izutsu, Wada et al. 2001). According to DMS crosslinking and protection analysis experiments, RMF interacts with ribosomal proteins S13, L12, L2 and protects nucleotides in the PTC (Wada 1998; Yoshida, Maki et al. 2002; Yoshida, Yamamoto et al. 2004). On the contrary, the x-ray structure of 70S ribosomes with RMF revealed that RMF overlaps with messenger RNA (mRNA) and Shine-Dalgarno sequence on 30S subunits, thereby preventing the interaction between the mRNA and the 16S ribosomal RNA (Polikanov, Blaha et al. 2012).

HPF (*yhbh* gene), (10 kDA, pI 6.62) is another protein associated with 100S formation (Maki, Yoshida et al. 2000). Deletion of HPF elevated the pool of 90S particles. Therefore, HPF has been proposed to bind to 90S particles and matures them to 100S particles (Ueta, Yoshida et al. 2005). However, deletion of HPF in *E. coli* shows no phenotype under optimal growth condition (Baba, Ara et al. 2006). HPF is present in nearly all-bacterial kingdoms. HPF exists in two versions: a short (about 95 amino acids) and long one (about 180 amino acids), which has an additional domain at the C-terminal (Ueta, Ohniwa et al. 2008). Since RMF, a key player in 100S formation, is poorly conserved in bacterial kingdom, a longer version of HPF might take over the function of 70S dimerization. HPF binds to 30S subunit and overlaps with mRNA and tRNA binding site. As a consequence, HPF inhibits translation in vitro (Yoshida, Maki et al. 2002).

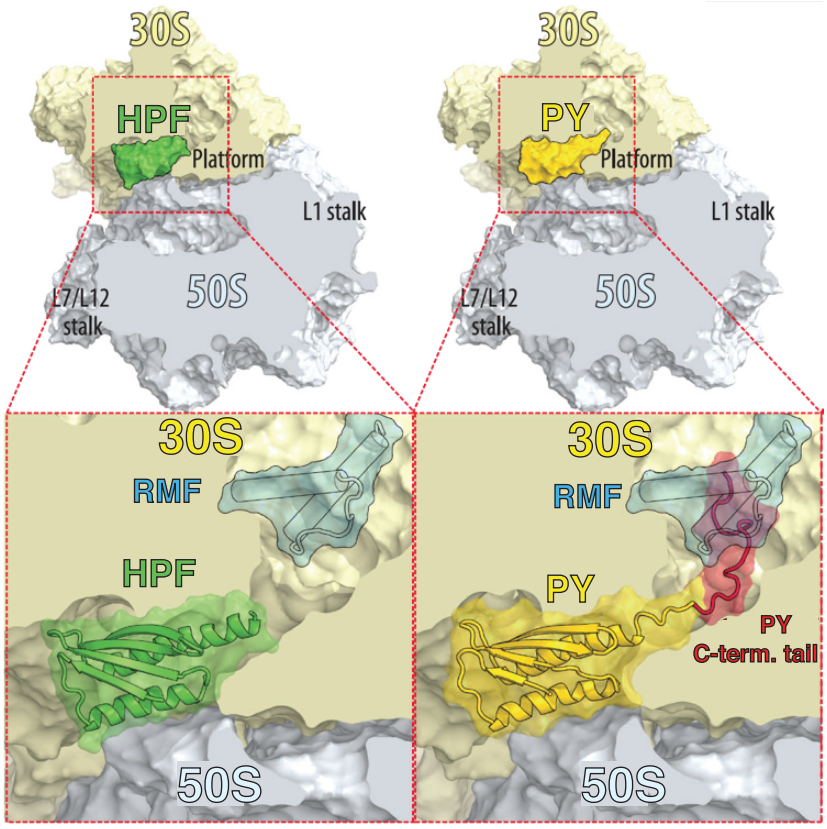

Figure 8. Crystal structure of RMF, HPF and PY bound to 30S subunit. HPF and PY have overlapping binding site. Binding of RMF and PY is mutually exclusive. Green – HPF, yellow – PY, light blue – RMF and red – C-terminal region of PY; according to (Polikanov, Blaha et al. 2012).

PY, encoded by the *yfia* gene (pI 6.23 and 12.6 kDa) is a paralog of HPF, sharing 40 % amino acid identity. Similarly to HPF, deletion of PY is viable in *E.*

coli. However, lack of PY increases fraction of 100S particle. This led to the conclusion that PY prevents 70S dimerization. (Ueta, Yoshida et al. 2005). PY binds to 100S particles and 70S ribosomes and as other 100S factors it inhibits poly(U) dependent poly(Phe) synthesis (Maki, Yoshida et al. 2000). PY is important during cold shock and prevents 70S dissociation at low Mg^{2+}. It inhibits translation at the aminoacyl-tRNA binding stage (Agafonov, Kolb et al. 2001). Furthermore, it reduces translation errors (Agafonov and Spirin 2004). PY binds to P an A sites on the 30S subunit overlapping with the binding site of HPF (Vila-Sanjurjo, Schuwirth et al. 2004; Polikanov, Blaha et al. 2012). However a part of PY reaches out the biding site of RMF. This explains why PY and HPF have opposite function, although being closely related.

In *E. coli* the occurrence of 100S particles has been reported from the semi-logarithmic phase until a prolonged stationary phase (Wada, Igarashi et al. 1995). On the other hand, in *Staphylococcus aureus* 100S particles has been observed from early logarithmic phase till stationary phase (Ueta, Wada et al. 2010). Although 100S particles have been proposed to protect bacterial ribosome under prolonged stress conditions, the half-life of 100S has its limit. After approximately four days in stationary phase 100S dissociates into 70S (Wada 1998; Wada, Mikkola et al. 2000). Moreover, the role of 100S as ribosome protector, has been questioned under elevated temperature (Niven 2004) and high acidity (El-Sharoud and Niven 2005; El-Sharoud and Niven 2007).

1.3.2 Ribosome Silencing Factor S (RsfS)

Another example of non-canonical translation factors has been recently described, Ribosome Silencing Factor S (RsfS / former name Ybeb/RsfA). RsfS, encoded by the *ybeb* gene, belongs to the family of highly conserved proteins containing DUF143 domain. RsfS is present in nearly all bacteria as well as in mitochondria and chloroplasts of eukaryotes (Häuser, Pech et al. 2012). Several groups reported an interaction of this protein with ribosomes (Butland, Peregrin-Alvarez et al. 2005; Gavin, Aloy et al. 2006; Titz, Rajagopala et al. 2008; Wanschers, Szklarczyk et al. 2012). Others showed that RsfS associates with the large ribosomal subunit (Häuser, Pech et al. 2012; Rorbach, Gammage et al. 2012; Wanschers, Szklarczyk et al. 2012). Further investigations demonstrated

that RsfS and its eukaryotic orthologous binds to the ribosomal protein L14 in bacteria and man (Häuser, Pech et al. 2012). The interaction seems to be conserved, because mutations of three most conserved residues of L14 leads to loss of interaction with RsfS (Häuser, Pech et al. 2012).

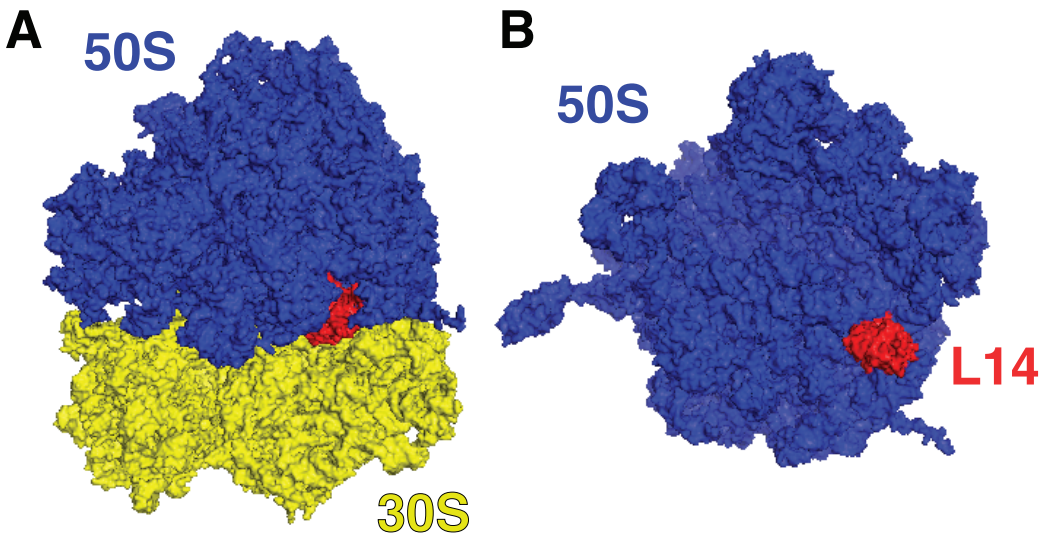

Figure 9. Topography of ribosomal protein L14 on the ribosome – a binding target of RsfS. A) Position of L14 on 70S ribosome: L14 – red; 50S – blue; 30S – red. B) Position of protein L14 of 50S subunit; view at the interface region. PDB files: 1VSA, 2OW8.

Figure 9 shows the position of protein L14 on the ribosome. Although RsfS is well conserved, deletion of this gene is not lethal in bacteria (Baba, Ara et al. 2006). Interestingly, lack of RsfS ortholog in plant *Zea mays* causes the so-called albino-strip-leaf phenotype and was identified as a defect in plastid biogenesis (Jenkins 1924; Walbot and Coe 1979). Depletion of mtRsfS (*c7orf30*) causes an assembly defect in mitochondrial respiratory system (Fung, Nishimura et al. 2012; Rorbach, Gammage et al. 2012). Although the sedimentation profile of mitochondrial ribosomes after silencing of mtRsfS is unchanged, several large ribosome subunit proteins are less expressed upon depletion of this factor (Fung, Nishimura et al. 2012). Interestingly, the reciprocal study - where L14 is depleted - leads to a phenotype equivalent to a mtRsfS depletion (Fung, Nishimura et al. 2012).

1.4 The aim of study

In *E. coli* the number of ribosome ranges from 3,000 to 7,000 depending on the growth phase. The highest density of ribosomes occurs during logarithmic

growth phase, when cells double in approximately 20 min. When cell experiences stress conditions or transits into the stationary phase, the growth rate reduces accompanied by depleting the pool of active ribosomes. We have a limited knowledge about the mechanism of ribosome silencing. The 100S factors have been proposed to play a key role in down-regulation of protein synthesis during the transition to the in stationary phase. However, 100S particles were reported not only during the stationary phase, but also in early logarithmic phase (Wada, Igarashi et al. 1995; Ueta, Wada et al. 2010). Furthermore, 100S particles disappear in *E. coli* after 4 days of incubation in stationary phase, most probably converted into 70S ribosome (Wada 1998; Wada, Mikkola et al. 2000). Here we present a study of the mechanism of ribosome silencing in bacteria using *in vivo* and *in vitro* methods. Our data indicate that RsfS is a key protein for ribosomal silencing.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and biological components

Amersham:

ECL Plus Western Blotting Detection Kit, (RPN2132)

Bovine Serum Albumine (BSA, RNase/DNase Free), (27-8914-02)

Poly(U), (27-444002)

Beckman:

Ready Value (Liquid Scintillation Cocktail), (PN 586602)

Ultracentrifuge tubes (Ultra-Clear) SW40 and SW60, (344060, 344058)

Biorad:

2x Lämmli Sample Buffer, (161-0737)

2x Native Sample Buffer, (161-0738)

Sodium-dodecyl-sulfate (SDS), (161-0301)

Calbiochem:

HEPES, Free Acid, ULTROL® Grade

Difco:

Bacto™ Agar, (214010)

Bacto™ Peptone, (211677)

Bacto™ Yeast Extract, (212750)

Invitrogen:

Agarose Electrophoresis Grade, (15510-019)

Sucrose, (15503-022)

TEMED, (15524-010)

Fermentas:

2x Loading Dye Solution for RNA electrophoresis, (#R 0641)

6x Loading Dye Solution, (#R 0611)

GeneRuler.™ 1kb DNA Ladder, (#SM 0311)

GeneRuler™ 100bp DNA Ladder RNA ladder, (SM 0241)

RNA Ladder, Low Range (#SM 0411)

T4 DNA Ligase, (#EL 0015)

Fluka:

Spermidine trihydrochloride, (85578)

Spermine tetrahydrochloride, (85605)

Merck:

2-Propanol, (1.09634.2500)

β-Mercaptoethanol, (8.05740.0250)

Acetic acid glacial 100 %, (1.00063.2500)

Ammonium acetate, (1.01116.1000)

Ammonium chloride, (1.01145.1000)

Ammonium persulfate (APS), (1.01201.1000)

Boric acid, (1.00165.1000)

Chloroform, (1.02445.1000)

Diethyl ether, (1.00926.500)

Ethanol, (1.00986.2500)

Ethidium bromide (1 %), (1.11608.0030)

Ethylenediaminetetraacetic acid (EDTA), (1.08418.1000)

Glycerol 100 %, (1.05819.1000)

Glycine, (1.04201.1000)

Hydrochloric acid 32 %, (1.00319.2500)

Magnesium acetate, (1.04936.1000)

Methanol, (1.06002.2500)

Potassium acetate, (1.04820.1000)

Potassium chloride, (1.04936.1000)

Potassium hydroxide solution 1 mol/L, (1.09108.1000)

Sodium acetate trihydrate, (1.06265.1000)

Sodium carbonate monohydrate, (1.06386.1000)

Sodium chloride, (1.06404.1000)

Sodium citrate, (1.06448)

Sodium hydroxide, (1.06498.1000)

Sodium hydroxide solution, 1 mol/L (1.09137.1000)

Tris(hydroxymethyl) aminomethane, (1.08382.1000)

Trisodium citrate dehydrate, (1.06448.1000)

Urea, (1.08487.1000)

Millipore:

Filters 0.45 µm, (HAW02500)

New England BioLabs:

Restriction endonucleases with buffers

T4 DNA Ligase, (M0202S)

PerkinElmer:

Filter-Count™ (complete LSC-cocktail)

Promega:

Steady-Glo® Luciferase Assay System

Dual-Glow Luciferase Assay System, (Promega #E2920)

Roche Pharmaceuticals:

1,4-Dithiothreitol (DTT), (1 583 786)

DNase I, RNase-free, (10 776 785 001)

dNTPs: dATP, dCTP, dGTP, dUTP, (1 051 440, 1 051 458, 1 051 466, 1 420 470)

Roth:

Ampicillin, (K029.2)

Chloroform, (AX984.2)

IPTG 25g, (2316.4)

Kanamycin A (KAN), (Roth, T832.3)

Phenol, (0040.2)

Rotiphorese® Gel 30, (37, 5:1) (3029.1)

Rotiphorese® Gel 40, (19:1) (3030.1)

Roti-Mark STANDARD 1 ml, (T851.1)

Sodium-dodecyl-sulfate (SDS), (4360.2)

Trichloroacetic acid (TCA), (8789.1)

Qiagen:

Ni-NTA Spin Kit (50), (31314)

Ni-NTA Agarose (100 ml), (30 230)

Plasmid Maxi Kit, (121163)

Plasmid Midi Kit (100), (12145)

QIAprep® Spin Miniprep Kit (250), (27106)

QIAquick® Gel Extraction Kit (50), (28706)

Santa Cruz Biotechnology:

Goat anti rabbit HRP conjugate

Sartorius:

Nitrocellulose filter, (11306)

Schleicher and Schüll:

Glass Fiber Filters Æ23 mm, (10 370 021)

Serva:

Aluminiumoxid Alcoa A-305, (12293)

Coomassie® Brilliant Blue G-250, (17524)

Coomassie® Brilliant Blue R-250, (17525)

Sigma:

Albumin, bovine, (A-7906)

Ficol, 400

GTP Tris salt

Lysozyme, (L-6876)

ReadyMix.™ Taq PCR Reaction Mix with $MgCl_2$, (P-4600)

Roche:

Rapid Translation System 500, RTS 500 E. coli HY Kit, (3 246 949)

Whatman Ltd., England

Paper Filters

2.1.2 Laboratory equipment

Beckmann Coulter DU®640B Spectrophotometer

Beckmann Coulter Optima™L-90K ultracentrifuge

Beckmann L7-55 ultracentrifuge

Biocomp Gradient Master

Biorad Gene Pulser

Biorad Electrophoresis Chamber

LKB BROMMA Microperpex Peristaltic Pump

LAS – 1000 CCD camera (Fuji film)

Luminometer Centro LB 960 (Berthold technologies, Germany)

New Brunswick Scientific GmbH Innova 4400 incubator shaker

Sorvall RC 5B plus centrifuge

UVICORD S photometer

Wallac 1409 Liquid Scintillation Counter

2.1.3 Bacterial strains

BW25113 *Δ(araD-araB)*567
*ΔrsfS, ΔybeB774::*kan, JW5090-2 (b0637)
Δhpf, *ΔyhbH731::* kan, JW3170-3
Δrmf, *Δrmf-769::*kan, JW0936-1
Δpy, *ΔyfiA760::*kan,JW2578-1
CAN20-12E
BL21(DE3)

2.1.4 Plasmids

pBAD24-lacZ-HA based on pBAD24HA for translation tests in vivo
pRedET (Gene Bridges) to knock out *rsfS*, *hpf, rmf and py* genes in *E. coli*
pCP20 to remove kanamycin cassete in *ΔrsfS*, *Δhpf, Δrmf and Δpy* strains
pHGWA (Gateway System) for expression of RsfS-His and and C7orf30-His
pET28a for expression of HPF, RMF and PY

2.1.5 Primers

RsfS –Flag
GAACTGGAAAAACTCTGGAGTTCCATGGAAGACTACAAGGATGACGATGACA
AGTAA

RsfS-His
GAACTGGAAAAACTCTGGAGTTCCATGGAACACCACCACCACCACCATTAA

RMF knockout reverse
CAAAAGGCGAAACCTCCGCAATGCGGAGGTTTCTTTTTAAAGAGACAGAATA
ATACGACTCACTATAGGGCTC

RsfS knockout forward

ATACTGACAGACCATTTTTATCTATTTGATTCACCCAGGGGGAAAACTTGAATT
AACCCTCACTAAAGGGCG

RsfS knockout reverse

ATTTTCGTTCCCACGGCGACAAGTTGCAGCTTCACGCATTAACTCCAGAGTAA
TACGACTCACTATAGGGCTC

2.1.6 Buffers

Polyacrylamide gels

Solution	**Reagents**	**Concentration / Volume**
Coomassie Blue R-250 Staining Solution (0.05 %)	Coommassie® Brillant Blue R-250	0.05 % (w/v)
	Methanol	50 % (w/v)
	Acetic acid glacial 100 %	10 % (w/v)
Coomassie Blue R-250 Destaining Solution	Methanol	25 % (w/v)
	Acetic acid glacial 100 %	8 % (w/v)
Polyacrylamide gel 15 % for protein electrophoresis (separating gel)	1.5 M Tris solution, pH 8.8	3 ml
	Rotiphorese® Gel 30 (37.5:1)	6 ml
	APS solution 10 %	50 µl
	TEMED	10 µl
	H_2O	ad 12 ml
Polyacrylamide gel 5 % for protein electrophoresis (stacking gel)	0.5 M Tris solution, pH 6.8	1.25 ml
	Rotiphorese® Gel 30 (37.5:1)	0.85 ml
	APS solution 10 %	75 µl
	TEMED	15 µl
	H_2O	ad 5 ml
Tris solution, 0.5 M,	Tris	0.5 M

pH 6.8	Hydrochloric acid 32 %	Adjust pH ~ 6.8
Tris solution, 1.5 M,	Tris	1.5 M
pH 8.8	Hydrochloric acid 32 %	Adjust pH ~ 8.8
10X SDS Electrophoresis	Tris	60 g
Buffer	Glycin	288 g
	SDS	20 g
	H_2O	ad 2L
SDS loading dye	Lämmli SDS loading dye	1 ml
	β – mercaptoethanol	100 mM

Agarose gels

Solution	**Reagents**	**Concentration / Volume**
10X TBE Buffer	TRIS	108 g
	Boric acid	55 g
	EDTA	9.6 g
	H_2O	Ad 1000 ml
50X TAE Buffer	TRIS	242 g
	Glacial acetic acid	57.1 ml
	EDTA 0.5 M	100 ml
	H_2O	Ad 1000 ml
6X DNA Sample Buffer	Ficoll 400	15 % (w/v)
	Bromophenol Blue	0.25 % (w/v)
	Xylene cyanol	0.25 % (w/v)
Electrophoresis buffer	10X TBE/TAE	100 ml
(1X TBE/TAE)	H_2O	Ad 1000 ml

Buffers for microbiological methods

Solution	Reagents	Concentration / Volume
Lysogen broth (LB) medium	Bacto™ Peptone	1 % (w/v)
	Bacto™ Yeast Extract	0.5 % (w/v)
	Sodium choride (NaCl)	1 % (w/v)
Lysogen Broth (LB) agar solid medium	Bacto™ Peptone	1 % (w/v)
	Bacto™ Yeast Extract	0.5 % (w/v)
	Sodium choride (NaCl)	1 % (w/v)
	Bacto™ Agar	1.5 % (w/v)
M9 medium, pH 7	Na_2HPO_4 x 7 H_2O	60 g
	KH_2PO_4	30 g
	NaCl	5 g
	NH_4C	10 g
	$MgSO_4$	1 mM
	$CaCl_2$	0.1 mM
	Glucose	0.4 % (w/v)
	Thiamine	100 µg/ml
		Ad 1000 ml
Medium E supplemented with trypton and glucose	K_2HPO_4	10 g
	$NaHNH_4$ PO_4 4x H_2O	3.5 g
	Citric acid 1 x H_2O	2 g
	$MgSO_4$ – 7 x H_2O	0.2 g
	Trypton	20 g
	Glucose	5g
	H_2O	Ad 1000ml

Buffers for studies of ribosomal functional states

Solution	Reagents	Concentration / Volume
Binding buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_{4.5}N_{150}SH_4Spd_2Spm_{0.05}$)	Magnesium acetate [$Mg(Ac)_2$]	4.5 mM
	Ammonium acetate (NH_4Ac)	150 mM
	β – mercaptoethanol	4 mM
	Spermidine	2 mM
	Spermine	0.05 mM
	H_2O	Ad 10 ml
Tico buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_6N_{30}SH_4$)	Magnesium acetate [$Mg(Ac)_2$]	6 mM
	Ammonium acetate (NH_4Ac)	30 mM
	β – mercaptoethanol	4 mM
	H_2O	Ad 10 ml
HMK buffer	Hepes-KOH, pH 7.5	20 mM
	Magnesium acetate [$Mg(Ac)_2$]	6 mM
	Potasium chloride (KCl)	150 mM
	β – mercaptoethanol	4 mM
	H_2O	Ad 10 ml
Mix I	Hepes-KOH, pH 7.5	60 mM
	Magnesium acetate [$Mg(Ac)_2$]	10.5 mM
	Ammonium acetate (NH_4Ac)	690 mM
	β – mercaptoethanol	12 mM
	Spermidine	10 mM
	Spermine	0.25 mM
	H_2O	Ad 10 ml
Mix II	Hepes-KOH, pH 7.5	100 mM

