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KASPAR REIER

Quantity, stability and disparity of ribosomal components in *Escherichia coli* stationary phase

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Institute of Molecular and Cell Biology, University of Tartu, Estonia.

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I – Lilleorg, S.*, **Reier, K.***, Pulk, A.*, Liiv, A., Tammsalu, T., Peil, L., Cate, J. D., and Remme, J. (2019). Bacterial ribosome heterogeneity: Changes in ribosomal protein composition during transition into stationary growth phase. Biochimie 156, 169–180.
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III – **Reier, K.**, Liiv, A., and Remme, J. (2023). Ribosome Protein Composition Mediates Translation during the *E. coli* Stationary Phase. Int J Mol Sci. 2023; 24(4):3128.

My contribution to the articles is as follows:

Ref I – Co-designed and performed the experiments, analyzed the data and participated in the writing of the manuscript.

Ref II – Designed and performed the experiments, analyzed the data and participated in the writing of the manuscript.

Ref III – Designed and performed the experiments, analyzed the data and participated in the writing of the manuscript.

LIST OF ABBREVIATIONS

E. coli	–	*Escherichia coli*
r-protein	–	ribosomal protein
LSU, 50S	–	large ribosomal subunit
SSU, 30S	–	small ribosomal subunit
CP	–	central protuberance
NPET	–	nascent peptide exit tunnel
PTC	–	peptidyl transferase center
DC	–	decoding center
SILAC	–	stable isotope labeled amino acids in culture
PNP	–	polyribonucleotide nucleotidyltransferase
GTP	–	guanosine-5'-triphosphate
mRNA	–	messenger ribonucleic acid
tRNA	–	transfer ribonucleic acid
rRNA	–	ribosomal ribonucleic acid
Pka	–	peptidyl-lysine N-acetyltransferase
ZUR	–	zinc uptake regulator
MCT	–	malonyl CoA-acyl carrier protein transacylase
EDTA	–	ethylenediaminetetraacetic acid
SDS	–	sodium dodecyl sulfate

INTRODUCTION

Ribosomes are molecular complexes present in every living organism. These complexes perform a function called translation – synthesis of proteins based on genetic instructions. Proteins themselves are important to the functioning of cells: some act as enzymes with catalytic activity, while others have structural or mechanical functions. Intriguingly, approximately 40% of ribosome mass is proteins, meaning the product of their primary function is necessary for their own existence and function. Most of these ribosomal proteins (r-proteins) interact with ribosomal RNA (rRNA) cooperatively to form functional ribosomes. R-proteins play a relevant role in the functionality of the ribosome, performing various objectives, such as mRNA placement (bS1), binding to GTP binding factors (bL7/bL12), interaction with elongating peptide chains (uL4, uL22) and association with chaperones for protein formation (uL23, uL29). In bacteria, the ribosome r-protein composition has been shown to undergo changes both *in vivo* and *in vitro* (Robertson, Dowsett et al. 1977, Subramanian and van Duin 1977, Pulk, Liiv et al. 2010). These variations in ribosome r-protein composition have been hypothesized to act as a possible mechanism for repair or fine-tuning of translation regulation via ribosome specialization (Pulk, Liiv et al. 2010). Outside of the ribosome, some r-proteins have been implicated in the post-transcriptional regulation of their own polycistronic mRNA (Nomura, Gourse et al. 1984). Given that ribosomal proteins constitute a substantial portion of the proteome, understanding their diverse roles within the ribosome and the cell as a whole is essential for gaining deeper insights into translation machinery.

Cell culture growth is usually divided into different stages: lag phase with null growth rate, acceleration phase with increasing growth rate, exponential phase with constant growth rate, early stationary phase with decreasing growth rate and prolonged stationary phase where growth rate is either null or negative. This thesis sheds light on the translation apparatus and how it adapts to prolonged stationary phase in *E. coli*, from the viewpoint of r-proteins. Three published articles are disserted with the intent to answer these questions: “How does the r-protein content of ribosomes and proteome change in response to stationary phase?” (ref. I, II and III), “Does the ribosome-associated protein content in ribosomes and proteome change during stationary phase?” (ref. I and III), “Does the stability of r-proteins remain unified during stationary phase?” (ref. II) and “Dynamics of ribosome content during stationary phase?” (ref. II and III).

1. REVIEW OF THE LITERATURE

1.1 Structure of the bacterial ribosome

Escherichia coli ribosome is an RNA-protein complex with a total molecular mass of 2.4 MDa. Approximately two-thirds of this mass can be attributed to ribonucleic acid (RNA), while the remaining one-third corresponds to ribosomal proteins (r-proteins). Within the ribosomes, both RNA and r-proteins contribute to the catalytic activity of the active sites, with RNA playing a central role in catalytic activity and the r-proteins providing essential structural support to these sites.

A mature ribosome denoted as 70S consists of two unequal subunits: the large subunit (LSU, 50S) and the small subunit (SSU, 30S) (Figure 1). Each subunit harbors specific active sites with distinct functions. For instance, the LSU contains the peptidyl transferase center (PTC), which facilitates the catalytic formation of peptide bonds between an elongating amino acid chain and amino-acetylated tRNA (aa-tRNA). In contrast, the SSU encompasses the decoding center (DC), which plays a crucial role in facilitating specific interactions and the binding of aa-tRNA to ribosomes, based on the messenger RNA primary sequence.