	Magnesium acetate [$Mg(Ac)_2$]	22.5 mM
	Ammonium acetate (NH_4Ac)	750 mM
	β – mercaptoethanol	20 mM
	Spermidine	10 mM
	Spermine	0.25 mM
	H_2O	Ad 10 ml
Mix III	Hepes-KOH, pH 7.5	66.7 mM
	Magnesium acetate [$Mg(Ac)_2$]	12.6 mM
	Ammonium acetate (NH_4Ac)	500 mM
	β – mercaptoethanol	13.4 mM
	Spermidine	9.96 mM
	Spermine	0.26 mM
	H_2O	Ad 10 ml
Mix II enzymatic	Hepes-KOH, pH 7.5	40 mM
	Magnesium acetate [$Mg(Ac)_2$]	8.3 mM
	Ammonium acetate (NH_4Ac)	300 mM
	β – mercaptoethanol	8 mM
	Spermidine	5 mM
	Spermine	0.125 mM
	H_2O	Ad 10 ml

Buffers for Western Blot analysis

Solution	**Reagents**	**Concentration / Volume**
Transfer buffer	Tris	25 mM
	Glycine	192 mM
	Methanol 100 %	20 % (w/v)
	H_2O	ad 1000 ml
5X PBS pH 6.5	Na_2HPO_4 (anhydrous)	57.5 g

	NaH_2PO_4 (anhydrous)	14.8 g
	NaCl	29.2 g
	H_2O	ad 1000 L
1X PBS-T	5X PBS	50 ml
	Tween 20	100 µl
	H_2O	ad 250 ml
Blocking buffer	1X PBS-T	50 ml
	Milk powder	5 % (w/v)

Buffers for sucrose gradient centrifugation

Solution	**Reagents**	**Concentration / Volume**
Polysome preparation buffer	Hepes-KOH, pH 7.5	20 mM
	Magnesium acetate [$Mg(Ac)_2$]	4.5 mM
	Ammonium acetate (NH_4Ac)	150 mM
	β – mercaptoethanol	4 mM
	Spermine	0.05 mM
	H_2O	Ad 10 ml
100S preparation buffer	Tris	20 mM
	Magnesium acetate [$Mg(Ac)_2$]	15.2 mM
	Ammonium acetate (NH_4Ac)	100 mM
	EDTA	0.8 mM
	DTT	3 mM

2.1.7 Software

Adobe Illustrator CS5

ImageJ 1.440

ImageQuant 5.2

iTOL 2.2

MatLab 2011

Microsoft Office 2011

Pico Log Recorder (Software for Pico ADC-16 module)

Plot 0.997

Plot Digitalizer 2.5.1

PyMol 0.99 (DeLano Scientific LLC, USA)

2.1.8 Kits

ECL Plus Western Blotting Detection Kit, (Ammersham # RPN2132)

High Pure Plasmid Isolation Kit, (Roche # 11754785001)

Invisorb Spin DNA Extraction Kit, (Invitek, Berlin)

Ni-NTA Agarose (100 ml), (Qiagen, Hilden # 30 230)

Qiagen Maxi Prep® Tip 500, (Qiagen, Hilden)

Qiagen Midi Prep® Tip 100, (Qiagen, Hilden)

QIAquick® PCR Purification Kit, (Qiagen, Hilden)

RTS 100 E. coli HY Kit (Roche #03186148001)

Dual-Glow Luciferase Assay System (Promega #E2920)

2.2 Analytical methods

2.2.1 Transmission electron microscopy: Negative staining

The electron microscope is a tool for obtaining a magnified image of an object. It has higher resolution and magnification than an optical microscope due to 100,000 times shorter wavelengths of electrons comparing to visible light photons (350 - 700 nm).

Cells from wild type (WT) and *ΔrsfS* strains were inoculated separately in 5 ml of LB medium and incubated at 37 $^{\circ}$C with shaking (160 rpm) overnight (O/N). After the culture reached the stationary growth phase, cells were centrifuged and resuspended in 1 ml of LB medium to increase cell density. In parallel grids for negative staining were prepared. First a copper 400-mesh hexagonal grid was covered with a carbon film. To make the surface of the carbon film hydrophilic a glow-discharge was performed in a vacuum chamber. Subsequently 5 µl of culture

was applied to the carbon grid resulting in binding of bacteria. After 1 min incubation the supernatant was taken away with filter paper. To contrast bacterial cells, a drop of ammonium molybdate solution (0.2 % in distilled water, pH 7) was placed on covered grid for 5 seconds. Finally the excess of ammonium molybdate was removed by filter paper and ready grids were analyzed in the electron microscope (CM Spirit, FEI) using EMMENU4 software.

2.2.2 Polymerase Chain Reaction (PCR)

PCR uses DNA polymerase to amplify a DNA fragment of interest in an exponential manner. This method is based on thermal cycling, between temperatures favoring: i) DNA melting, ii) primer annealing and iii) enzymatic DNA replication. Two oligonucleotides, complementary to the template on both 3'- and 5'- sides are used as primers to ensure the specificity of amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

PCR was performed with ReadyMix™ Taq PCR Reaction from Sigma. The mix includes Sigma's Taq DNA Polymerase (an enzyme active under high temperature), $MgCl_2$, 99 % pure deoxynucleotides and buffer in a 2X optimized reaction concentrate. Primers were added to the final concentration of 1 mM and the reaction required a minimum of 10 ng of template DNA. Standard PCR cycle was initiated with denaturation step at 94 ºC for 5 minutes, followed by a denaturation at each cycle, i.e. 94 ºC for 30 second. This was then followed by annealing of the primers at the temperature of choice in such a way that it should be 5-8 ºC less than melting temperature (T_m) of both primers. Extension was performed at 72 ºC and the time required was 1 minute for the amplification of a fragment with about 1 kb. This cycle was repeated for 30 times, which, was finally followed by the extension step at 72 ºC for 10 minutes. After the reaction was over, the temperature was reduced to 4 ºC.

2.2.3 Measurement of concentration of nucleic acids

We measure the light absorbance by nucleic acids (RNA for ribosomes) at a wavelength of 260 nm to calculate the concentration of a particle of interest, such 30S, 50S and 70S ribosome. The measurement is carried out in a micro

cuvette (Beckman Coulter) with a volume of 100 µl whereas the optical path has a length of 1cm.

According to (Sambrook, Fritsch et al. 1989) we use the following approximation of calculation of the concentrations of specific particles:
1 A_{260} of double stranded DNA = 50 µg/ml
1 A_{260} of single stranded DNA or RNA = 40 µg/ml
1 A_{260} of oligonucleotide = 20 µg/ ml

We used the following equivalences for ribosomal particles and tRNA:
1 A_{260} of 70S = 24 pmol ($\varepsilon = 4.2 \times 10^7\ M^{-1} \times cm^{-1}$)
1 A_{260} of 50S = 36 pmol ($\varepsilon = 2.8 \times 10^7\ M^{-1} \times cm^{-1}$)
1 A_{260} of 30S = 72 pmol ($\varepsilon = 1.4 \times 10^7$ M-1 x cm^{-1})
1 A_{260} of tRNA = 1.5 nmol

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA or RNA depending mainly on their molecular weight. Due to phosphate residues bearing a negative charge the nucleic acids migrate in the electric field towards the anode with a positive charge. The agarose content of a gel ranges from 0.4 % to 2 %, depending on the difference in molecular weight of separated molecules. Higher concentration of agarose increases the resolution power but decreases the separation time. **Table 1** the resolution power of agarose gels depending on the concentration of agarose.

For preparation of agarose gels 100 ml of 1x TBE or TAE buffer were mixed with the proper amount of agarose (e.g. 1 g for 1 %) and shortly heated in a microwave, until there were no undisolved agarose pieces were in the solution. The solution was slightly cooled 5 µl of a 1 % ethidium bromide solution was added. The mixture was then poured into a gel chamber and a pocket-forming comb was inserted. After the gel was solid, the comb was removed and the chamber was being filled with buffer. TBE buffer is used for analysis and TAE used for purification and cloning procedures. Boric acid present in TBE buffer blocks enzymes used for cloning.

Agarose concentration [%]	Resolution power [kb]
0.4	30 – 2.5
0.8	15 – 1
1	10 – 0.5
1.25	6 – 0.4
1.5	3 – 0.2
2	2.5 – 0.1

Table 1. The resolution power of agarose gels.

The electrophoretic separation was carried out at 5-10 V/cm. After separation the gel was analyzed under UV-light which excitates ethidium bromide, an intercalator binding to the DNA double strands. The gel analysis was carried out in a Herolab gel documentation system.

2.2.5 Polyacrylamide gel electrophoresis

The SDS polyacrylamide gel electrophoresis is used to separate protein mixtures under denaturing conditions. SDS PAGE consists of two gels: stacking and separating one. The stacking gel concentrates the protein-SDS complexes, thereby increasing the resolution of SDS PAGE. In the separating gel proteins are separated according their molecular mass. SDS denatures the proteins and shields their charge therefore the structure of proteins does not affect the migration in the gel. The resulting charge of the SDS protein complex is dependent on the molecular weight of the protein, because each SDS molecule shields one aminoacid. For the SDS PAGE a Mini Protean II System from Biorad was used. **Table 2** and **Table 3** show the resolution power of SDS PAGE gels.

Concentration of acrylamide [%]	Resolution power [kDa]
6	60-120
8	40-140
10	20-80
12	15-70
15	10-15

Table 2. The resolution power of SDS-PAGE.

Reagent	Volume [µl]
Acrylamide 30 %	2000
Tris 1.5 M pH 8.8	1300
H_2O	1600
SDS 10 %	100
APS 10 %	20
TEMED	2.5

Table 3. Composition chart of a 12 % Separation Gel.

First, the separating gel was poured and overlayered with isopropanol, to produce a smooth, completely leveled surface on the upper edge of the separating gel. After polymerization the over-layer was removed, the stacking gel was poured and a comb inserted. Samples were prepared with 5x loading dye and all complemented with MilliQ water to the same volume. Then, the samples were incubated at 95 °C for 8 min for complete denaturation and prior to loading quickly cooled on ice. The electrophoresis was carried out at first for 10 min at 80 V and followed by 150 V for 30 min. Gels were stained by incubation in Coomassie staining solution for 15 minutes. Destaining was carried out by boiling the gel for 2-5 minutes in a microwave.

2.2.6 Analytical sucrose gradient centrifugation

Sucrose gradient centrifugation is a technique that allows the separation of complexes based on the sedimentation coefficient (S). We used this technique to separate 70S, 50S and 30S ribosomal subunits. A sucrose gradient (10-30% w/v) was prepared in an Ultra-Clear or polyallomer tube (14 x 95 mm Beckman) using Biocomp Gradient Master 107ip. The reaction mix was overlaid on the gradient and centrifugation was performed in SW 60 or SW 40 rotors (Beckmann). In the SW 40 up to 10 A_{260} units of pure ribosomes or ribosomal subunits per tube can be loaded. In case of SW 40 1 A_{260} of ribosomes is optimal. The centrifugation was performed in an ultracentrifuge (Beckman). After centrifugation the gradient was fractionated while monitoring the absorbance at 260 nm.

Running time and rpm were optimized for ideal separation:

Subunit profile in SW 60, 38,000 rpm and 240 min.
Polysome profile in SW 60, 38,000 rpm and 120 min.
Polysome profile in SW 40, 18,000 rpm and 16 h.
Subunit profile in SW 40, 22,000 rpm and 18 h.

After centrifugation the gradient was fractionated while monitoring the absorbance at 260 nm.

2.2.6.1 Preparation of polysomes

Wild type and *ΔrsfS* mutant strains, were inoculated in 5 ml of LB and (mutant culture contained kanamycin – 50 mg/µl) at 37 ºC and with 150 rpm agitation O/N. Following overnight cultivation, culture were diluted in 100 ml LB to OD_{600} = 0.05 and incubated at 37 ºC with 150 rpm agitation. The OD_{600} was measured in regular time intervals until it reached approximately OD_{600} = 0.5. Then cell cultures were fast cooled by adding approximately 100 ml of ice and then poured into a pre-chilled centrifuge bottles. The mixture was shaken energetically for 5 seconds and immediately put in an acetone bath containing dry ice and again shaken for 15 seconds. Subsequently, bottles were placed in the pre-chilled centrifuge rotor and cells were pelleted down at 5,000 rpm for 10 min. The supernatant was decanted and each pellet was resuspended gently in 1 ml of 1X Polysome Preparation Buffer containing lysozyme (0.4 mg/ml) and transferred to 2 ml Eppendorf tubes containing 5 µl of DNase. The samples were mixed gently, frozen in liquid nitrogen and kept at -80 ºC for two hours. Then frozen samples were thawed at 4 ºC and centrifuged at 10,000 rpm for 5 minutes in pre–chilled centrifuge. The supernatant was collected in fresh tube. Following the measurement of A_{260} samples were diluted to A_{260} = 3 - 4 and loaded on a 10 - 30 % gradient prepared in Binding Buffer in SW60 tubes. Samples were then centrifuged at 35,000 rpm for two hours. Polysome profiles were monitored with 2138 UVICORD S photometer from LKB BROMMA.

2.2.6.2 Preparation of 100S particles

Wild type and *ΔrsfS* cells were inoculated in 100 ml of LB/M9/M9 supplemented with 2 % casamino acids or medium E supplemented with 0.5 %

glucose and 2 % trypton. Strains were grown (mutant culture contained kanamycin – 50 mg/µl) at 37 ºC and with 120 rpm agitation overnight. Following overnight cultivation, cultures were centrifuged at 5,000 rpm for 10 min. The supernatant was decanted and each pellet was grinded 60 seconds in porcelain mortar using aluminium oxide (Alcoa, 1 g cells + 2 g aluminium oxide). The disrupted cells were resupended in 100S Buffer (20 mM Tris, 15 mM Mg, 100 mM NH_4Ac) and transferred to 2 ml Eppendorfs tube containing 5 µl of DNase I. Then the Eppendorfs were centrifuged at 10,000 rpm for 5 minutes in pre–chilled centrifuge. Supernatant was collected in fresh tube. Following the measurement of ribosome concentration at A_{260}, samples were diluted to A_{260} = 2 and loaded on 5 - 20 % sucrose gradient prepared in 100S Preparation Buffer. Samples were centrifuged in SW60Ti rotor at 38,000 rpm 80 min at 4 ºC. Ribosome profiles were monitored with 2138 UVICORD S photometer from LKB BROMMA at 260 nm.

2.2.7 Cold Trichloroacetic-acid (TCA) precipitation

Samples derived from sucrose gradient centrifugation were diluted (1 : 1 proportion) in Binding Buffer. Subsequently TCA was added to the final concentration of 10 %. Then samples were incubated on ice for 1 hour and centrifuged at 14,000 rpm for 1.5 hour. The supernatant was discarded and the pellet was resuspended gently in equal volume of acetone. A second centrifugation at 14,000 rpm for 1 hour was performed. After removing the supernatant, samples were incubated at 37 ºC until the acetone evaporated. Then samples were resuspended in SDS loading dye containing 50 % of polysome SDS loading dye, denatured at 80 ºC for 5 min and applied to an SDS PAGE gel.

2.2.8 Western blot

First the membrane was incubated in methanol for few seconds, then washed in MilliQ water for 5 minutes and equilibrated in transfer buffer before the blotting procedure. Gels from SDS PAGE were equilibrated in transfer buffer for 20 min. Proteins were transferred from polyacrylamide gel to PVDF membrane using the BioRad Mini Trans-Blot^{TM} system. Transfer proceeded at 100 V for 1 hour. The efficiency of transfer in gel was verified by Coomassie blue staining. Then the membrane was blocked with Blocking Buffer with gentle agitation over

night at 4 °C. Subsequently, the membrane was incubated in PBS-T with gentle shaking three times for 10 min order to remove Blocking Buffer. Primary antibodies (diluted 1 : 10,000 in Blocking Buffer) were added to the membrane and incubated with gentle shaking for 2 hours. Next, the excess of antibodies was removed by incubating with PBS-T three times for 10 min. Then the membrane was incubated with secondary antibodies (diluted 1: 10,000 in Blocking Buffer) for 1 hour followed by three times washing step in PBS-T. To develop the membrane an ECL-Kit for Western blotting detection was used according to the manual of the supplier. Chemiluminescence detection is then performed with a LAS-1000 camera (Fuji Film).

2.3 Preparative methods

2.3.1 Ribosome preparation

2.3.1.1 Isolation of tight coupled 70S from *E. coli*

The isolation of ribosomes was carried out according to the method described in Bommer et al. (Bommer, Burkhardt et al. 1996). The isolation of ribosome was carried out in Tico buffer ($H_{20}M_6N_{30}SH_4$), (Hapke and Noll 1976). For large scale preparation 200 g of frozen cells of the strain CAN/20-12E were dissolved in 400 ml of Tico buffer. Then cells were disrupted using a microfluidizer. Disrupted cells were centrifuged at 16,000 rpm for 45 min in an SA-600 rotor. The pellet consisting mainly of cell debris was discarded and the supernatant holding ribosomes and soluble enzymes (also called as S-30) was centrifuged further at 22,000 rpm for 20 h in a 45Ti-Rotor to pellet the 70S-ribosomes. The pellet was then resuspended in Tico buffer and centrifuged again for 10 min to remove insoluble aggregates. The supernatant (S100) was shock frozen and kept for further purification processes. The “crude 70S” particles were aliquotized and also shock frozen in liquid nitrogen.

2.3.1.2 Isolation of 50S and 30S subunits

Ribosomal subunits were obtained by dissociation of 70S ribosomes. First crude 70S were purified from factors-contaminations by zonal centrifugation in a

linear sucrose gradient (0-40 %) in Tico buffer. The centrifugation was carried out in a zonal rotor Ti15 at 22,000 rpm for 17 h at 4 °C. Subsequently, the gradient was pumped out by feeding 50 % sucrose solution into the rotor while running at 3,000 rpm. The gradient was then pushed out by the sucrose solution. The 70S peak containing fractions was pooled and centrifuged in a Ti45 rotor at 24,000 rpm for 24 h to pellet the tight-coupled 70S free of factors. The pelleted 70S (tight-coupled) were dissolved in dissociation buffer and a second zonal run with the same conditions mentioned above was performed. After the zonal run 50S and 30S containing fractions were pooled and pelleted overnight at 34,000 rpm for 22 h in a Ti 45 rotor. Subunits were then dissolved either in Tico buffer or in reassociation buffer to form reassociated 70S, used for the functional assays. Crude 70S or tight-coupled 70S cannot be used for this purpose since they still contain mRNA fragments and tRNA.

The concentration of the subunits was determined by measuring their optical density at A_{260}. Subunits were then aliquotized, shock frozen and stored at -80 °C. The typical yield is 600 A_{260} units of 30S and 800 A_{260} units of 50S per 3,000 A_{260} tight-coupled 70S.

2.3.1.3 Preparation of reassociatiated 70S

Crude 70S and isolated tight couples are obtained by isolation of translating ribosomes in the cell (polysomes), therefore they usually hold fragments of mRNAs and tRNAs. To remove mRNA and tRNA these ribosomes have to be dissociatied as described in chapter 2.3.1.2. Afterwards subunits are reassociatied under high magnesium concentrations to recover 70S particles (Blaha, Burkhardt et al. 2002). Reassociated ribosomes are more efficient in the binding of tRNAs as well as in their activity in poly(U) dependent poly(Phe) synthesis.

First, 50S and 30S subunits were mixed together in a ratio of 2:1 (6000 A_{260} of purified 30S with 3000 A_{260} of 50S). A high excess of 30S was used in this incubation step to minimize the amount of free 50S. The mixture was diluted to a final concentration of 140 A_{260}/ml and incubated for 60 min at 40 °C. After the incubation step the possible ribosome aggregates wre pelleted down at 10,000 rpm for 15 min. The reassociated 70S were then applied to the zonal gradient in reassociation buffer. The zonal run was centrifuged in linear sucrose gradient

(0-40 %) for 17 h at 18,000 rpm and 4 °C. The gradient was fractionated, the 70S peak collected and the re-associated particles pelleted in a Ti 45 rotor for 24 h at 24,000 rpm. The pellets were finally dissolved in Tico buffer and their concentration measured at A_{260}. The ribosomes were then aliquotized in 50 µl aliquots shock frozen and stored at -80 °C.

2.3.2 Ribosome crosslinking with DMS

To create ribosomes unable to dissociate, the compound dimethyl-suberimidate (DMS) was used according to Moll et al. (Moll, Hirokawa et al. 2004) with modifications. A typical reaction producing cross-linked ribosomes was performed in $H_{20}M_6K_{30}SH_4$. The buffer was supplemented with DMS to a f.c. of 10 mM and the pH adjusted to 8.5 – 9. Ribosomes were diluted in this buffer yielding a concentration of 0.3 µM. Cross-linking was allowed for 2.5 hours at 30 °C and stopped by the addition of 0.1 volume of 1 M Tris-HCl, pH 6.7. Hereafter the sample was dialyzed against 100 volumes of $Tris_{10}N_{60}M_{10}SH_4$ for 45 min. To purify the dissociation resistant 70S fraction from ribosomes that were not cross-linked during the reaction time, the sample was placed on a 10-30 % sucrose gradient in $H_{20}M_1N_{60}SH_4$ and centrifuged. The dissociation resistant fraction was collected, pelleted and dissolved in $H_{20}M_6N_{30}SH_4$. A_{260} was measured, the sample aliquotized and stored at -80 °C. An analytical sucrose gradient centrifugation (SW 60 rotor at 45,000 rpm for 2 h 15 min) was performed under low magnesium concentration (1 mM), where native and cross-linked ribosomes were compared in their dissociation behavior.

2.3.3 Protein overexpression

RsfS and its mitochondrial paralog C7orf30 were expressed using the Gateway System compatible plasmid pHGWA. The gene coding for *E. coli* RsfS (b0637) was expressed as an N-terminal His-tag fusion in E. coli BL21 (DE3). First, a single colony of BL21 (DE3) with RsfS expression plasmid was inoculated in LB medium with 50 µg/ml of ampicillin and grown overnight at 37 °C. Next day the culture was diluted to OD_{600} 0.05 and grown in 4 flasks containing 1 L of LB medium at 37 °C with 50 µg/ml of ampicillin. The expression was induced at OD_{600} = 0.4 with 0.1 mM IPTG and carried out for 2 h at 30 °C to decrease the formation

of inclusion bodies. The cells were disrupted with microfluidizer and centrifuged for 1h at 30,000 rpm in Type 60Ti rotor. The supernatant was collected. The soluble protein was purified via nickel-nitrilotriacetic-acid-agarose (Qiagen, according to the manufacturer's manual) and anion exchange chromatography (Source 15Q, GE Healthcare). The purified protein was dialyzed against 20 mM Hepes, 6 mM Mg-acetate, 150 mM K-acetate, 4 mM β-mercaptoethanol, pH 7.6 at 0 °C. The human mitochondrial RsfS (C7orf30; amino acids 23–234) was expressed N-terminal His tag fusion and the protein purified like the *E. coli* RsfS paralog.

RMF, HPF and PY were expressed as N-terminal His-tag fusion using pET28a (kanamycin resistance) plasmid in BL21 (DE3) cells. First each strain was inoculated from a single colony to in 100 ml LB and grown overnight at 37 °C. Next day the pre-culture was diluted in 1:100 proportion in 2 L of LB with 50 µg/ml of kanamycin. At OD_{600} = 0.4 the expression of proteins was induced with 1mM IPTG. Then cells were incubated for 2h at 37 °C. The cells were disrupted with microfluidizer and centrifuged for 1h at 30,000 rpm in Type 60Ti rotor. The supernatant was collected. The soluble protein was purified *via* nickel-nitrilotriacetic-acid-agarose (Qiagen, according to the manufacturer's manual) and anion exchange chromatography (Source 15Q, GE Healthcare). The purified protein was dialyzed against 20 mM Hepes, 6 mM Mg-acetate, 150 mM K-acetate, 4 mM β-mercaptoethanol, pH 7.6 at 0 °C.

2.1 . Microbiological and genetic methods

2.1.1 Preparation of electro-competent cells

For preparation of electro-competent cells 500 ml LB was inoculated with the appropriate strain and grown with mild shaking at 37 °C till OD_{600} of 0.7. Cells were then harvested in sterile centrifuge bottles at ~6,000 rpm for 15 min. The cells were then resuspended in 100 ml of cold distilled and deionized water or MQ, and centrifuged again at 6,000 rpm for 15 min. The washing step was repeated twice. Cells were dissolved in 4 ml of cold 10 % (v/v) glycerol and aliquotized in volumes of 40 µl. Aliquots were shock frozen in liquid nitrogen and stored at -80 °C.

2.1.2 Transformation of a plasmid *via* electroporation

Cells for electroporation were thawed on ice for 10 minutes. A plasmid (0.1 to 1 ng) was added to the cells, mixed and left for 5 min on ice. The mixture was transferred to a cooled electroporation cuvette (0.2 cm gap size; Biorad #165-2092). The electroporation was carried out in an electroporation device (Biorad Gene Pulser / Pulse control System) with the following settings: voltage = 1.5 kV, resistance = 200 Ω and capacitance = 25 µF. The time constant should range between 3 to 4 ms. After applying the pulse LB medium without antibiotic was added and the cells were incubated at 37 °C in an incubator shaker for 1 hour. Approximately 200 µl was plated on LB agar plates with the selective antibiotics and plates were incubated O/N in an incubator.

2.1.3 Growth competition assay

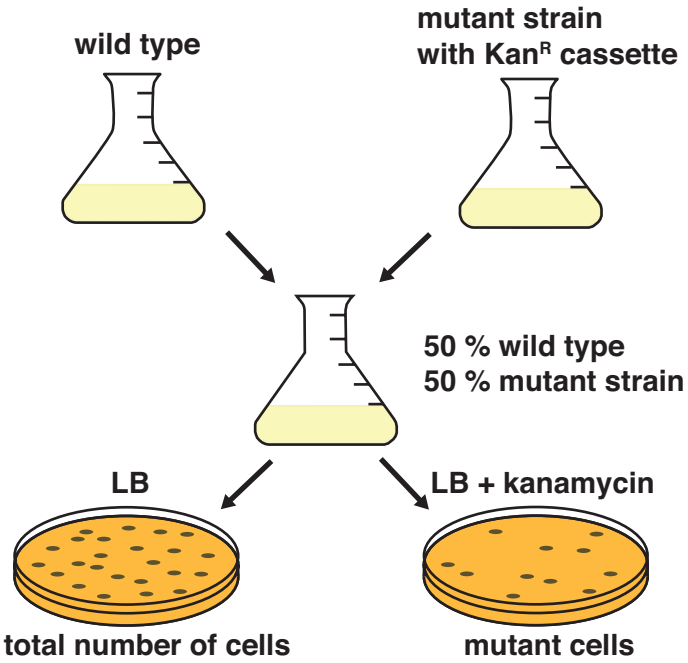

Figure 10. The principle of growth competition assay. Wild type and mutant strain are mixed in 1:1 ration and the fitness of mutant is estimated as number of colonies on LB kan$^+$ plate (mutant colonies only) versus LB plate (WT and mutant colonies).

For growth competition assays the same amount of cells from overnight cultures of wild type and mutant strain were mixed, yielding a final OD_{600} of 0.01 in a volume of 5 ml, and incubated under mild shaking either in LB or M9 medium with 0.4 % glucose. Aliquots were withdrawn every 3 h or 6 h or 24 h (depending

on the growth rate) and OD_{600} was measured. Simultaneously, dilutions to approximately 5,000 cells/ml (according to the assumption that 1 OD_{600} corresponds roughly to 10^9 cells) were made and 100 ml of each was plated in duplicates on either LB plates or LB plates containing 25 mg/ml kanamycin. The number of colonies (mutant strain contained a kanamycin (kan^R) cassette, WT not) was counted after incubation at 37°C for overnight. Generation time was calculated according to the formula (ln(OD_{600} before incubation) - ln(OD_{600} after incubation))/ln(2) and presented in a graph as a function of generation number. The table below contains incubation parameters for growth competition assay.

For viability competition experiment in stationary phase (LB medium) mutant and wild type strains were separately grown overnight. Subsequently two cultures were diluted to OD_{600}= 0.005 and incubated with shaking till 0.5 OD_{600}. Then two cultures were mixed and the fitness of mutant strain was monitored as numbers of colonies on LB plates (mutant and wild type colonies) and LB plates containing kanamycin (only mutant colonies) after 2, 6, 9, 21, 32, 52, 78 hours of incubation at 37 °C.

2.1.4 Growth curves during media transition

For the media shift wild type and mutant strains were grown overnight in LB medium (rich) and then diluted in either LB (rich) or M9 medium (poor) yielding an OD_{600} = 0.005. Cultures were incubated at 37 °C with shaking (200 rpm) and the growth was monitored measuring the OD_{600} over a time of up to 40 hours. For rescuing the phenotype of *ΔrsfS* strain during the transition from rich to poor medium *ΔrsfS* cells lacking the kanamycin resistance gene and wild type cells were transformed with a plasmid containing a gene coding for RsfS fused with a C-terminal His-tag under control of either the native promoter or the IPTG inducible tac-promoter and with the corresponding empty plasmid. The transformed strains were grown overnight in rich (LB) medium at 37 °C and then diluted in poor M9 medium yielding a start OD_{600} = 0.005 and incubated like described above. At several time points samples were withdrawn and the expression of RsfS was analyzed by Western blot using an antibody against the His-tag. The intensity of the RsfS-His bands was quantified using ImageQuant 5.2 and normalized for correction of the input to a non-altered protein band of the

Coomassie blue stained gel.

2.1.5 Single gene deletion and removal of kanamycin cassette

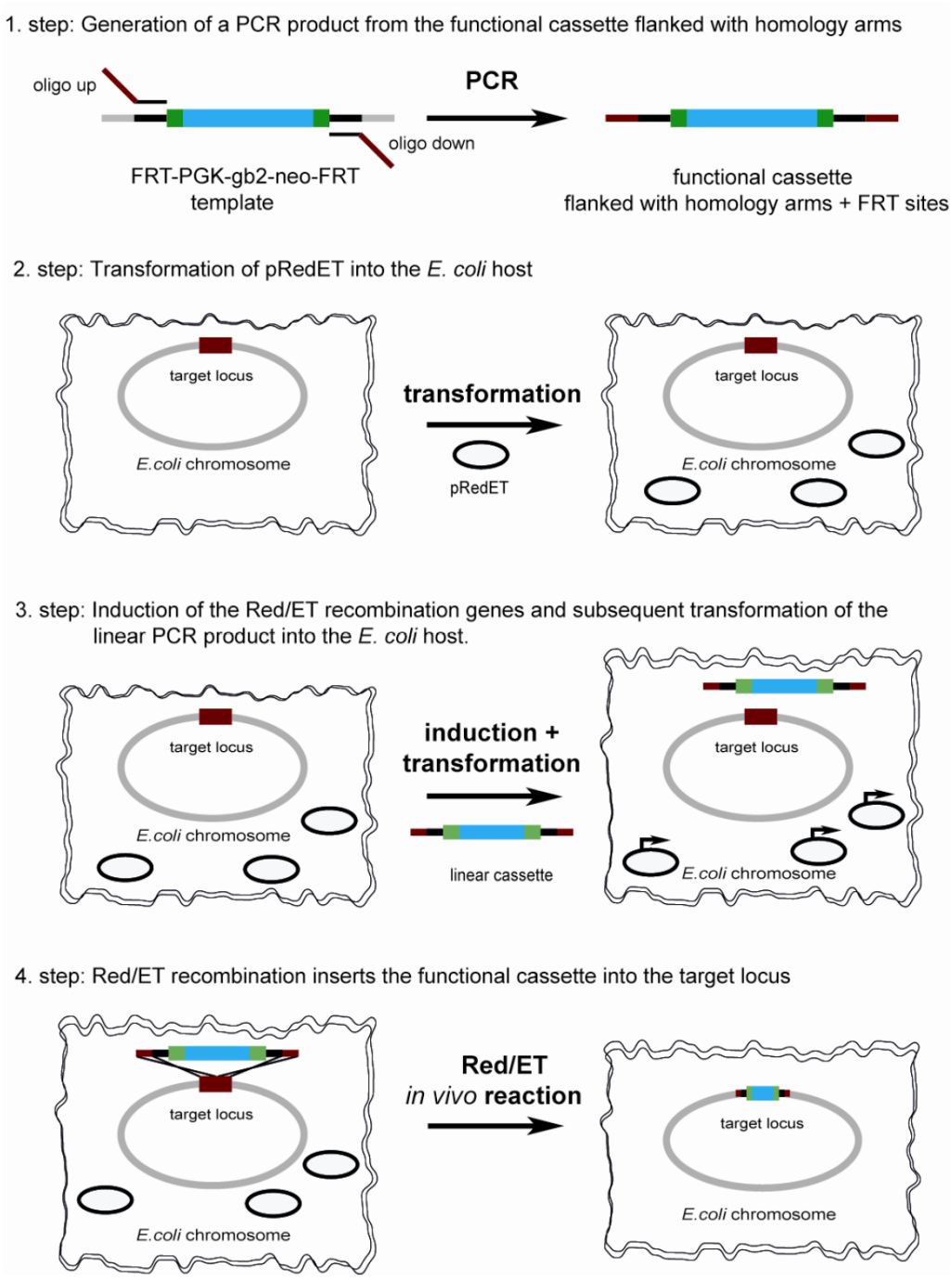

Figure 11. The principle of Red/ET recombination kit. According to the manual "Quick & Easy *E. coli* Gene Deletion Kit" (Gene Bridges).

We used Red/ET recombination kit to remove the kanamycin cassette of *ΔrsfS* strain. In Red/ET Recombination, also referred to as λ-mediated

recombination, target DNA molecules are precisely altered by homologous recombination in *E. coli*, which express the phage-derived protein pairs, either RecE/RecT from the Rac prophage, or Redα/Redβ from λ phage.

These protein pairs are functionally and operationally equivalent. RecE and Redα are 5‘- 3‘ exonucleases, and RecT and Redβ are DNA annealing proteins. A functional interaction between RecE and RecT, or between Redα and Redβ is also required in order to catalyze the homologous recombination reaction. Recombination occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine. The recombination is further assisted by l-encoded Gam protein, which inhibits the RecBCD exonuclease activity of *E. coli*.

The principle of RedET recombination is depicted in the Figure 11. In the first step 50bp long homology arms corresponding to the sequences flanking the insertion site on the chromosome are added to the functional cassette by PCR. In parallel, the *E. coli* strain, which will be modified, is transformed with the expression plasmid pRedET (step 2). The expression of genes mediating Red/ET is induced by the addition of L-arabinose. After induction, the cells are prepared for electroporation and the PCR product carrying the homology arms is electroporated (step 3). Red/ET recombination inserts the functional cassette into the target locus (step 4). Only colonies carrying the inserted modification (replacement of a gene by the FRT-PGKgb2- neo-FRT cassette) will survive kanamycin selection on the agar plates. Optionally, the kanamycin selection marker can be removed from the chromosome by transforming the cells with FLP expression plasmid. Expression of the site-specific FLP recombinase removes the selection marker from the target locus leaving a single FRT site as footprint behind.

2.1.6 Measurement of β-galactosidase expression *in vivo*

ΔrsfS, *Δhpf*, *Δpy* and *Δrmf* strains and wild type (BW25113) were transformed with a β-galactosidase reporter plasmid, pBAD24-lacZ-HA (based on pBAD24HA) (Guzman, Belin et al. 1995; Titz, Hauser et al. 2007), and selected on LB agar containing 50 µg/ml ampicillin. All strains were grown overnight in LB in the presence of 50 µg/ml ampicillin.

Logarithmic phase expression

After overnight incubation the cultures were diluted 1:100 and grew with mild shaking shaken at 37°C until OD_{600} = 0.4. Then β-galactosidase expression was induced with 0.2 % arabinose. Every hour 300 µl suspension was withdrawn, 100 µl of which was loaded into a well of a 96-well plate (flat bottom) and OD_{600} was measured using ELISA spectrophotometer. The rest of aliquots were centrifuged at 12,000 rpm for 5 min and pellets were resuspended in 20 µl loading buffer (2X) Tris-glycine SDS and incubated at 95 °C for 5 min to denature proteins. Samples were loaded on SDS-polyacrylamide gel (10 %) and the electrophoresis was carried out using 150 V for 45 minutes. The β-galactosidase expression was quantified as relative protein-band intensity using ImageJ software.

Stationary phase expression

After overnight incubation the cultures were diluted 1:100 and grew with mild shaking shaken at 37°C until stationary phase. Then β-galactosidase expression was induced with 0.2 % arabinose. Every hour 300 µl suspension was withdrawn, 100 µl of which was loaded into a well of a 96-well plate (flat bottom) and OD_{600} was measured using ELISA spectrophotometer. The rest of aliquots were centrifuged at 12,000 rpm for 5 min and pellets were resuspended in 20 µl loading buffer (2X) Tris-glycine SDS and incubated at 95 °C for 5 min to denature proteins. Samples were loaded on SDS-polyacrylamide gel (10 %) and the electrophoresis was carried out using 150 V for 45 minutes. The β-galactosidase expression was quantified as relative protein-band intensity using ImageJ software.

2.1 *In vitro* assays

2.1.1 Poly(U) dependent oligo(Phe) synthesis

2.1.1.1 The principle of poly(U) dependent oligo (Phe) synthesis

The elongation activity of the ribosome was checked using a modification of the poly (U)-dependent poly(Phe) synthesis system described by Traub and Nomura (Traub and Nomura 1969; Bartetzko and Nierhaus 1988), and/or the AcPhe-tRNA primed poly(Phe) synthesis described by Bartetzko and Nierhaus

(Traub and Nomura 1969; Bartetzko and Nierhaus 1988), modified according to Szaflarski and Nierhaus (Szaflarski, Vesper et al. 2008).

First, ribosomes were programmed with mRNA, which was carried out by incubation of ribosomal subunits with an excess of mRNA. In parallel, a ternary complex containing radioactively labeled precharged tRNA, elongation factors EF-Tu, EF-Ts and GTP as a source of energy has to be formed by incubation for few minutes. Afterwards, programmed ribosomes and tRNA complex are combined and supplemented with EF-G, an essential factor for translation elongation. Oligo(Phe) synthesis was carried out, usually at 37°C in Binding Buffer - $H_{20}M_{4.5}K_{150}SH_4$ $Spd_2Spm_{0.05}$. Oligo(Phe) synthesis was stopped by adding 1 ml cold TCA, supplemented with few drops 1 % Bovine Serum Albumine (BSA) followed with vigorous shaking for 1 min. BSA in solution acts as precipitation carried. Precipitated oligo(Phe)-tRNA wes then incubated at 90 °C for 15 min to hydrolyze $tRNA^{Phe}$ from oligo(Phe)-tRNA complex. Free oligo(Phe) was then left on ice for 5 min and filtrated through glass filters. These filters were washed 3x with 10 % TCA and twice with 5 ml of diethyl ether / ethanol (1:1) to remove the TCA and to dry the filters. The amount of oligo(Phe) was measured using scintillation counter and calculated as function of the amount of radioactivity retained on the filters and presented as a number of Phe per single ribosomes (ν).

2.1.1.2 Oligo(Phe) synthesis initiated with ribosomal subunits

2.1.1.2.1 Oligo(Phe) synthesis in the presence of bacterial RsfS

18 pmol 50S ribosomes were incubated with 180 µg poly(U) mRNA with or without 360 pmol RsfS in 90 µl for 10 min at 37 °C in Binding Buffer $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. Reaction was further incubated with 10 pmol of 30S ribosomes for 10 min at 37 °C and then analyzed in poly(U) dependent oligo(Phe) synthesis and sucrose gradient centrifugation (look chapter 2.2.6.). 15 µl of the reaction was used for oligo(Phe) synthesis. 15 µl of ternary complex containing 30 pmol [^{14}C]-Phe-$tRNA^{Phe}$, 45 pmol EF-Tu, 45 pmol EF-Ts, 3 mM GTP and was preincubated 5 min at 37 °C. Then 2.4 pmol of EF-G together with the ternary complex mix were added to programmed ribosomes yielding 30 µl in Binding Buffer $H_{20}M_{4.5}K_{150}SH_4$ $Spd_2Spm_{0.05}$. Oligo(Phe) synthesis was carried out at 30 °C for 2 min and 12.5 µl aliquots were precipitated with TCA, incubated at 90 °C in

the presence of 2 drops of 1 % (w/v) BSA. The samples were cooled to 0 °C, filtered through glass filters and the radioactivity adsorbed on the filters was measured in scintillation counter. 60 µl of the reaction was mixed with 40 ml $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ and loaded onto a 10 - 30 % sucrose gradient prepared in the same buffer. Centrifugation was carried out at 42,000 rpm for 4 h in an SW60 rotor. The gradient was pumped out from bottom to top and the A_{260} was measured to obtain the ribosome profile.

2.1.1.2.2 Oligo(Phe) synthesis in the presence of mitochondrial RsfS

The corresponding assay with mitochondrial components from pig liver was performed in $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. Mitochondrial RsfS (mtRsfS) was pre-incubated with 2.5 pmol large subunit 39S in 80 molar excess over ribosomes, before the same amount of 28S subunits were added. Likewise 2.5 pmol 55S ribosomes were incubated with the same amount of RsfS. Mitochondrial EF-G1 was added in a 0.8-fold excess over ribosomes. Then 37.5 pmol of [^{14}C]-Phe-tRNA^{Phe} was added followed by mitochondrial factors mtEF-Tu and mtEF-Ts, both in an excess of 1.5 over Phe-tRNA. The total volume was 100 µl. The main incubation was carried out 20 min at 30 °C and the amount of synthesized oligo(Phe) was measured by filtration through glass filters as described in chapter 2.1.1.2.1.

2.1.1.2.3 Oligo(Phe) synthesis in the presence of RsfS, RMF, HPF, and PY (cooperativity assay)

Preincubation step:

3 pmol of 50S ribosomes were incubated with 30 µg poly(U) with or without 60 pmol RsfS, HPF, PY or RMF for 10 min at 37 °C in Binding Buffer $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. The reaction was further supplied with 3 pmol 30S ribosomes, yielding 15 µl and incubated for 10 min at 37 °C.

Poly(U) dependent oligo(Phe) synthesis at 30° C:

The ternary complex mix containing 30 pmol [^{14}C]-Phe-tRNA^{Phe}, 45 pmol EF-Tu, 45 pmol EF-Ts, 3mM GTP and was preincubated 5 min at 37 °C. Then 2.4 pmol of EF-G was added yielding 15 µl and combined with 15 µl of preincubated ribosomes with factors to a total volume of 30 µl in Binding Buffer $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. This was finally incubated at 30° C for 10 min and

10 µl aliquots were precipitated with TCA, incubated at 90°C in the presence of 30 µl of 1 % (w/v) BSA and filtered using glass filters. Activity of [^{14}C]-Phe-tRNA^{Phe} was counted in scintillation counter.

Poly(U) dependent oligo(Phe) synthesis at 25 °C:

The reaction was carried out as described above except that ternary complex mixture contained 45 pmol [^{14}C]-Phe-tRNA^{Phe}, 67.5 pmol EF-Tu, 67.5 pmol EF-Ts, 3 mM GTP. The complete mixture was incubated at 25 °C for 5 min.

2.1.1.3 Oligo(Phe) synthesis with reassociated 70S

The oligo(Phe) synthesis with reassociated 70S ribosomes was performed in the following way: 3 pmol 70 S ribosomes were incubated with 30 µg poly(U) and 6 pmol Ac-Phe-tRNA for 10 min at 37 °C. 60 pmol RsfS was added to the programmed ribosomes. The reaction was carried out in Binding Buffer. the mixture in total volume of 20 µl was incubated for 5 min at 37 °C and oligo(Phe) synthesis was measured by filtration through glass filters as described in chapter 2.1.1.2.1.

2.1.2 Dissociation test of 70S programmed with tRNA by RsfS

40 pmol of 70S ribosomes were preincubated with 160 µg of poly(U) mRNA and 80 pmol [^{14}C]-Ac-Phe-tRNA^{Phe}. The preincubation was carried out in Binding Buffer for 15 min at 37 °C. Then the occupancy of [^{14}C]-Phe-tRNA^{Phe} was determined by nitrocellulose filtration as following. Briefly, an aliquot of the mother reaction was diluted in 2 ml of ice cold Binding Buffer $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ and filtered immediately through a nitrocellulose filter, which was in parallel equilibrated in the same buffer (Nirenberg and Leder 1964). The filter was then washed two times with 2 ml of Binding Buffer and 2ml of ether-ethanol. The amount of Ac-Phe-tRNA^{Phe} bound to the ribosomes was calculated as function of the amount of radioactivity retained on the filters and this value was normalized to pmol bound per pmol of ribosomes in the reaction (v). Then the mother reaction was split in two and the first half was incubated with 400 pmol of RsfS in the 200 µl of Binding Buffer. The second half was incubated with Binding Buffer only. The incubation was carried out for 20 min at 30 °C. Afterwards an aliquot of the

reaction was taken and the occupancy of tRNA was determined by nitrocellulose filtration as described above. In parallel, 125 µl of both reactions were loaded on 10-30 % sucrose gradient in Binding Buffer. Then the samples were centrifuged in SW40 rotor for 20 hours at 22,000 rpm. The ribosome profile was analyzed by spectrometer at 260 nm. The fractions corresponding to ribosomal subunits and 70S were precipitated by cold TCA and resuspended in sample buffer containing 200mM TRIS. The proteins were separated on 15 % SDS PAGE, transferred to PVDV membrane and immunoblotted against ribosomal protein L2 and mitochondrial RsfS (c7orf30). We note that we used anti mitochondrial RsfS antibody for detection of bacterial RsfS.

2.1.3 Rapid translation system (RTS)

The RTS 100 system is a coupled transcription translation system for DNA-templates or linearized DNA fragments containing a T7-promoter. **Table 4** shows the lyophilisates, supplied with the system, which need to be reconstituted and combined: *E. coli* lysate, energy components, amino acids, reaction buffer and control DNA.

Compound	Description	Reconstitution procedure
E. coli lysate	Contains all components of transcription and translation	Add 0.36 ml of reconstitution buffer, mix carefully, never vortex
Reaction mix	Substrate mix for reaction	Add 0.3 ml of reconstitution buffer, mix carefully, never vortex
Amino acid mix	Mix of 19 amino acids	Add 0.36 ml of reconstitution buffer, mix carefully, never vortex
Methionine	Separate methionine for radiolabelling of the product with ^{35}S-Methionine	Add 0.33 ml of reconstitution buffer, mix carefully, never vortex
Reconstituti	Buffer to reconstitute 1-4	1.6 ready solution

on buffer	from lyophilisate	
DNA template	(with the Kit control GFP vector is supplied)	Vector is solved in 50 µl MilliQ

Table 4. Composition of RTS kit according to the manual.

Rapid translation system has been performed according to the protocol with slight modifications. Standard volume of reaction batch containing reconstitution buffer, reaction mix, amino acids and DNA has been increased from 10 µl to 20 µl. Due to different buffer used for hibernation factors and RsfS, adaptation buffer adjusting reaction to binding buffer ($H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$) has been used. As a template DNA plasmid coding for a bicistronic renilla and firefly-luciferase mRNA was used. Reaction was stopped and quenched after 1h of incubation at 30 °C. Chemoluminescence was measured for 30 seconds.

2.1 *In silico* methods

2.1.1 Distribution of RsfS, RMF, HPF and PY within bacterial kingdom.

669 bacterial species, each representing a different genus, were analysed by BLASTP search for the presence of RsfS, HPF (short), HPF (long), PY and RMF using the sequences NP_415170.4, NP_417670.1, ZP_03100261.1, NP_417088.1 and NP_415473.1 as queries. Distribution higher than 90 % within each phylum was taken as representative. The tree of life was build with the iTOL software.

3 Results

3.1 Search for a phenotype of *ΔrsfS* strain

3.1.1 *ΔrsfS* shows adaptation problems during transition from rich to poor medium

RsfS is well conserved in bacteria and plastids-containing eukaryotes. However, deletion of RsfS is viable and results in no obvious growth disadvantage in *E. coli* under optimal growth condition (LB, 37 °C), (Baba, Ara et al. 2006). To find the function of RsfS we first sought to determine the condition under which RsfS plays an important role for cell viability. To this end we used an assay, which compares the wild type and the *ΔrsfS* strain under competitive condition. In growth competition we mix equal amounts of WT and mutant cells and monitor the fitness of mutant at constant time intervals. The fitness of mutant strain is estimated by plating out the mutant – wild type mixture on LB and kanamycin-containing LB agar plates. After overnight incubation we calculate the number of colonies on the plates and compare the number of colonies of LB + kanamycin plate *versus* the number of colonies on the LB sister-plate. The colony-ratio of the LB kanamycin$^+$ / LB plates corresponds to the fraction of mutant in the culture, because only mutant cells have an antibiotic resistance cassette in place of the studied gene. Growth competition is a more sensitive method then measurement of growth rates of WT and mutant strains in separate flasks. The possible growth defect in growth competition is enhanced, because we can compare the number of cells of wt and mutant strains directly in the mixture.

In the first growth competition we tested the importance of RsfS at 15 °C in rich medium (LB). First we inoculated WT and *ΔrsfS* strain in LB and incubated overnight at 37 °C with mild shaking. At the next day both cultures were diluted 100 times, mixed in a single flask in 1:1 proportion and grown at 15 °C with mild shaking. Every 6 hours we plated out the culture on agar and calculated the fraction of mutant. To continuously monitor the condition of the mutant in the mixture we diluted the culture before reaching stationary growth phase in fresh LB. **Figure 12** demonstrates that WT and mutant strain grow at similar rate *i.e.* RsfS seems to play no role in cell viability at low temperature. This suggests that RsfS is

unlikely a cold shock protein.

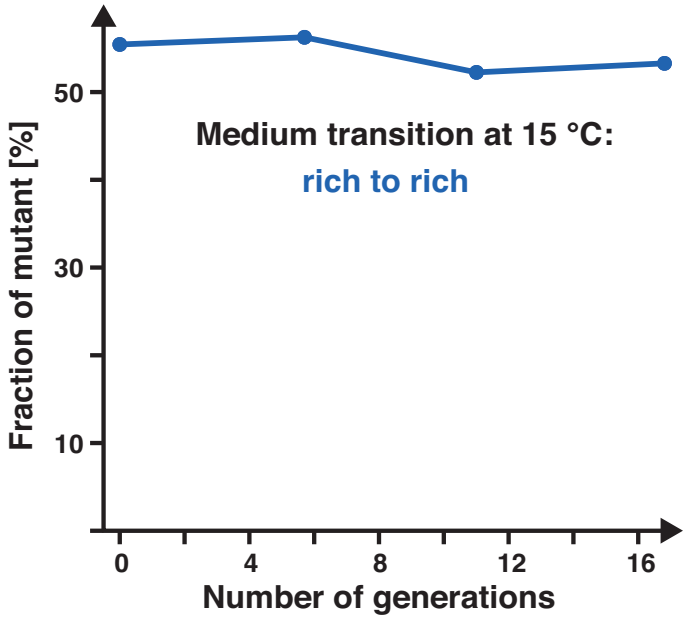

Figure 12. Deletion of RsfS shows no growth disadvantage at low temperature in LB medium. WT and *ΔrsfS* strains were mixed in 1:1 ratio and cultivated in LB at 15 °C. Every 6 generations the culture was diluted in fresh medium for continuous growth and the mixture was by plated out on LB and LB + kanamycin plate. The fraction of mutant was estimated based on number of colonies in LB + kanamycin dived by number of colonies on LB plate.

The competition of wild type and mutant at low temperature demonstrated no importance of RsfS for cell viability. However, we consider this experiment as an important control, because i) our experimental approach works, and ii) a substitution RsfS gene with kanamycin cassette has no effect on growth rate. After excluding the importance of RsfS under low temperature stress, we studied the fitness of *ΔrsfS* under nutrition starvation. We again used growth completion assay, only this time we diluted the mixture of wild type and mutant in different media at 37 °C. First, we studied conditions of mutant after the transition from LB to LB medium, which represents a rich medium. In contrast to the growth competition at low temperature, *ΔrsfS* mutant was losing the competition with wild type, showing only a modest but continuous detrimental effect of RsfS deletion. The fraction of mutant strain decreased to 10 % within 35 generations (**Figure 13, blue line**). We note that such a decrease corresponds to only about 4 % of growth-rate difference between wild type and mutant. Therefore, we consider this effect as modest but clearly indicating the resolution power of growth competition.

Next, we analyzed the RsfS deletion after transition from rich (LB) to minimal medium (M9), which represents a poor medium. Astonishingly, transition from rich to poor medium caused a severe growth defect of *ΔrsfS* strain. The wild type overgrew *ΔrsfS* strain within only five generations (**Figure 12**, red line). Contrary to rich to poor transition, the opposite transfer (poor to rich medium) was better tolerated by the mutant strain. Interestingly, the supplement of minimal medium with aminoacids completely rescued this striking growth defect of the RsfS mutant in the rich to poor transition (**Figure 12**, orange line). Taken together RsfS is important when we transfer *E. coli* cells from a nutrition-rich medium to medium containing only basic ions and glucose.

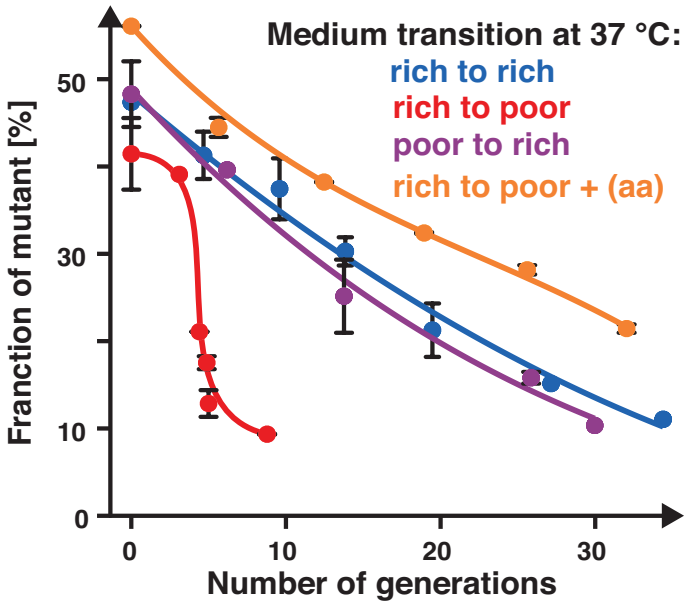

Figure 13. Under competitive condition *ΔrsfS* loses the viability after transition from rich to poor medium. We mixed wild type and mutant cells and monitored the fitness of mutant at 37 °C after shifting from LB medium to i) LB – blue line, ii) M9 – red line , iii) M9 supplemented with aminoacids – orange. Transition from M9 to LB is indicated with magenta. At given time points the mixtures were plated out on LB and LB + kanamycin plate and the fraction of mutant was estimated based on the number of colonies in LB + kanamycin dived by the number of colonies on LB plate.

The strong viability defects observed during transition from rich to poor medium should be visible also in a direct determination of the doubling times of wild type versus mutant in separate cultures. To this end, we inoculated WT and *ΔrsfS* strain in separate flasks, grew them overnight and diluted in the media used for growth competition. First, we monitored the growth after transition in rich

medium. In rich medium the generation time of wild type and mutant strain was almost indistinguishable (30 and 32 min, respectively; **Figure 14,** orange and magenta). Next, we checked the mutant strain after transition from rich to poor medium. We expected decreased doubling time of mutant strain comparing to the WT strain. However, to our surprise, a change from rich to poor medium revealed a substantial difference: initially the *ΔrsfS* mutant strain showed a WT-like growth for about 7 h, but then growth was stopped for about 14 h before it resumed almost with the same doubling time as the WT strain (130 versus 120 min). The growth block for many hours suggests that the lack of the RsfS gene poses a serious adaptation problem for the cells after a transition from rich to poor medium.

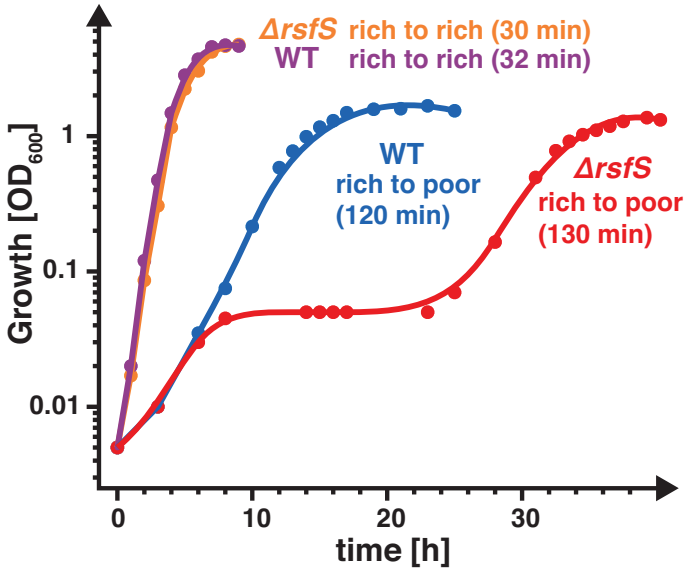

Figure 14. *ΔrsfS* strain shows growth adaptation problem after transition from rich to poor medium. Wild type and mutant were cultivated O/N in separate flasks containing LB at 37 °C with mild shaking. Then the strains were diluted in LB (wild type - orange, *ΔrsfS* - magenta) or M9 media (wild type - blue, *ΔrsfS* - red). In brackets shown the doubling time of each strain.

3.1.2 Deletion of RsfS decreases the cell viability during stationary growth phase.

We demonstrated that deletion of RsfS after transition from rich to poor reduces the ability of the mutant to adapt to the new environment. During transition from rich to poor medium cells have to re-arrange the metabolism in a fine-tuned manner. Similar situation occurs when cells enter stationary growth phase. To this end we tested, whether RsfS is important in stationary growth phase.

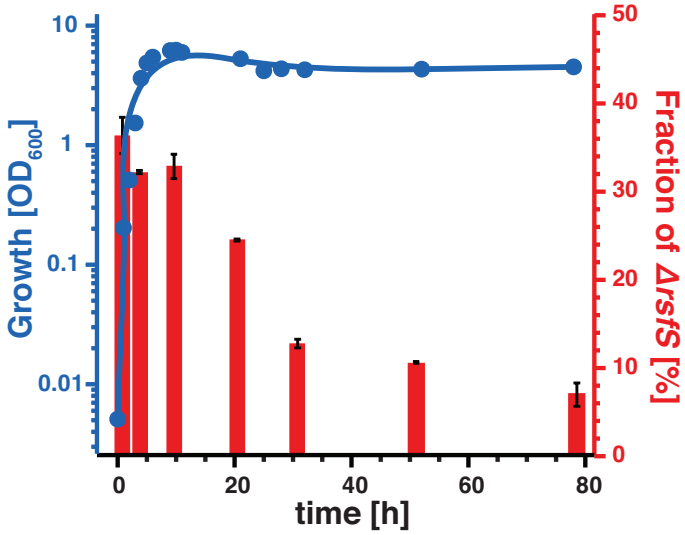

Figure 15. *ΔrsfS* strain loses the viability as compared with wild-type cells during stationary growth phase. Both strains were first grown separately and mixed 1:1 after 2 h of incubation; then we monitored the fitness of mutant by striking out on both LB plates and LB + kanamycin plates. The fraction of *ΔrsfS* survivors is indicated by red bars; in blue the growth curve of (WT and *ΔrsfS*) cells.

Similarly to transition rich to poor experiment we mixed WT and *ΔrsfS* cells during logarithmic growth phase (OD_{600} = 0.5) and at this point we started monitoring the viability of mutant strain for the next 40 hours without refreshing the medium. Before the mixed WT and mutant reached stationary phase (after around 7h) the fraction of mutant cells remained constant at about 35 %, but thereafter the viability of mutant cells sharply declined to less than 10 % indicating that the mutant cells have serious problems to survive stationary growth phase (**Figure 15)**. We conclude that RsfS plays an important role for cell viability during stationary growth phase.

3.1.3 *ΔrsfS* cells show increased translation activity during stationary growth phase

RsfS binds to ribosomal protein L14 and this interaction is universally conserved (Häuser, Pech et al. 2012). Given that the deletion of RsfS has causes a viability defect during stationary phase we assumed that the observed phenotype might be reflected in an aberration of protein synthesis. To determine the effect of RsfS deletion on protein synthesis we transformed wild type and *ΔrsfS* strains with a plasmid containing the β-galactosidase gene. We chose this

gene because it can be positively regulated with L-arabinose and blocked with glucose. Furthermore, β-galactosidase is a large protein therefore can be easily separated from cellular proteins in the cell lysate using gel electrophoresis. First, we grew both strains at 37 °C in LB medium containing ampicillin to prevent plasmid loss. For analysis of logarithmic growth phase after overnight incubation we diluted the wild type and mutant culture and grew them in LB medium at 37 °C until early logarithmic growth phase (OD_{600} = 0.4). Subsequently, we induced the expression of β-galactosidase by adding 0.2 % of L-arabinose to the culture. Then every hour we measured OD_{600} and took an aliquot for measuring the amount of β-galactosidase. For stationary-phase analysis we induced the β-galactosidase expression after overnight incubation and collected the samples every hour. After we measured OD_{600} we pelleted down the cells, lysed them and separated the proteins using SDS PAGE. After electrophoresis we stained the gel and calculated the β-galactosidase expression by measuring the intensity of β-galactosidase band on SDS PAGE. We normalized the intensity of the β-galactosidase band to a reference band and purified β-galactosidase loaded on SDS PAGE.

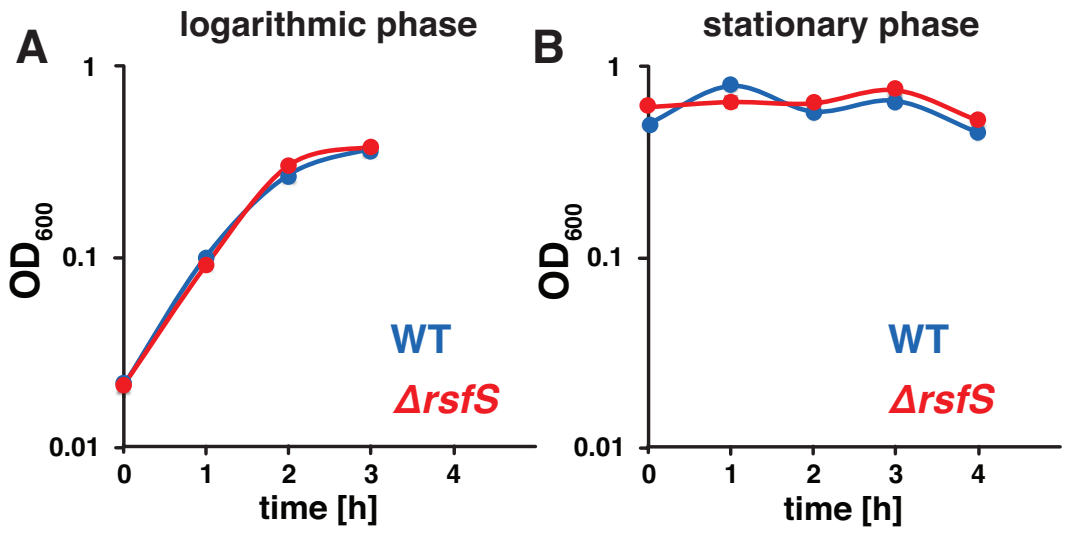

Figure 16. Growth curves of *ΔrsfS* and WT strains during expression of β-galactosidase in logarithmic and stationary phase. Expression of reporter gene was induced at 0 h. **A**) Logarithmic growth phase. **B**) Stationary growth phase.

When we expressed β-galactosidase during logarithmic growth phase we found similar levels of β-galactosidase (**Figure 17**) for wild type and mutant strains. In addition, the wild type and mutant cells grew with similar rates (**Figure**

16A). Subsequently, we analyzed the β-galactosidase expression in the early stationary growth phase. The cell density of wild type and mutant was at comparable levels (**Figure 16B**). However, the β-galactosidase expression was strongly repressed in wild type cells comparing to logarithmic phase (**Figure 17**). In contrast, the *ΔrsfS* strain accumulated 2 times more β-galactosidase than wild type in the stationary phase. These results demonstrate that RsfS acts as a negative modulator of protein translation *in vivo* in the stationary growth phase.

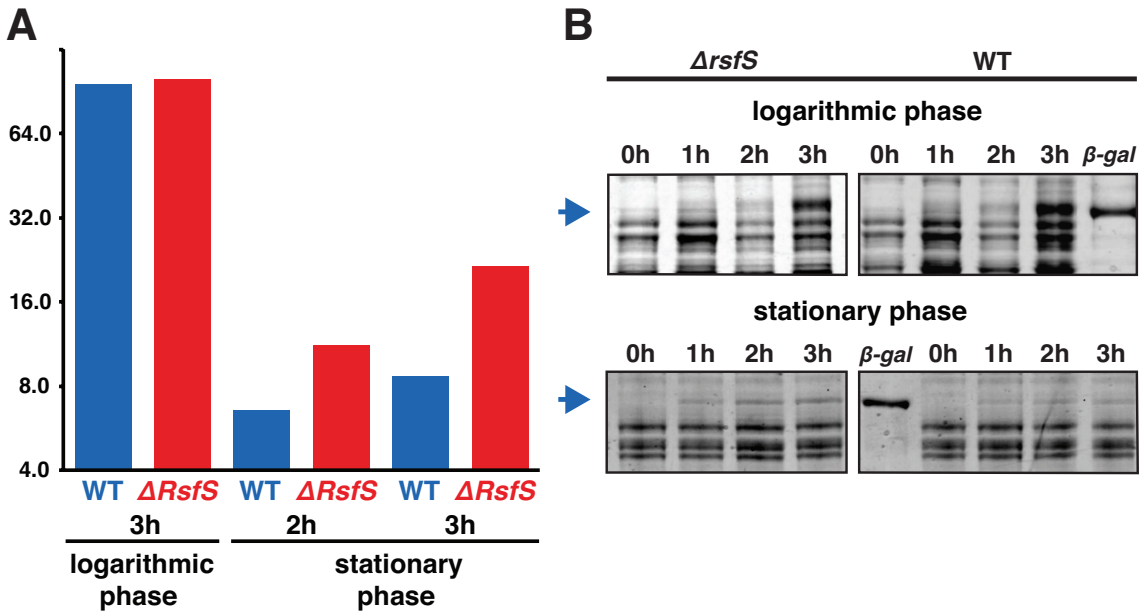

Figure 17. *ΔrsfS* cells have higher translation activity in stationary phase than wild-type cells. We monitored the protein synthesis using a plasmid containing inducible β-galactosidase reporter gene. We measured the expression of β-galactosidase (arrow) during logarithmic and stationary phase. *ΔrsfS* mutant expressed more reporter protein than WT during stationary phase. Under logarithmic growth phase there is no difference in β-galactosidase expression. **A**) Expression of β-galactosidase calculated as a band intensity on SDS Page **B**) SDS PAGE electrophoresis of cell lysates. Numbers indicate hours after the induction of β-galactosidase expression.

3.1.4 Are the demonstrated effects of RsfS deletion directly related to the lack of RsfS?

Bacterial cytokinesis is driven by the septal ring apparatus, assembly of which in *E. coli* is directed to the mid-cell region by the Min-gene system. Cells lacking the *minCDE* operon suffer from non-productive polar divisions, but have a nearly normal growth rate (Akerlund, Bernander et al. 1992; Donachie and Begg 1996). However, the cell growth is severely affected, when *ΔminCDE* is combined

with a mrdB mutation resulting in the loss of rod-like cell shape (Corbin, Yu et al. 2002). The *mrbB* operon starts with the RsfS gene and substitution of RsfS gene with a transposon cassette led to aberrant shape of *E. coli* cells (Bernhardt and de Boer 2004). Interestingly, a complementation of *ΔrsfS* with the downstream gene *mrdB* rescued the phenotype meaning that deletion of the first gene in the operon leads to a polarity effect causing defects in expression of downstream genes (Bernhardt and de Boer 2004). Therefore, it is essential for our conclusions to test, whether or not the phenotypes we demonstrated here are a result of reduced expression of downstream genes. To this end, we designed an experiment, where we rescue the observed phenotypes due to the deletion of RsfS gene by supplying an external RsfS gene.

3.1.4.1 Cell morphology of *ΔrsfS* strain.

To test whether the RsfS deletion causes a polarity effect, we first compared cell morphology of wild type and *ΔrsfS* cells. For this purpose we used negative-stained cells harvested from logarithmic phase in LB medium. We studied the specimens using an electron microscope. **Figure 18** shows dividing cells of wild type and mutant strain. Both wild type and mutant cells had similar shape.

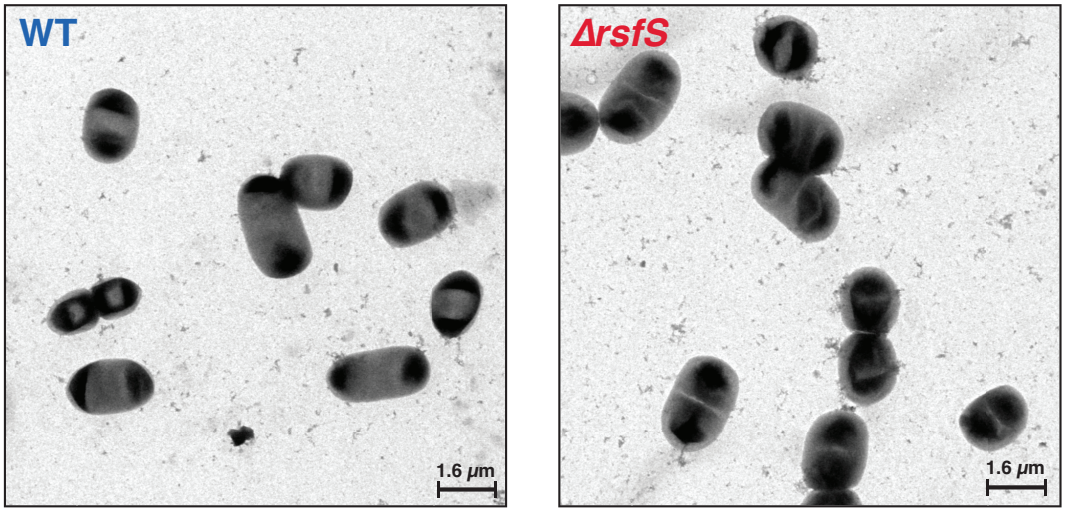

Figure 18. *ΔrsfS* cells show no aberrant cell shape as described in Bernhart et al. publication. Wild type and mutant cells were grown in LB medium, pelleted down and prepared for Electron Microscope analysis using negative staining.

3.1.4.2 Rescue assay

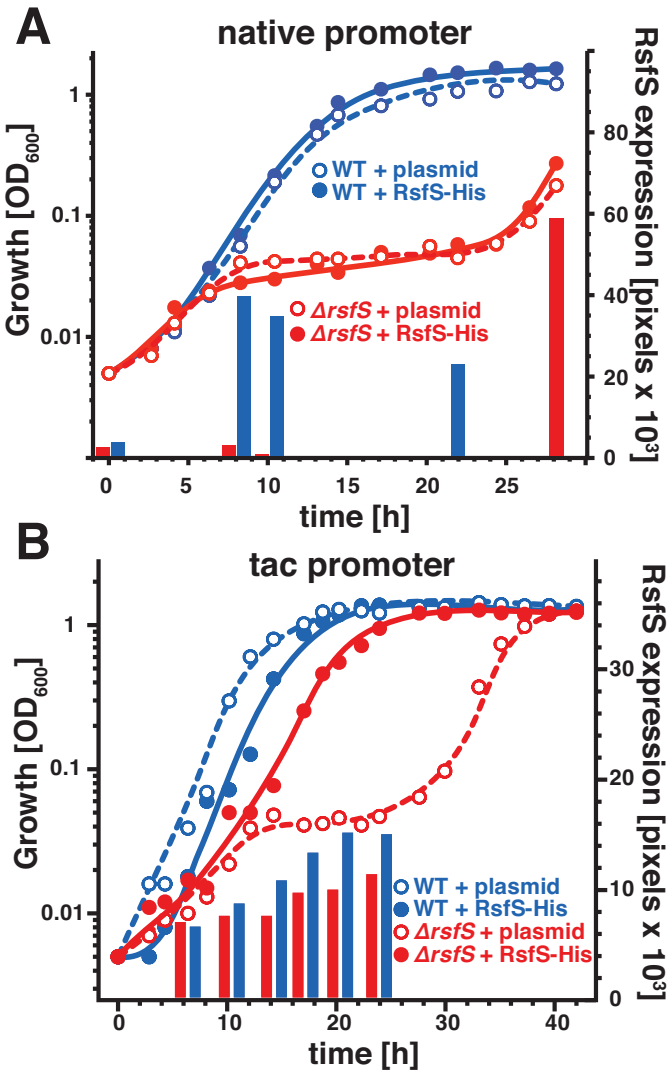

Figure 19. Forced expression of RsfS-His in *ΔrsfS* strain rescued the phenotype after transition from rich to poor medium. We monitored the growth rate and expression of RsfS-His in wild type and mutant cells. **A**) Wild type and mutant strain were both transformed with a plasmid containing *rsfS*-His under native promoter and the same plasmid but without *rsfS*-His gene. **B**) We transformed both strains with a plasmid expressing RsfS-His under the tac promoter and the same plasmid without *rsfS*-His gene. Red line, *ΔrsfS*; blue line, WT; solid line, plasmid with RsfS-His; dashed line, empty plasmid; bars – relative expression of RsfS-His measured by immunoblotting against His Tag.

To heal the effect of RsfS deletion we designed two rescue assays. In these two experiments we sought to complement the mutant phenotype observed in Figures 13 and 14 by introducing a plasmid carrying the RsfS gene. First, we removed the kanamycin cassette (using pCP20 plasmid), which was in place of the chromosomal RsfS gene and introduced a plasmid with the RsfS gene under the native promoter. The expressed RsfS carried a His tag at the C-terminus to

monitor the expression by immunoblotting against anti-His. **Figure 19A** demonstrates that the mutant phenotype could not be cured. The Western blot analysis of RsfS expression revealed that after the shift to the poor medium RsfS-His expression was insufficient, whereas taking up growth after 30 h was accompanied by a strong RsfS expression (see red bars in Figure 8A). Therefore, to overcome this problem we performed the same experiment but now with the RsfS gene under a tac promoter in order to force the expression of RsfS. The induced RsfS expression could indeed heal the mutant phenotype (**Figure 19B**; red closed circles).

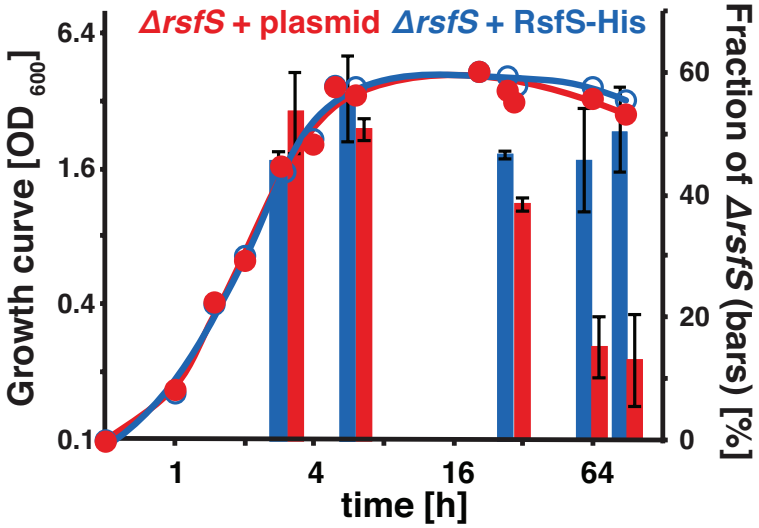

Figure 20. Expression of RsfS-His in *ΔrsfS* strain rescued the viability defect during stationary growth phase. Mutant and WT cells were first grown separately and mixed in 1:1 proportions after the culture reached stationary phase. We monitored the fitness of mutant by plating out the culture on both LB and LB + kanamycin plates and calculating the number of colonies on these plates. (indicated by bars). Red, *ΔrsfS* + empty plasmid; blue, *ΔrsfS* + plasmid with RsfS-His

To rescue to the phenotype of *ΔrsfS* in the stationary phase we used a plasmid containing RsfS-His under tac promoter. Prior to the experiment we inoculated following strains in LB: i) WT strain, ii) *ΔrsfS* strain containing a kanamycin cassette transformed with an empty plasmid but with ampicillin resistance cassette and iii) *ΔrsfS* strain resistant to kanamacin transformed with a plasmid (Amp^+) containing the RsfS-His under a tac promoter. *ΔrsfS* strains were grown with kanamycin and ampicillin to keep the plasmid in cells. After overnight incubation three strains were diluted in LB without antibiotics to an OD_{600} = 0.005.

At the OD_{600}= 0.4 we induced the expression of RsfS-His and grew the cultures till OD_{600} = 1.6. Then the WT culture was mixed in 1:1 proportion with i) *ΔrsfS* with the empty plasmid or ii) ΔrsfS with plasmid containing the RsfS-His gene. From this point we monitored the viability of *ΔrsfS* strain by plating out the mixtures on both LB and LB + kanamycin agar plates. **Figure 20** shows the growth rate (red and blue line) of two cultures and the fitness of mutant strain with and without complementation of RsfS-His (red and blue bars). Expression of RsfS-His in the *ΔrsfS* strain rescued the viability defect. We conclude that the observed phenotypes of *ΔrsfS* strain are due to the lack of RsfS gene rather than to a polarity effect on the downstream genes.

3.1.5 Lack of RsfS has no effect on the ribosome profile of *E. coli* cell lysates

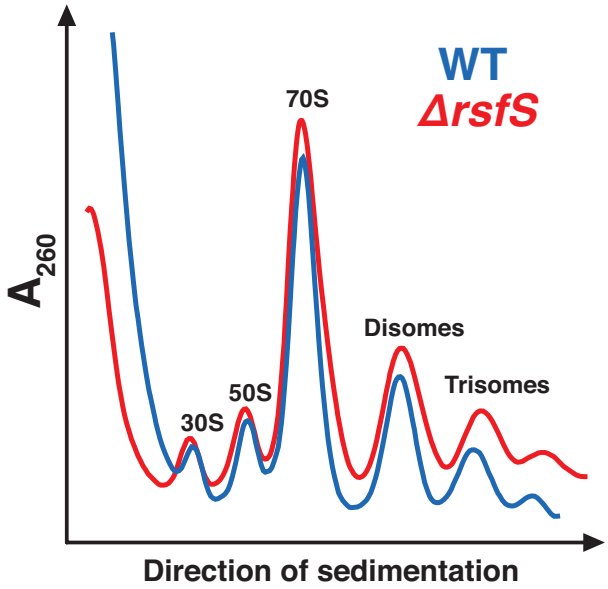

Figure 21. Ribosomal profile of WT and *ΔrsfS* strains. Cells were harvested during logarithmic phase. Red, WT; blue, *ΔrsfS*.

The lack of RsfS in *E. coli* poses a serious problem during stationary phase and after transition from rich to poor medium. During stationary phase the *ΔrsfS* strain shows an increased translation activity. Since RsfS seems to have an effect on protein synthesis we wondered whether the lack of RsfS would affect the ribosome profile *in vivo, i.e.* the proportions between 70S ribosomes, and 50S and 30S subunits. Furthermore we sought to determine, whether RsfS is involved in the assembly of ribosome.

To this end we analyzed cell lysates from logarithmic and stationary phase, using sucrose gradient centrifugation. This technique separates ribosomes and ribosomal subunits based on their different sedimentation coefficient. First we checked the ribosome profile during logarithmic growth phase. **Figure 21** shows the ribosomal profiles of wild type and *ΔrsfS* cells. We observed subunits, 70S and polysomes, (disomes and trisomes), *i.e* two and three ribosomes respectively, occupy one mRNA. Such a pattern is an indication of ribosomes in the elongation stage of translation. The ribosome pattern of wild type and *ΔrsfS* strains looked very similar, suggesting that RsfS does not affect protein synthesis during stationary phase.

Next we compared the profiles of wild type *ΔrsfS* cells during stationary growth phase, where we found a phenotype for RsfS deletion. Briefly, we prepared cell lysates as described for logarithmic phase, except that we harvested wild type and mutant cells after overnight incubation. Ribosomal profile of cells harvested during stationary growth phase differs from logarithmic growth phase. **Figure 22A** shows a ribosome profile of cell lysates derived from stationary phase. We observed a fraction of ribosome corresponding to 100S particles, a common feature of ribosome profile during stationary growth phase. 100S is a dimer of 70S, connected *via* heads of 30S subunits, which has no translation activity. Formation of 100S is governed by three factors: RMF, HPF and PY. RMF initializes the dimerization and HPF subsequently stabilizes it. The PY on the other hand turns 100S particle into inactive 70S ribosomes. Given that 100S particles are inactive in translation, they have been proposed to play a key role in down-regulation of protein synthesis during stationary phase (Wada, Yamazaki et al. 1990). Figure **22A** shows the representative experiment of wild type and *ΔrsfS* cells grown in M9 medium. We found a distinct 100S peak, which migrated in sucrose gradient next to 70S. The profiles of wild type and *ΔrsfS* cells looked similar when cells were cultivated in LB, M9, M9 supplemented with casamino acids or LB. In striking contrast, as shown in Figure **22B**, cultivation of *ΔrsfS* in medium E (Vogel and Bonner 1956) supplemented with trypton reduced the 100S peak. Interestingly, medium E supplemented with trypton is almost identical to M9 supplemented with casamino acids. The latter contains only free amino acids, whereas trypton digestion contains peptides.

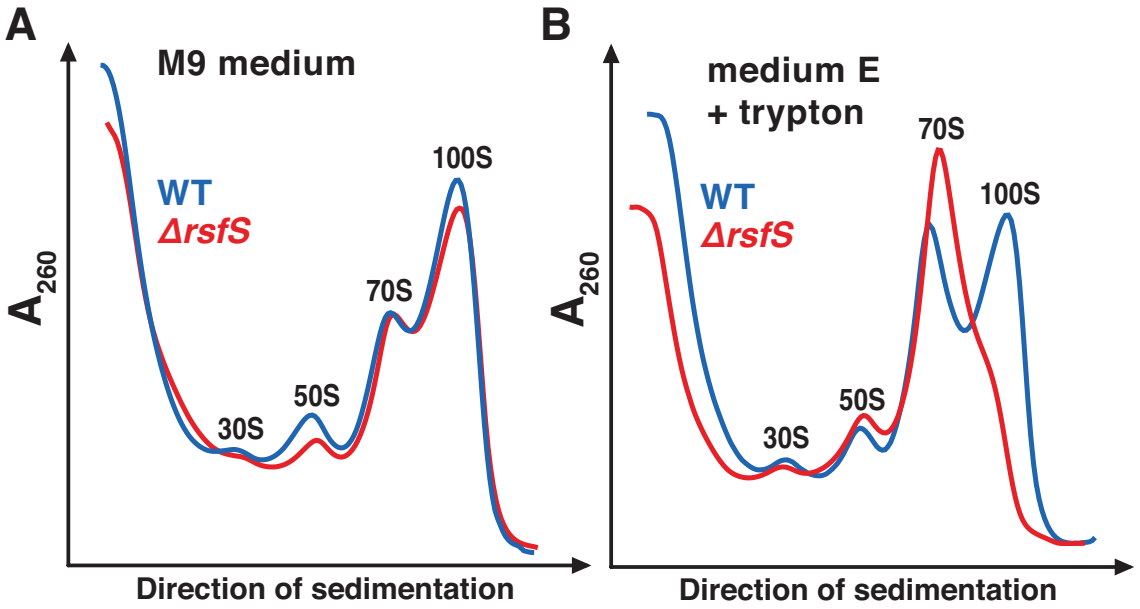

Figure 22. In the absence of RsfS during stationary phase 100S peak is diminished when medium E supplemented with trypton is used for cultivation. We harvested the cells in early stationary growth phase, disrupted in mortar using Alcoa and loaded the lysates on a sucrose gradient. **A**) Cells grown in M9 medium; **B**) Cells grown in medium E supplemented with 2 % trypton (Vogel and Bonner 1956)

Medium E contains citric acid monohydrate as a source of sugar, whereas M9 contains glucose. Furthermore, medium E lacks ammonium and calcium ions. We conclude that in RsfS might regulate the formation of 100S particles under specific conditions as medium E. However, the profiles of 70S, 50S and 30S during stationary phase showed no difference between *ΔrsfS* and wild type cells (**Figure 22A**). This suggests that RsfS is not involved in assembly of ribosome.

Ribosomes harvested in stationary phase are less active than those collected in logarithmic phase. Since 100S particles have no translation activity, it has been proposed to be an obligatory feature of stationary-phase ribosomes. However, there are reports of 100S particles formed already in the logarithmic phase. Furthermore, 100S particles are turned into 70S ribosomes after 3 days of incubation in stationary phase. By chance we observed that the formation of 100 particles is not an essential feature of stationary growth phase. When cells carrying a plasmid with inducible β-galactosidase, 100S formation was abolished (**Figure 23**). We note that before we collected cells for sucrose gradient centrifugation we kept the plasmid uninduced. This suggests that in *E. coli* 100S formation is not an obligatory feature in the early stationary phase.

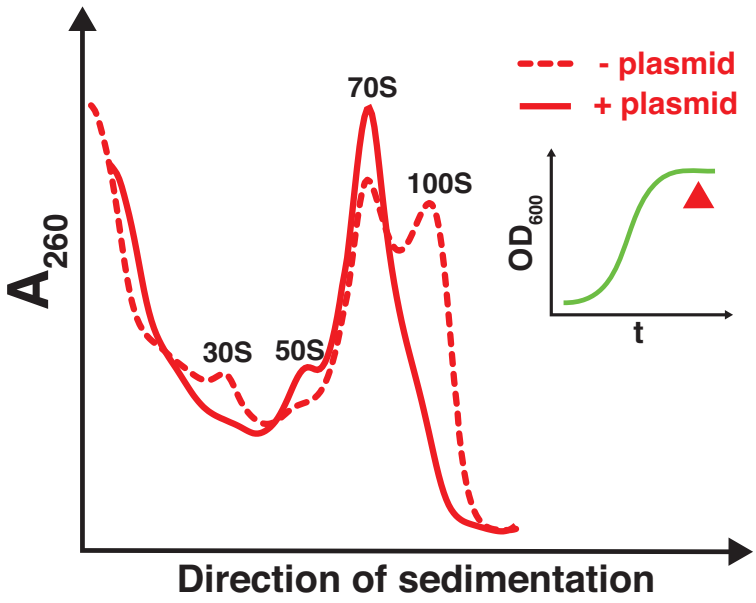

Figure 23. 100S is not an obligatory feature of stationary-phase ribosomes. Cells with and without plasmid were harvested after overnight incubation. We used Alcoa grinding to prepare the lysate. The insert shows the harvesting point.

3.2 Effects of RsfS on translation *in vitro*

The increased translation activity of *ΔrsfS* strain during the stationary growth phase suggests that RsfS is negatively regulating the protein synthesis. To test this hypothesis we used two different *in vitro* translation systems. The first one, called Rapid Translation System (RTS), is a coupled transcription translation assay based on the bacterial cell lysate supplemented with factors and energy necessary for transcription and translation. We used the Renilla luciferase gene on a plasmid, as reporter for translation activity. We added ten molar excess of RsfS over ribosome during the translation reaction for 30 min at 30 °C. **Figure 24A** shows that RsfS blocked about 90 % of the Renilla synthesis under these conditions. Since RsfS binds to the 50S subunits, we wondered, whether the 70S integrity is affected by the presence of RsfS. To this end, we checked sister aliquots from translation assay using sucrose gradient centrifugations. As showed in **Figure 24B** RsfS completely dissociated the ribosomes into subunits.

As a second translation system we used a resolved poly(U) dependent oligo(Phe) synthesis. In comparison to RTS, oligo(Phe) system contains only ribosomes, poly(U) mRNA, precharged [^{14}C]Phe-tRNA^{Phe}, translation elongation

factors and GTP as the source of energy. We initiated translation in this system by programming of 70S with homopolymeric mRNA coding for phenylalanine. Then we added radioactively labeled Phe-tRNA as ternary complex together with elongation factors and GTP as a source of energy. After incubation the translation mixtures were filtered through nitrocellulose filters. Then we measured and calculated the number of Phe incorporated per ribosome. We first tested the effect of RsfS on reassociated 70S in oligo(Phe) synthesis We incubated 70S programmed with mRNA and tRNA with RsfS prior to the main incubation. The result is shown in **Figure 25**. To our astonishment RsfS blocks Phe synthesis only by 20 % in contrast to 90 % inhibition of the RTS system.

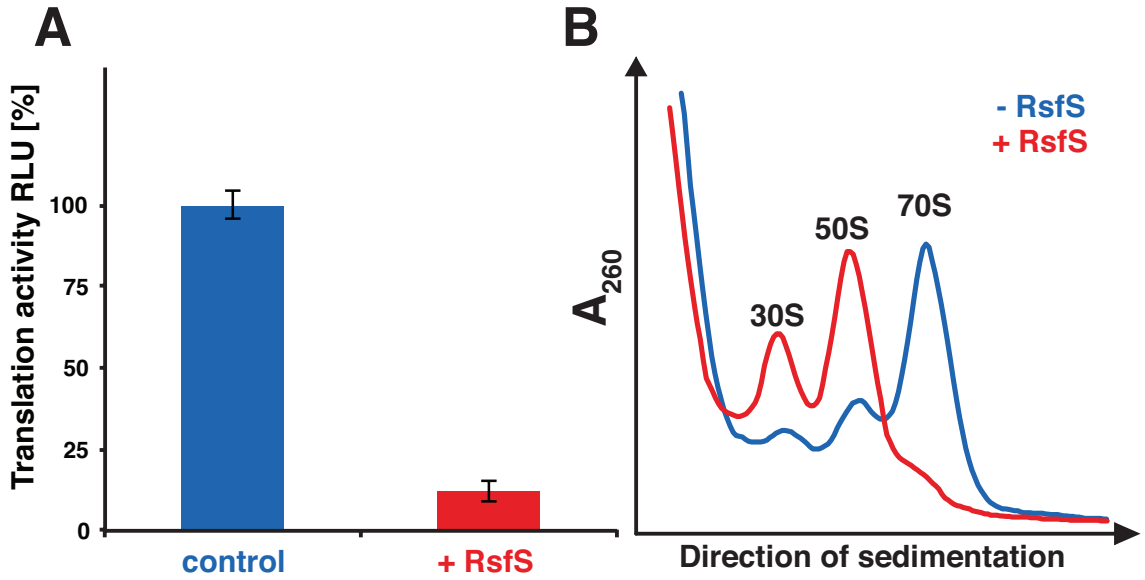

Figure 24. RsfS blocks in vitro translation and dissociates 70S ribosome into subunits. We tested the effect of RsfS in Rapid Translation System (RTS). **A**) We used Renilla luciferase as a reporter of translation activity in the presence RsfS (10X excess over ribosome) **B**) A sister aliquot of the reaction was loaded on a sucrose gradient and centrifuged. RsfS dissociated the 70S ribosomes into subunits.

To explain this discrepancy we preincubated RsfS with 50S subunit and subsequently added 30S to the system. Then we added mRNA, tRNA and factors and initiated translation. Interestingly, in this case RsfS inhibited translation down to 20 % similarly to the RTS-system test (**Figure 26A**). The sucrose gradient analysis of an aliquot derived from oligo(Phe) synthesis demonstrated that RsfS blocks translation by preventing association of ribosomal subunits to 70S (**Figure 26B**).

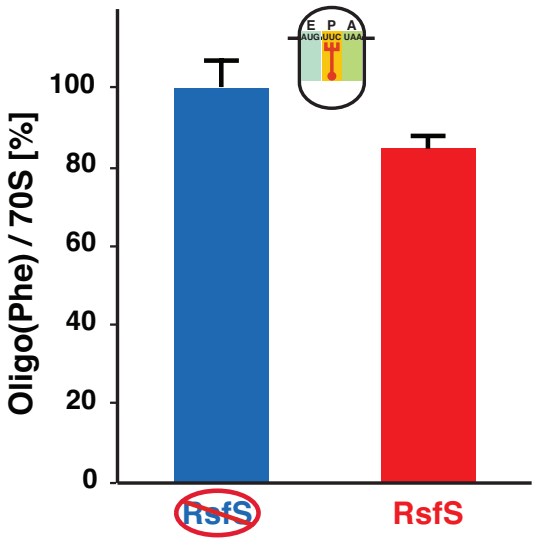

Figure 25. RsfS blocks oligo(Phe) synthesis only by 20 % when incubated with reassociated 70S loaded with mRNA and Phe-tRNA. RsfS was added to 70S ribosomes after they were charged with tRNA and mRNA. We used 20 times molar excess of RsfS over 70S. Oligo(Phe) synthesis was for 10 min at 37 °C.

RsfS is found in bacteria, mitochondria and chloroplasts of eukaryotes. Binding of RsfS to protein L14 is universally conserved. RsfS blocks the association of 70S in bacteria. We wondered if similar mechanism is valid also for mitochondrial ribosome. To this end, we checked the effect of mitochondrial RsfS on mitochondrial ribosomes using oligo(Phe) synthesis assay. We preincubated mitochondrial large 39S subunits with RsfS and subsequently added small 28S subunits together with the ternary complex Phe-tRNA•mtEF-Tu•GTP. **Figure 27A** demonstrates that RsfS blocked oligo(Phe) synthesis down to 20 %, similarly to experiments with bacterial system (**Figure 26A**). When we tested the effect of mitochondrial RsfS on reassociated 55S (mitochondrial ribosome), RsfS inhibited oligo(Phe) by 50 %, which was stronger than in case of its bacterial homolog (**Figure 27B**).

Taken together RsfS shifts the equilibrium of 70S towards dissociation in the lysate based translation system (RTS), and thus inhibits the translation. On the other hand RsfS blocks 70S association rather than promoting dissociation in a more resolved system containing only essential translation factors, poly(U) and precharged Phe-tRNA. However, both the analysis using both systems demonstrated that RsfS is a potent translation inhibitor.

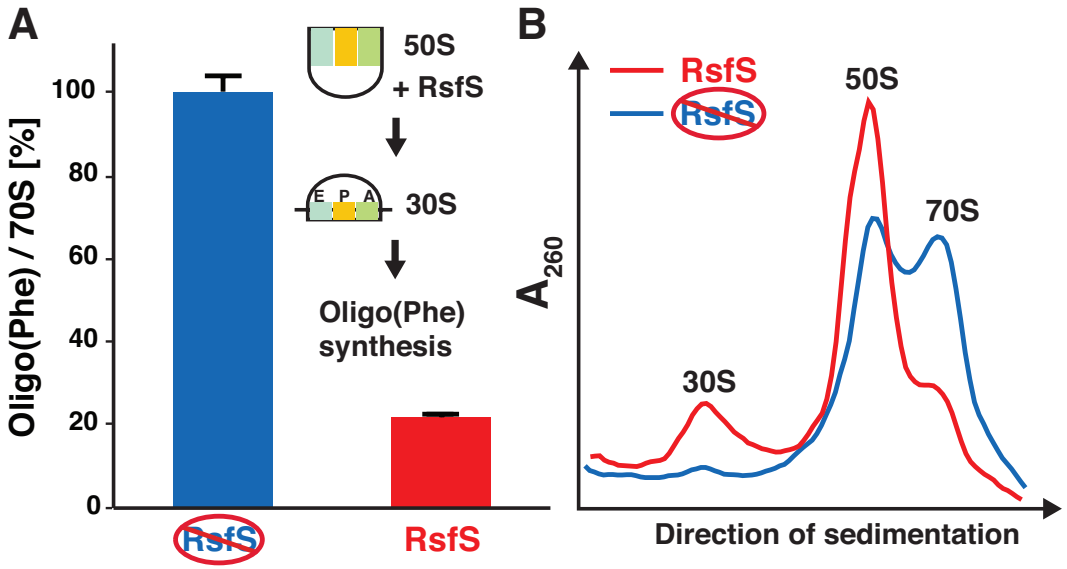

Figure 26. RsfS blocks translation by preventing the association of 70S. We preincubated RsfS with 50S, and then added 30S, mRNA, [^{14}C]Phe-tRNA, GTP and elongation factors. We performed oligo(Phe) synthesis with 20-fold molar excess of RsfS over ribosome for 10 min at 37 °C. **A**) The amount of synthesized Phe in the presence and absence of RsfS. **B**) Ribosome profiles of sister aliquot from the oligo(Phe) synthesis in the presence of RsfS analyzed with sucrose gradient centrifugation.

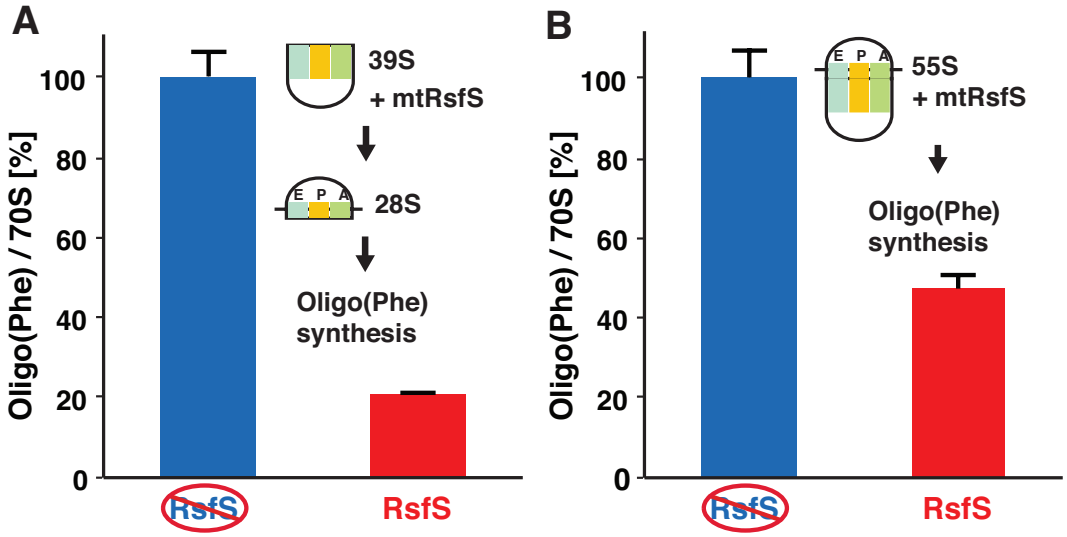

Figure 27. Mitochondrial RsfS (*c7orf30*) blocks translation of mitochondrial ribosome. We analyzed the effect of mitochondrial RsfS on mitochondrial ribosome in oligo(Phe) synthesis using mitochondrial elongation factors. **A**) Mitochondrial large subunit was preincubated with RsfS prior to oligo(Phe) synthesis. **B**) Mitochondrial 55S ribosome was incubated with RsfS prior to oligo(Phe) synthesis. We used 20-fold molar excess of mitochondrial RsfS over ribosomes and carried out the reaction for 20 min at 30 °C.

3.3 RsfS: Dissociation or anti-association factor?

RsfS inhibits translation in cell lysate based systems and promotes dissociation of 70S ribosome into subunits. However, this result is in the contrast to oligo(Phe) system. The RTS and oligo(Phe) system, although both being *in vitro* translation systems, differ substantially. RTS, which is based on *E. coli* lysate, contains all factors involved in protein synthesis. Therefore it is considered to be close to *in vivo* conditions. On the other hand oligo(Phe) system contains only precharged Phe-tRNA, poly(U), elongation factors and GTP. We sought to study the discrepancy between the effects of RsfS on these two systems.

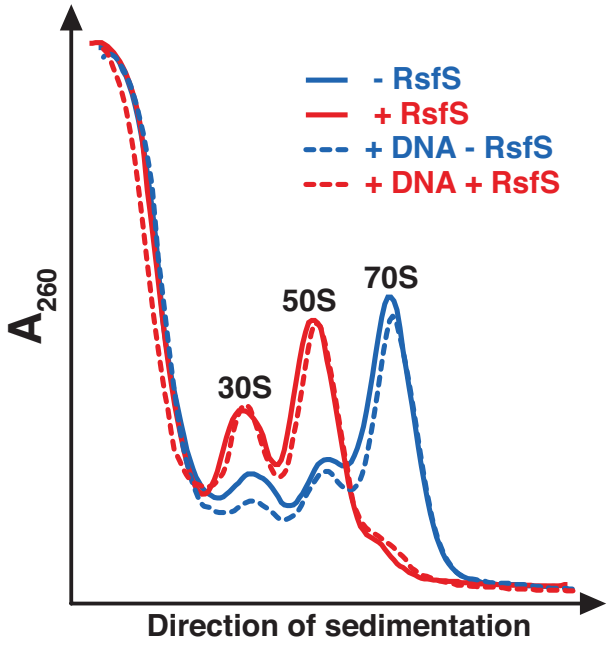

Figure 28. RsfS promotes dissociation of 70S into subunits in the Rapid Translation System(RTS). RsfS dissociaties 70S ribosome regardless whether it is active in translation(+ DNA) or idle (-DNA). Without DNA translation reaction cannot be initiated. We carried out the reaction for 20 min at 30 °C

We assumed that in the RTS system RsfS blocks the translation either by blocking the association of 70S or it actively dissociates 70S ribosome into subunits. The former would be possible after the recycling of 70S ribosomes or before the initiation of translation. This would eventually lead to a continuous depletion of the pool of active 70S eventually hampering the translation. The latter possibility would be independent of translation stage. To analyse these possibilities we introduced a modification to RTS system. We assumed that if we

omit DNA templates from the RTS reaction mixture, the transcription, and subsequent translation will not be initiated. Therefore, any dissociation of 70S by RsfS will be due to active dissociation of 70S rather than anti-association. **Figure 28** shows the sucrose gradient of samples derived from the RTS experiment. As a control we used RTS supplied with DNA with and without RsfS. We could reproduce the effect of translation inhibition (solid lines) by RsfS. Now when we omitted DNA we found strong peak of 70S, indicating that in the RTS most of ribosomes are associated. However, when we removed DNA and added RsfS most of 70S ribosomes dissociated into subunits. This experiment demonstrates that RsfS is not only blocking association but shifts the equilibrium of 70S towards dissociation in cell lysate based system.

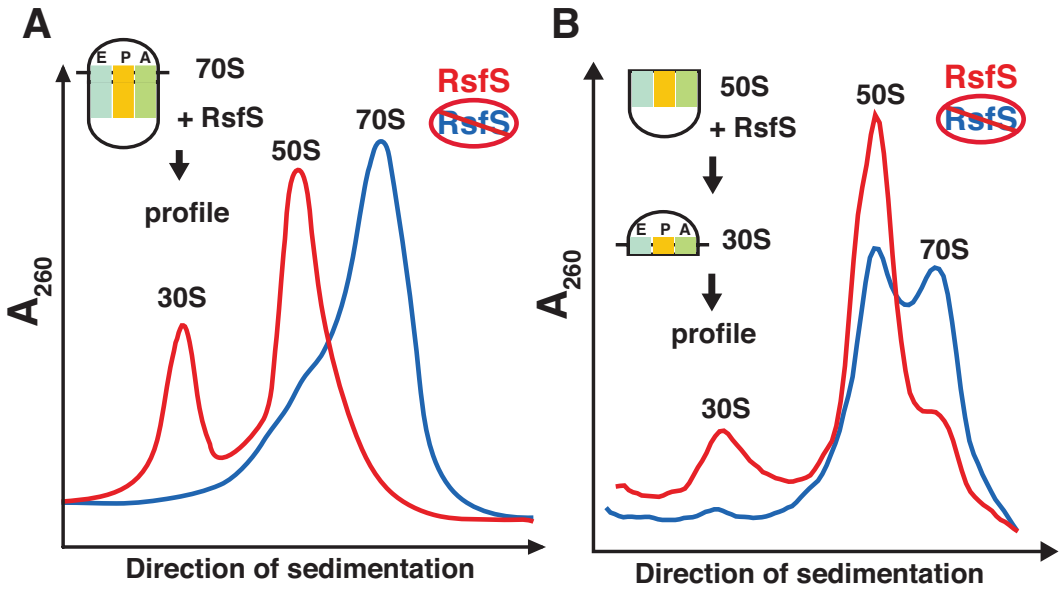

Figure 29. RsfS dissociates 70S ribosomes and prevents association of ribosomal subunits A) Non-programmed 70S incubated with RsfS. **B**) 50S incubated with RsfS prior to addition of 30S. Both reactions were incubated for 10 min at 37 °C.

If RsfS is both an anti-association and a dissociation factor, then both effects should be evident after incubation of RsfS with the ribosome alone. To test this we studied the effect of RsfS on reassociated 70S ribosomes *i.e.* ribosomes free of any additional factors and tRNAs. We incubated RsfS in a 10 molar excess over 70S for 10 min at 37 °C in Binding Buffer containing 4.5 mM Mg^{+2}. This time, in contrast to oligo(Phe) synthesis assay (**Figure 25**), RsfS dissociated 70S almost completely (**Figure 29A**). Simultaneously, we checked the binding of RsfS to

ribosome by immunoblotting of fraction corresponding to 50S from the sucrose gradient. RsfS comigrated with 50S subunit (**Figure 30**). Figure **29B** shows the anti association test of RsfS. We incubated RsfS with 50S and subsequently added 30S. After the incubation the sample was analyzed in sucrose-gradient centrifugation. We found that RsfS blocks anti-association of ribosome when incubated with 50S alone. Our results suggest that RsfS dissociates 70S without an energy source or additional factors. After dissociation RsfS forms a stable complex with 50S.

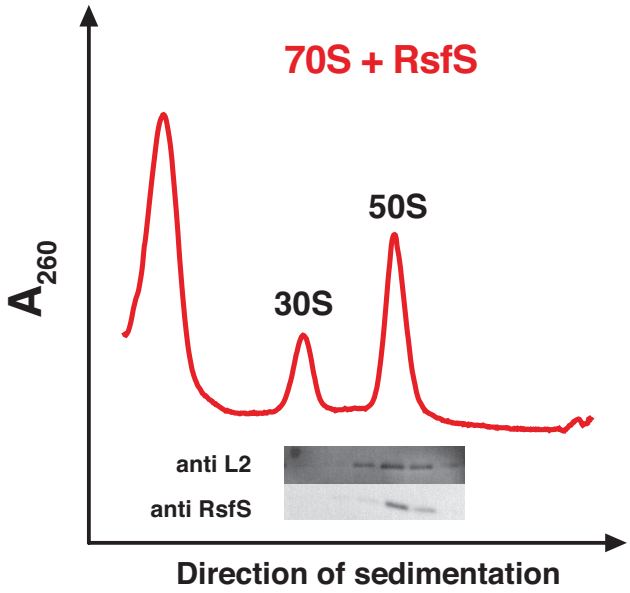

Figure 30. RsfS dissociates 70S and forms a stable complex with 50S subunits. RsfS was incubated with empty reassociated 70S, loaded on a sucrose gradient and fractions corresponding to subunits were analyzed by immunoblotting against ribosomal protein L2 and RsfS.

However, the only partial dissociation of 70S ribosomes by RsfS in oligo(Phe) synthesis is contrasting the complete dissociation in the RTS system. Since there were only few additional translation factors, we assumed that the presence of Phe-tRNA in the reaction mixture might reduce the dissociation-activity of RsfS. To test this hypothesis we used radioactively labeled precharged Acetyl-Phe-tRNA and monitored the binding of tRNA to the ribosome using nitrocellulose filters. AcPhe-tRNA bound to ribosomes will not pass the nitrocellulose filter, therefore the radioactivity on that filter will indicate the occupancy of ribosomes with tRNAs. In parallel we checked the ribosomal profile of programmed 70S with a tRNA at the P site with and without RsfS using sucrose-gradient centrifugation. As a control

we tested 70S programmed with mRNA in the presence and absence of RsfS. As demonstrated before, the incubation with RsfS leads to full dissociation of empty 70S into subunits (**Figure 31A**).

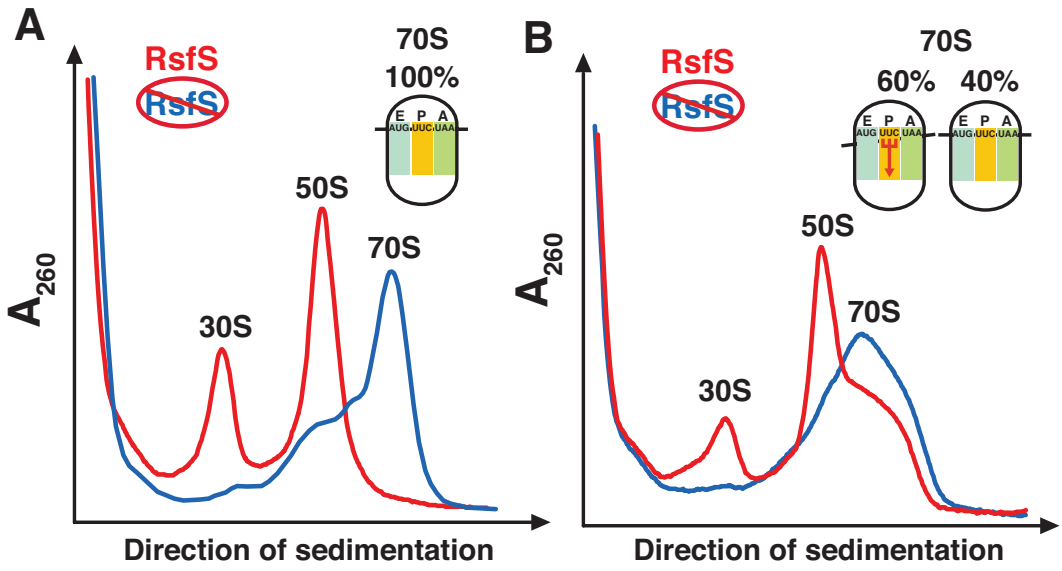

Figure 31. RsfS dissociates tRNA-free 70S. tRNA occupancy reduced the effect of ribosome splitting. A) RsfS was incubated with tRNA free 70S and analyzed on 10-30 % sucrose gradient. **B**) Poly(U) programmed 70S ribosomes were incubated with AcPhe-tRNA^{Phe}, followed by an incubation with RsfS. The mixture was incubated for 20 min at 30°C

In case of 70S with bound AcPhe-tRNA^{Phe} without RsfS we observed a broad peak of 70S, which could reflect the heterogeneity of 70S carrying a tRNA or being tRNA-free. Interestingly, RsfS could only partially dissociate 70S ribosomes carrying a AcPhe-tRNA^{Phe} (**Figure 31B**). In parallel, we monitored the occupancy of tRNA. First, we programmed 70S with mRNA and [^{14}C]AcPhe-tRNA^{Phe} in the absence of RsfS. After preincubation we measured the AcPhe-tRNA^{Phe} bound and found 60 % present on the 70S (**Figure 32**). Then we splitted the mother reaction into two aliquots and incubated them either with or without RsfS. We assumed that if RsfS dissociate 70S then AcPhe-tRNA^{Phe} should dissociate from the 50S subunit. The incubation without RsfS led to a higher occupancy of AcPhe-tRNA^{Phe}, which could mean that that prolonged incubation increased the binding of tRNA. On the other hand, RsfS failed to decrease the occupancy of AcPhe-tRNA^{Phe}. This suggests that RsfS has only limited capability of dissociating programmed ribosomes carrying AcPhe-tRNA^{Phe}.

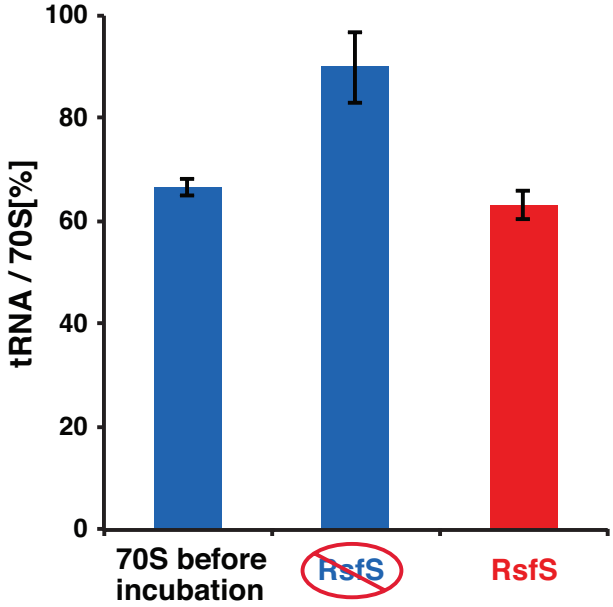

Figure 32. RsfS does not dissociate a ribosome when it is programmed with tRNA. We first programmed 70S with mRNA and tRNA, checked occupancy and incubated with or without RsfS. tRNA occupancy remains at 60 % after incubation with RsfS. Occupancy was estimated by nitrocelulose filtration.

Finally, we wondered whether RsfS could inhibit translation without dissociating 70S ribosomes. If this hypothesis was true, it would mean that RsfS bound to 50S is not only causing a steric clash, thus preventing 70 association but it could induce a conformational change to the ribosome, resulting in inhibition of protein synthesis and 70S dissociation. To test this hypothesis, we used chemically crosslinked ribosomes. The stability of crosslinked ribosomes as well translation activity was checked prior to the experiment (Wittek 2009). As a control we checked the ribosome profile of 70S and crosslinked 70S (X-70S) in the presence of RsfS. As expected RsfS dissociated 70S but failed to split crosslinked 70S (**Figure 33A**). Western blot analysis revealed binding of RsfS to 50S subunits, when incubated with 70S. However, we could not find binding of RsfS to crosslinked 70S. In parallel, we subjected X-70S to oligo(Phe) synthesis with and without RsfS. After 40 min of incubation we found no difference in Phe incorporation (**Figure 33B**). This suggests that the mechanism of translation inhibition is a result of subunit dissociation.

We conclude that RsfS is a dissociation and anti association factor. It blocks translation, when 70S ribosomes are free of AcPhe-tRNA and forms a stable complex with 50S subunits. RsfS does not seem to block translation, when

70S ribosomes are unable to dissociate or RsfS is unable to form a stable complex with crosslinked 70S ribosomes.

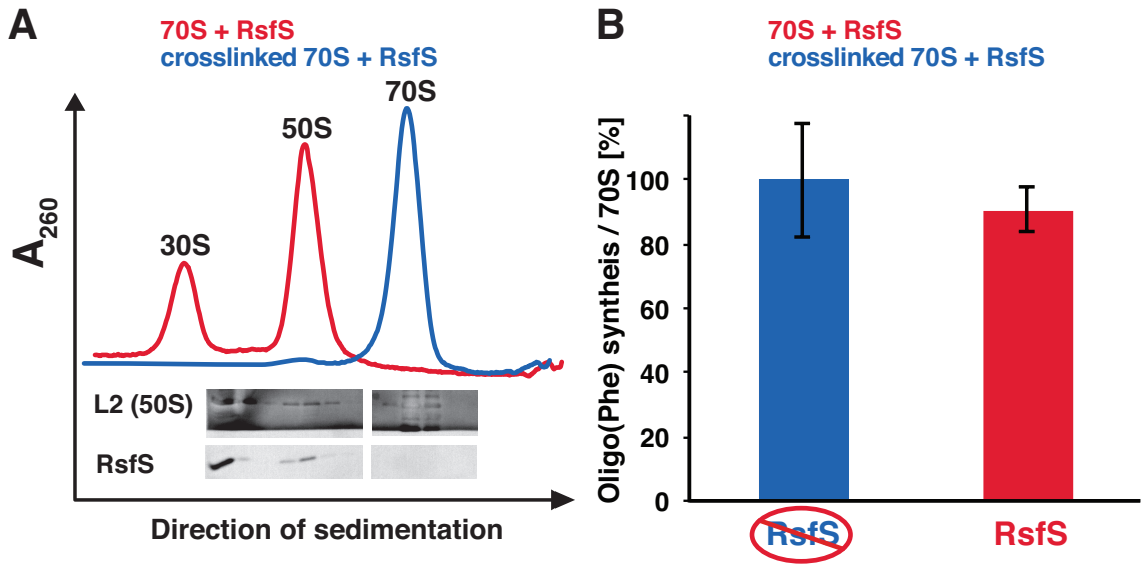

Figure 33. RsfS does not form a stable complex with non-dissociable 70S ribosomes (X-70S) and does not inhibit translation when X-70S is used. **A**) Ribosome profile of 70S and X-70S incubated with RsfS. **B**) Oligo(Phe) synthesis of X70S preincubated with and without RsfS

3.4 Comparison of RsfS, RMF, HPF and PY

RsfS is a 50S binding protein, which turns off translation by dissociating 70S ribosomes or preventing association of ribosomal subunits during stationary growth phase and after transition from rich to poor medium. RsfS is not the only factor dimming translation in stationary phase. Three factors are involved in transient deactivation of 70S: Ribosome Modulating Factor (RMF), Hibernation Promoting Factor (HPF) and Protein Y (PY). RMF promotes dimerization of 70S, which has a sedimentation coefficient of 90. HPF stabilizes the 70S dimer leading to the formation of 100S particles, which have no translation activity. PY, on the other hand, can turn 100S particles into 70S ribosomes, still having no translation activity. Since 100S factors and RsfS seem to have overlapping functions we studied all four factors and compared their functions. We call the three factors HPF, RMF and PY collectively 100S factors, because they influence the formation of 100S particles either positively (HPF, RMF) or negatively (PY).

3.4.1 Conservation and distribution of RsfS, RMF, HPF and PY

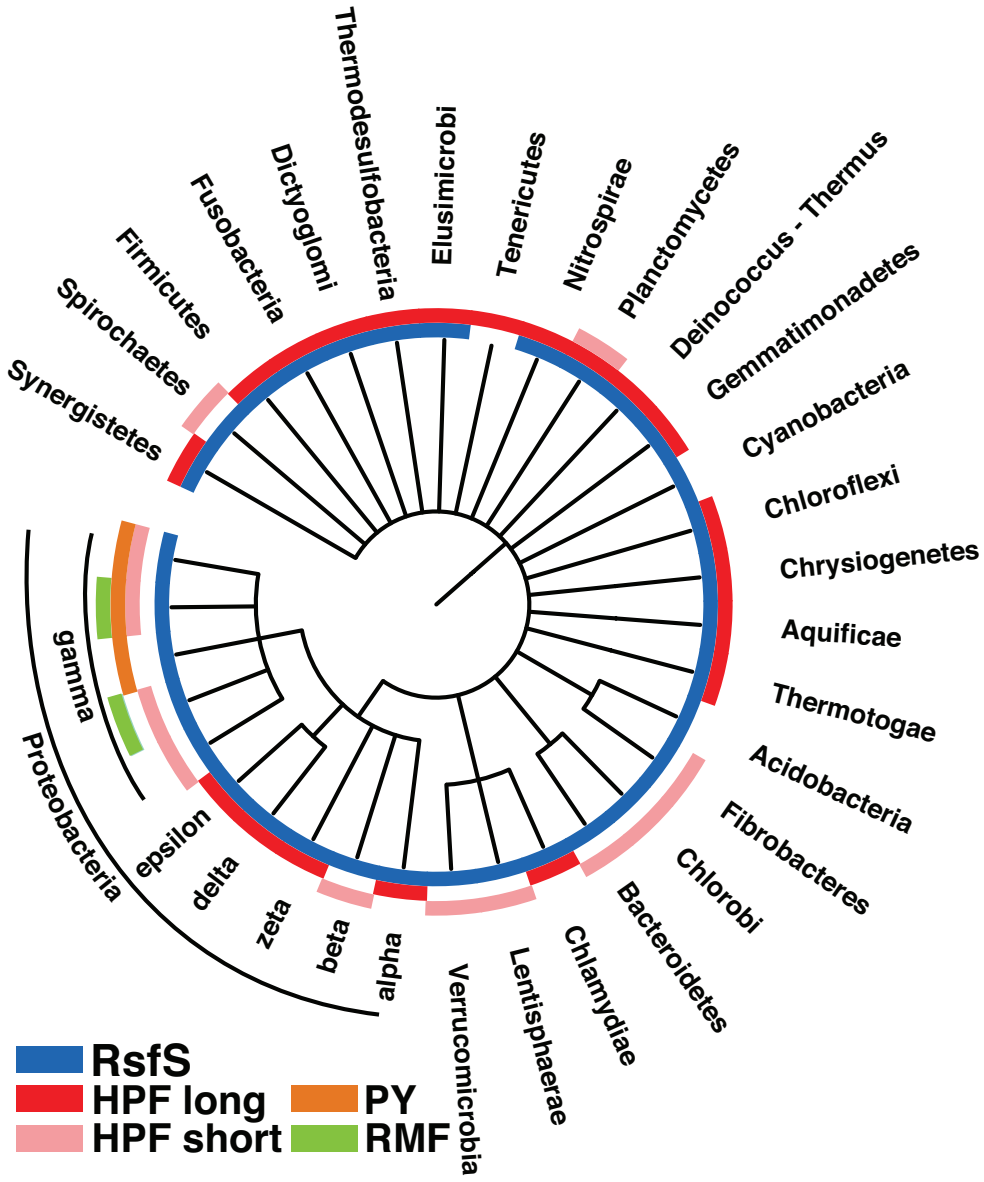

Figure 34. Distribution of silencing factors and RsfS in bacterial domain of life. Phylogenetic distribution of RsfS (Interpro entry IPR004394 [DUF143]), HPF, RMF and PY on the interactive tree of life (iTOL),(Letunic and Bork 2007). Pink color indicates shorter version of HPF, red the long HPF, green RMF and brown PY.

First, we analyzed the distribution of RsfS and 100S factors (**Figure 34**). Since 100S factors are present only in the bacterial domain of life, we restricted our analysis to bacteria. RsfS is present in almost all bacterial kingdoms (Häuser, Pech et al. 2012). HPF exists in bacteria in two versions – a long and a short one

(Ueta, Ohniwa et al. 2008). Long version (red, about 180 aa) is almost as disseminated as RsfS. Tenericutes, Gemmatimonadetes and some γ-proteobacteria lack the longer version and have the shorter one instead. *E. coli* used in this study contains the short form. PY (YfiA) and RMF seem to have only anecdotic importance in for bacterial life in general, because they exist only in a few γ-proteobacteria (Ueta, Ohniwa et al. 2008).

3.4.2 Comparison of phenotypes of *ΔrsfS, Δrmf, Δhpf* and *Δpy* strains.

When we knocked out the *rsfS* gene, we observed two strong phenotypes: i) After transition from rich to poor media the *ΔrsfS* strain showed a block of growth for about 15 h at 37 °C, after which the cells resumed a growth rate similar to that of WT cells.

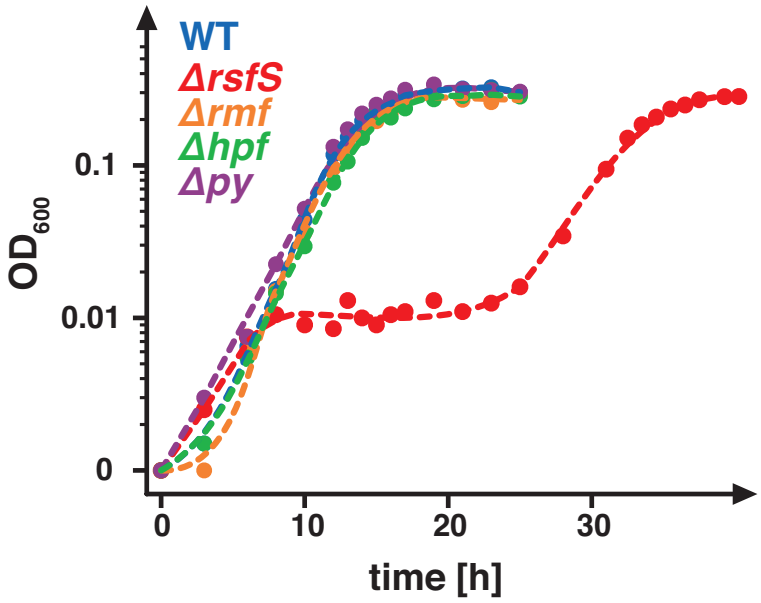

Figure 35. Growth curves of WT *ΔrsfS, Δhpf, Δrmf and Δpy* strains after transition from rich to poor medium. Strains were inoculated in LB medium and grown overnight at 37 °C. Then all strains were diluted in fresh M9 medium and the growth (OD_{600}) was measured until stationary growth phase.

Figure 35 shows that none of the deletion strains of the other silencing factors showed a similar phenotype: they all grew like WT cells after the transition. (ii) A second phenotype was observed, when the viability of cells were compared with WT cells in the stationary phase. We used a growth competition assay, where equal amounts of cells from overnight cultures of WT and the mutant strains

(kanamycin resistant) were mixed, thus maintaining the stationary-phase condition. In time intervals aliquots were taken and the both the total number of colonies and the colony number of the mutant strain were calculated. The latter was possible due to the fact that a kanamycin cassette was replacing the corresponding gene.

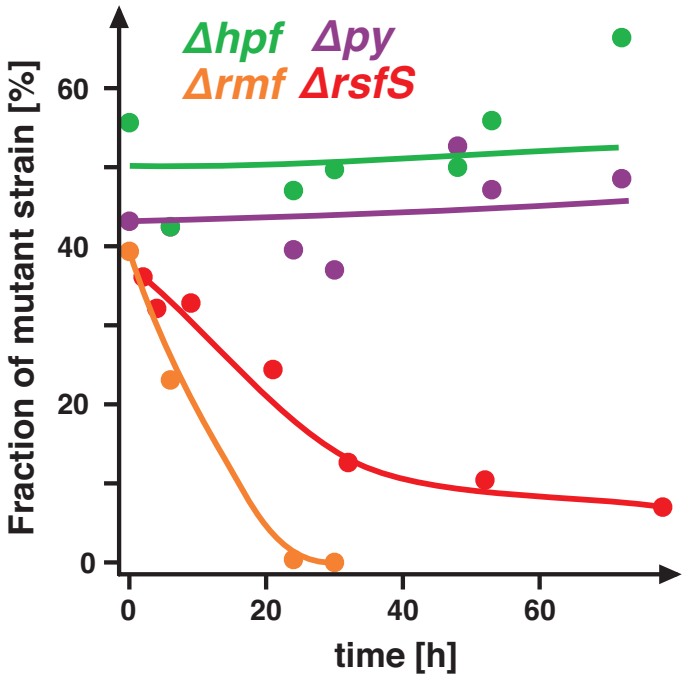

Figure 36. *ΔrsfS* and *Δrmf* strains show decreased viability in stationary growth phase. Equal numbers of *E. coli* wild type and mutant cells derived from an overnight LB-culture were mixed and grown in LB. Shown is the fraction of mutant cells in the total cell population. All strains had similar growth curves.

Plating an aliquot of the mixture in an LB plate in the presence of kanamycin allowed colony-growth of exclusively the mutant strain. Knocking down the genes for PY and HPF did not affect the viability during stationary phase, whereas the Δ*rmf* strain showed a handicap even more severe than that of the Δ*rsfS* strain (**Figure 36**); within 30 h at 37°C almost all the Δ*rmf* cells died.

3.4.3 Translational activity *in vivo*

We demonstrated that the lack of RsfS during the stationary phase showed a two times increase in induced β-galactosidase expression. This observation was used to explain the substantially impaired viability during this growth phase: the

energy drain caused by the increased protein synthesis in the absence of RsfS disorders the dormant state of the cell affecting strongly the viability.

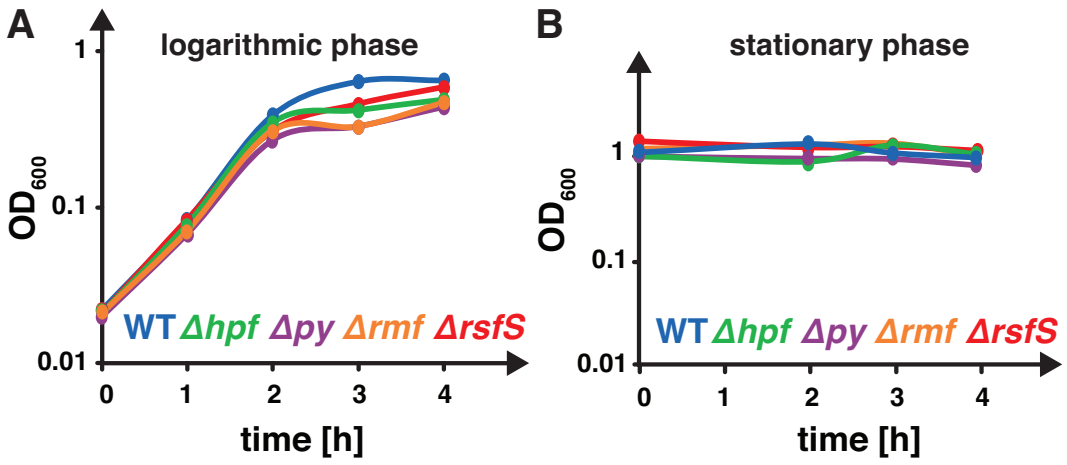

Figure 37. Wild type and mutant strains show similar growth rate when expression of β-galactosidase is induced. The β-galactosidase expression was induced at 0 h. **A**) Growth curve in logarithmic growth phase. **B**) Growth curve in stationary growth phase.

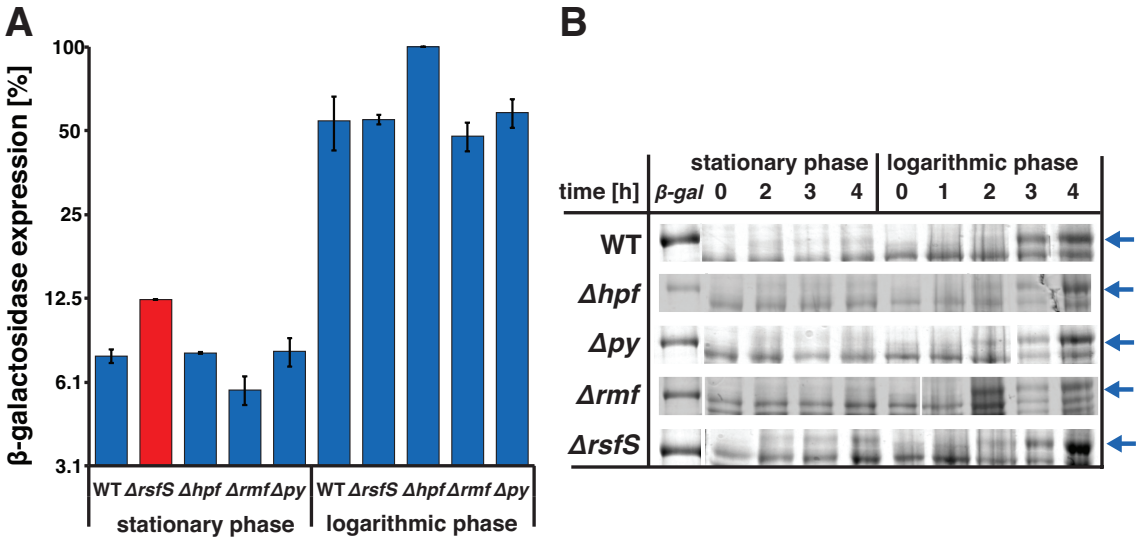

Figure 38. Only the *ΔrsfS* strain shows an increased translation activity during stationary growth phase. Comparison of of β-galactosidase expression in WT, *ΔrsfS, Δhpf, Δrmf and Δpy* strains in the logarithmic growth phase and the stationary growth phase. **A**) Expression of β-galactosidase as reporter to test translational activity of logarithmic and stationary phase cells in WT and mutant cells induced by 0.2 % arabinose. Shown expression of β-galactosidase was detected after 4 hours induction in logarithmic and stationary growth phase. The expression level was calculated from the band-intensity on a gel (Coomassie-stained SDS-PAGE) after normalization to a reference band. Error bar indicates variance from mean value. **B**) SDS PAGE gel of tested strains. Blue arrows indicate the β-galactosidase band.

Therefore, we compared the extent of induced β-galactosidase in logarithmic and stationary phase of WT cells and the mutant strains. First we compared the growth rate of tested strain in logarithmic and stationary growth phase (**Figure 37**). An increased protein synthesis during the stationary phase was only seen with the *ΔrsfS* strain, all other mutant strains showed a damping of protein synthesis similar to that of WT cells (**Figure 38**). It follows that RsfS is the key factor for silencing ribosomal activity during the stationary phase.

3.4.4 Translational activity *in vitro*

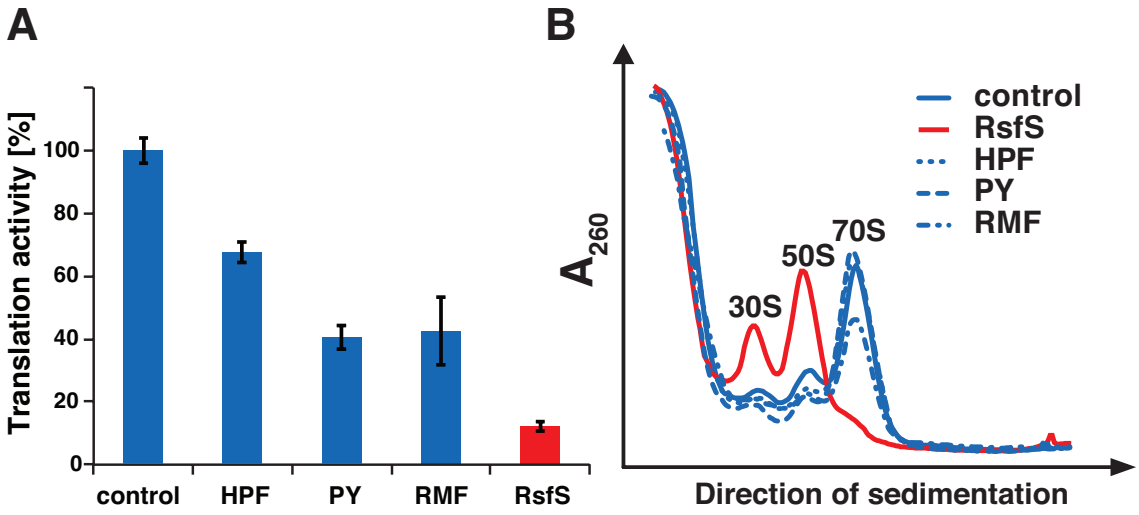

Figure 39. RsfS is a strongest translation inhibitor among tested proteins in lystate-based translation system(RTS). **A**) A test of inhibitory effect of RsfS, HPF, RMF, and PY on translation in the Rapid Translation System (RTS). Translation amounts of Renilla luciferase shown as relative luminescence units after 60 min of incubation at 30 °C. 10 molar excess of hibernation factors and RsfS over the ribosome was used. Error bars indicate standard deviation. **B**) A sister aliquot of the functional assays was subjected to a sucrose-gradient centrifugation.

A coupled transcription-translation system (RTS) was used to test the effects of the various silencing factors on the synthesis of Renilla luciferase (**Figure 39A**). The factors were added in a ten-molar excess over ribosomes. RsfS was the strongest inhibitor (about 90 % inhibition) followed by RMF and PY (both 60 %) and HPF (about 30 % inhibition). An aliquot of the RTS assay was subjected to a sucrose-gradient analysis (**Figure 39B**): only RsfS inhibited the activity *via* a dissociation of the 70S ribosomes. RsfS is a strongest translation inhibitor among tested proteins.

3.4.5 Cooperativity test of RsfS, RMF, HPF and PY

It has been demonstrated that HPF and PY bind to the same site on the ribosome. On the other hand, binding of RMF and PY is exclusive, because PY overlaps with binding pocket of RMF. We wondered, whether the silencing effect of RsfS is enhanced by the presence of the 100S factors. Therefore, we sought to test, the possible cooperativity between these factors for their translation inhibition during oligo(Phe) synthesis.

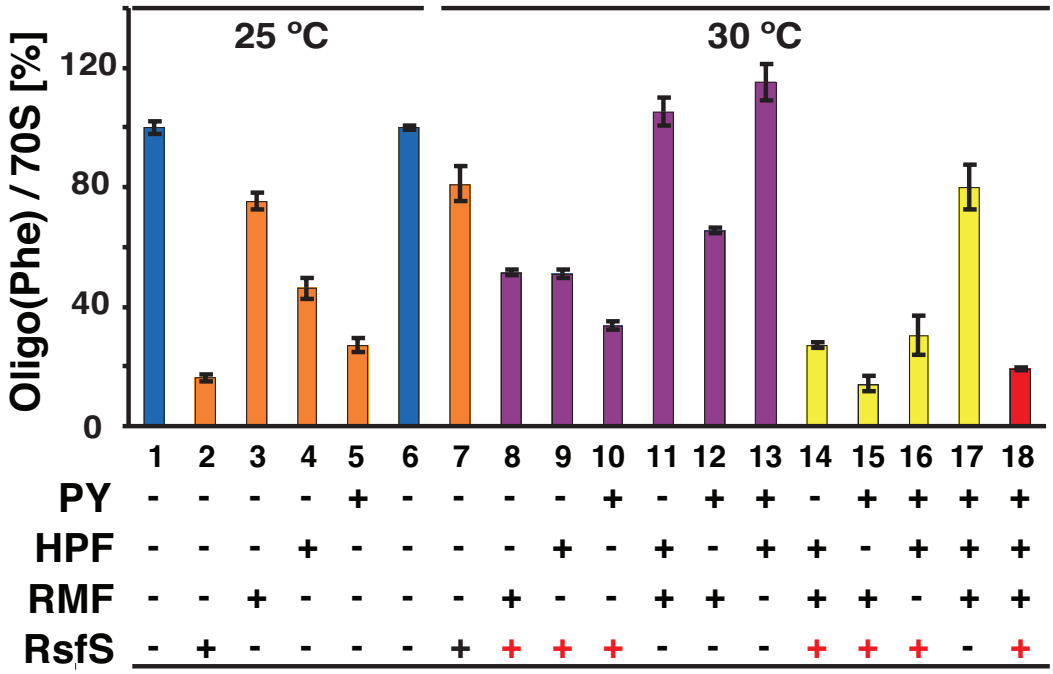

Figure 40. Effect of RsfS inhibition is supported by RMF, HPF and PY. Poly(U) dependent Oligo(Phe) synthesis with 100S factors and RsfS. Oligo(Phe) synthesis in a pure system containing pre-charged Phe-tRNAs (10 molar excess over ribosomes), 30S and 50S subunits and the purified factors EF-Tu, EF-Ts and EF-G plus/minus RsfA/HPF/RMF/PY from *E. coli*, 100 % corresponds to 5 Phe incorporated per ribosome. Red "+" indicates the presence of RsfS in the assay. Error bars indicate a variance from mean value.

We used again oligo(Phe) synthesis assay with purified elongation factors and precharged [^{14}C]Phe-tRNA^{Phe} (**Figure 39**). Comparing to RTS this *in vitro* system is more defined, therefore we expected a possible cooperativity to be more profound than in RTS. First, we tested all four factors individually using 20X excess over ribosomes at 25 °C. We note, that prior to the main incubation we preincubated the tested factors with 50S subunits. Considering that 100S factors bind to 30S subunits and RsfS to 50S, RsfS might be more outspoken in that assay. We observed strong inhibition of RsfS of more than 80 %, which left no

room for cooperativity test. Therefore, we raised the temperature to 30 °C, where under the incubation conditions RsfS (10 min instead of 5 min at 25 °C) reduced the activity for only 20 % leaving room for additional inhibition effects of the other factors. When we tested two out of four factors (violett bars) or three out of four factors (green bars), the strongest inhibition was always seen, when RsfS was present (red “+”). However, we note that the three factors RMF, HPF and PY supported strongly the RsfS effect (compare columns 7 and 18).

To conclude, under resolved condition of elongation, RsfS is the strongest inhibitor of translation. We note that elevated effect of RsfS in concert with other factors may stem from the preincubation step with 50S, but not with 30S. Translation effect in RTS and Oligo(Phe) showed that 100S and RsfS complement each other in inhibition of protein synthesis.

4 Discussion

In this thesis we sought to elucidate the function of the universally conserved protein RsfS, which associates with the ribosome. We found that deletion of RsfS in *E. coli* results in two distinct phenotypes. During the stationary growth phase the *ΔrsfS* strain loses the viability in competition with the wild type strain. Analysis of protein expression during stationary growth phase revealed that the *ΔrsfS* strain has increased translation activity comparing to the control strain. On the other hand, after transition from rich to poor medium the *ΔrsfS* strain shows 15 hours-long adaptation problems resulting in a block of growth during this period. *In vitro* RsfS blocks 70S association as well as dissociates 70S ribosome into 50S and 30S ribosomal subunits. Anti-association activity seems to be conserved, because both bacterial and mitochondrial RsfS are potent anti-association factors. However, when 70S carries an AcPhenylalanine-tRNA present at the P site, subunit dissociation mediated by RsfS is substantially weaker than in the case of empty 70S ribosome. After dissociation, RsfS forms a stable complex with 50S subunit, thereby preventing 70S association. Our comparison of factors related to 100S particles (RMF, HPF, PY) with RsfS showed that RsfS is the strongest translation inhibitor of all the tested translation silencers.

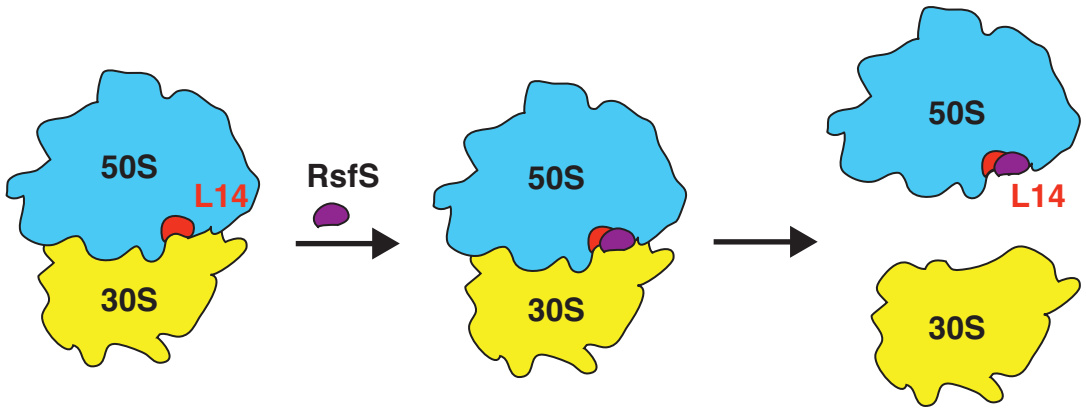

Figure 41. The function of RsfS. RsfS binds to 70S ribosome and induces dissociation of the ribosome into subunits. RsfS forms a stable complex with 50S, thereby preventing an association of 70S ribosome. RsfS is both anti-association and dissociation factor.

Furthermore, *Δrmf, Δhpf and Δpy* strains show no leaky translation during stationary phase, as observed in case of RsfS. Nevertheless, we note that deletion

of RMF reduces the viability of *E. coli* in the stationary growth phase to a higher extent than the deletion of RsfS. We conclude that RsfS is a universally conserved translation silencer, which works during stationary growth phase and after transition from rich to poor medium, *i.e.* whenever protein synthesis has to be down-regulated. **Figure 41** depicts RsfS binding to 70S and dissociating the ribosome into subunits.

4.1 Importance of 100S particles during stationary phase

Our tests of cell lysates in sucrose-gradient centrifugation suggest that 100S particles are not an obligatory feature of stationary phase in *E. coli*. The presence of a non-induced but propagated plasmid abolishes the 100S peak in *E. coli* strain, whereas the same strain lacking the tested plasmid shows a distinct 100S peak (**Figure 23**).

It is well documented, that the presence of a plasmid exerts a substantial effect on the metabolism of the *E. coli* (Diaz Ricci and Hernandez 2000). For instance, plasmid-harbouring strains have slower growth rate than plasmid free corresponding strains. The extent of such an effect depends on the plasmid-copy number per cell and the plasmid-dependent expressed proteins. In both cases, the presence of plasmid causes an energy drain, which has to be compensated by cellular metabolism and results in slower division rate. It remains to be tested the effects of the presence of a plasmid on the stationary-phase metabolism of the bacterial cell.

On the other hand, the formation 100S particles has been proposed to play a key role in ribosome silencing during stationary phase. 100S particle is a dimer of 70S ribosomes, where two 70S ribosomes contact each other via the heads of 30S subunits. The 100S particles do not seem to contain any tRNA, and ribosomes in state of 100S particles are inactive in translation (Ortiz, Brandt et al. 2010). Three factors - RMF, HPF and PY - are involved in regulation of 100S particle formation. RMF induces the formation of 70S dimers with sedimentation coefficient around 90S. The 90S particles are subsequently stabilized by HPF yielding a mature 100S particles (Ueta, Ohniwa et al. 2008). PY, on the other hand, transforms 100S particles into inactive 70S ribosomes. Previously, it was thought that the RMF dependent formation of 100S dimers is responsible for the

dormant state of the ribosomes during stationary growth phase (Wada, Yamazaki et al. 1990). However, 100S particles are observed not only during stationary growth phase but also in the logarithmic growth phase in *E. coli* (Wada, Yamazaki et al. 1990). Furthermore, a study of 100S particles in *Staphylococcus aureus* revealed, that 100S is mostly formed in logarithmic phase, which questioned its role in ribosome silencing (Ueta, Wada et al. 2010). In addition, the 100S fraction is noticeably reduced after prolonged incubation in stationary phase, suggesting that 100S particles have only transient role in protection of ribosome during prolonged stationary phase (Wada 1998; Wada, Mikkola et al. 2000). Our results show that abolition of 100S in early stationary seems not to play a role for cell survival. Moreover, given that RMF is essential for 100S formation, one would expect that lack of 100S particles would increase the translation activity of *Δrmf* strain. This was not the case as shown in the **Figure 38**, where we found comparable expression of β-galactosidase in the *Δrmf* and WT strains. However, we note that deletion of RMF, the only essential factor for ribosome dimerization, causes viability defect in the early stationary growth phase in *E. coli.* Moreover, *in vitro* RMF inhibits protein synthesis in poly(U) dependent oligo(Phe) system. On the other hand, RMF is poorly conserved in the bacterial domain, present only in a few γ-proteobacteria. *In silco* analysis suggest, that in strains lacking RMF the longer version of HPF could take over the function of RMF, because the binding sites of HPF and RMF are adjacent (Ueta, Ohniwa et al. 2008).

Taken together, our results suggest that 100S particle is not an obligatory element of early stationary growth phase. However, lack of RMF, a key player in 100S formation, decreases the viability in stationary growth phase.

4.2 Importance of RsfS

We demonstrated that RsfS is involved in down-regulation of protein synthesis after transition from rich to poor medium. In rich medium bacterial cells produce proteins at maximum rates to sustain cell division. Consequently, bacterial cells take up many metabolic precursors such as amino acids and thus block corresponding amino-acid synthesis pathways. In contrast, in poor medium protein synthesis must be down-regulated in a concerted fashion in order to save energy and resources, and at the same time many synthesis pathways such as

those for the synthesis of amino acids have to be switched on (Andersson and Kurland 1990; Dong, Nilsson et al. 1996). Our results suggest that RsfS plays a prominent role in this down-regulation by silencing ribosome activities. After a transition from rich to poor media the adaptation phase in the Δ*rsfS* strain lasts more than 15 hours before resuming the growth (**Figure 14**). Just adding casamino acids to the minimal medium relieves the strong growth defects of the *ΔrsfS* strain (**Figure 13**). Addition of amino-acids switches off most of the amino-acid synthesis pathways similar to the situation during the logarithmic phase in the presence of rich medium, when the silencing effect of RsfS is not strictly required. In contrast, during starvation and in the absence of ribosomal silencing (*ΔrsfS*), energy would be wasted affecting the conversion of the metabolic network, eventually causing deleterious growth defects.

Accordingly, protein synthesis is attenuated in the stationary growth phase, when RsfS is present (*i.e.* in wild type cells). In contrast, when RsfS is absent, we observe increased protein synthesis (**Figure 16**). Attenuation of protein synthesis by RsfS seems to be of utmost importance for reorganization the metabolic state on the way to the stationary phase, since the absence of this factor decreases the viability in the stationary growth phase (**Figure 14**), and it explains the well-known effect that ribosomes are much less active, when derived from the stationary rather than from log-phase cells.

4.3 Comparison of RsfS and other ribosome dissociating factors

We demonstrated that *in vitro* RsfS dissociates reassociated, tRNA-free 70S ribosomes into 50S and 30S subunits. Furthermore, 10 molar excess of RsfS over 70S ribosome is sufficient to block protein synthesis down to 10 % in cell lysate based system (**Figure 24A**). RsfS is not the only factor, which dissociates the tRNA-free ribosome. Ribosome Recycling Factor (RRF) together with EF-G (Gao, Zavialov et al. 2005) and IF3 (Singh, Das et al. 2005) have a similar function after termination of translation. According to the general view, translation is terminated with a help of termination factors RF1, RF2 and RF3 after the ribosome reaches stop codon on mRNA. RF1 or RF2 hydrolyse the ester bond between tRNA and the peptide. Then RF3 facilitates the dissociation of tRNA and

termination factors RF1 or RF2. When ribosome is free of factors and tRNA, it is recycled with a help of ribosome recycling factor (RRF), EF-G and initiation factor 3 (IF3). *In vitro* 90 % dissociation of high-salt-washed 70S ribosomes (possibly containing deacylated tRNA and mRNA) into ribosomal subunits requires 14 molar excess of RRF, 64 molar excess of IF3, 14 molar excess of EF-G and 6 excess of GTP (Hirokawa, Nijman et al. 2005). The presence of RRF, IF3, EF-G and GTP at similar concentrations dissociates reassociated 70S (tRNA and mRNA free 70S ribosome) only by 50 % (Hirokawa, Nijman et al. 2005). In contrast, only 10 molar excess of RsfS over reassociated 70 ribosomes turns most of the ribosomes into ribosomal subunits. We note that the incubation time and the temperature were comparable, except the Mg^{2+}, elevated to 8 mM comparing to 4.5 mM in our system. Why *in vitro* studies show that RsfS is a strongest 70S dissociation factor? The *in vivo* copy number of RsfS is not lower than RRF, EF-G and IF3: all these factors except EF-G have relatively low copy number per ribosome, around 1 per 10 ribosomes. Furthermore, deletion of RRF is lethal, in contrast to RsfS. *In vitro* experiments suggest that RRF together with IF3 and EF-G are not as potent as RsfS alone. Therefore, it is possible that RsfS enhances the ribosome dissociation and additionally blocks the reuse of subunits, which explains why in the absence of RsfS the translation activity is elevated (**Figure 16**). More experiments are required to study a possible interplay between termination factors and RsfS.

RsfS binds to the universally conserved ribosomal protein L14. Interestingly, there is another factor in eukaryotes that also binds to this particular ribosomal protein, namely the initiation factor eIF6. This protein shares no homology with RsfS and is thought to block ribosome association in Achaea and in Eukarya from yeast to man (Benelli, Marzi et al. 2009; Gartmann, Blau et al. 2010; Klinge, Voigts-Hoffmann et al. 2011; Greber, Boehringer et al. 2012). In contrast to RsfS, eIF6 is rather a 60S assembly factor that plays an essential role in the late pre-25S rRNA processing and the export of the 60S subunit from the nucleolus to the cytoplasm (Biswas, Mukherjee et al. 2011). Furthermore, the depletion of eIF6 is eventually lethal, contrary to RsfS.

4.4 RsfS in the eukaryotic world

We showed that mitochondrial paralog of RsfS (*c7orf30* or mtRsfS) has a silencing effect on translation *in vitro* when pre-incubated with the mitochondrial large subunit. However, a recent publication about mtRsfS suggest its involvement in the assembly of mitochondrial ribosome (Rorbach, Gammage et al. 2012). A depletion of mtRsfS impairs mitochondrial respiration system, *i.e* alters the expression of the proteins involved in the respiration complex 1, 3 and 4. Additionally, the analysis of ribosome profiles using sucrose gradient centrifugation and subsequent immunoblotting of ribosomal proteins after depletion of mtRsfS, shows decreased signal of few 50S proteins. The presented evidences are not convincing, because there is no direct evidence that depletion of RsfS results in the defect of ribosome assembly. The lack of L-proteins due to depletion of mtRsfS could be *a priori*, *i.e.* during ribosome assembly thus indicating an assembly defect, or *a posteriori*, *i.e.* lack of RsfS causes a conformational change on the 50S subunit resulting in release of ribosomal proteins. We already observed a paradigm for such an *a posteriori* effect: expressing a leaderless mRNA *in vivo* in the presence of the antibiotic kasugamycin triggers the release of up to 10 S proteins (Schluenzen, Takemoto et al. 2006). Our comparison of ribosomal profiles from the logarithmic-growth-phase of the *ΔrsfS* and WT strains shows no difference in ribosomal profile (**Figure 21**). Moreover, assuming that *ΔrsfS* strain has a defect in ribosome assembly, such a defect would be reflected in the protein synthesis during logarithmic or stationary growth phase. Our results revealed no difference in translation efficiency during logarithmic growth phase and increased translation activity during stationary growth phase in the absence of RsfS *in vivo* (**Figure 16**).

Another recent paper about mtRsfS is in favor of hypothesis suggesting an involvement of mtRsfS in the large subunit biogenesis (Fung, Nishimura et al. 2012). The authors knocked down the mtRsfS using siRNA and analyzed the ribosomal profile of mitochondrial ribosome in sucrose gradient centrifugation. They found no difference in the sedimentation of ribosomes, in agreement with our findings. However, an immunoblotting of ribosomal proteins showed a depletion of several ribosomal proteins (L11, L12, L32 and L44). Interestingly, the proteins of small subunit and majority of 50S showed no decreased expression. Notably, a

down-regulation of protein L14 phenocopied the phenotype of mtRsfS depletion. Result of Fung et. Al. suggests that mtRsfS might be involved in assembly of the mitochondrial ribosome. It remains possible that mtRsfS has multiple roles in mitochondria or that its role in ribosome assembly is indirect. On the other hand, upon binding to ribosomal protein L14, RsfS might induce a conformational change in the ribosome, which might lead to dissociation of some ribosomal proteins.

4.5 Open questions and outlook

RsfS blocks protein synthesis by binding to the 50S subunit or 70S ribosome and prevents association of 70S ribosome or actively dissociates 70S ribosome. This may happen during stationary growth phase or after a shift to poor medium *i.e.* whenever protein synthesis has to be down-regulated. RsfS is a potent translation inhibitor in 10 molar excess over the ribosome *in vitro*. Therefore, one would assume that the copy number of RsfS per ribosome is relatively high in the stationary growth phase comparing to logarithmic growth phase. However, this is not the case, because according to mRNA expression-level tests, mRNA coding RsfS is expressed mainly during logarithmic phase. Furthermore, single molecule analysis of proteins expression *in vivo*, suggests that there is on average 1 copy of RsfS per 10 ribosomes, similarly to termination factors RF3 and RF1 (**Table 5**).

Protein	**Protein / ribosome ratio**
EF-Tu	10
RF3	0.15
RF1	0.1
RsfS	0.3

Table 5. Protein to ribosome ratio of *E. coli* factors. RsfS has a relatively ratio during logarithmic phase. Calculated from the data of (Taniguchi, Choi et al. 2010).

There are few possible explanations of the discrepancy between RsfS activity in the stationary growth phase and the higher expression level during logarithmic growth phase in *E. coli*. RsfS might need additional factors to

down-regulate translation. This possibility might be supported by the fact that addition of RsfS to cell lysate based in vitro translation system (RTS) blocks translation, in contrast to more resolved oligo(Phe) synthesis. As demonstrated with oligo(Phe) system the presence of Ac-Phe-tRNA on the ribosome hampers the ribosome-dissociation activity of RsfS (**Figure 31A**). However, cell-lysate based system (RTS) should contain a significant fraction of ribosomes with tRNAs present at the P site.

RsfS forms a stable complex with 50S subunit (**Figure 30**). Binding test of RsfS to chemically cross-linked 70S did not reveal any binding (**Figure 33A**), but we have to note that the chemical crosslinking could have destroyed the binding site of RsfS. Therefore, a complex of RsfS and 50S for cryo-electron-microscope analysis would help us to understand, if RsfS induces any conformational change on the ribosome or if the silencing is based primarily on steric clash of 50S-RsfS complex and 30S. Furthermore, more experiments are necessary to elucidate when RsfS leaves 50S-RsfS complex. Another open question concerns the overexpression of RsfS *in vivo*. We could not see an effect on the growth rate of WT strain when RsfS was overexpressed. However, we note that at higher levels RsfS might be directed to the inclusion bodies. Finally, one should exclude *via* 2D-electrophoresis, whether binding of RsfS to bacterial 50S subunits triggers a release of some large ribosomal proteins as reported for mitochondrial ribosomes.

We have identified a phenotype of RsfS deletion. We found that this proteins is silencing the ribosomal activity *via* 70S dissociation and preventing subunit association, which is essential for viability during stationary phase and very important during the transition form the poor to a rich medium. These results led to an armada of new questions, which hopefully will be answered during postdoctoral training.

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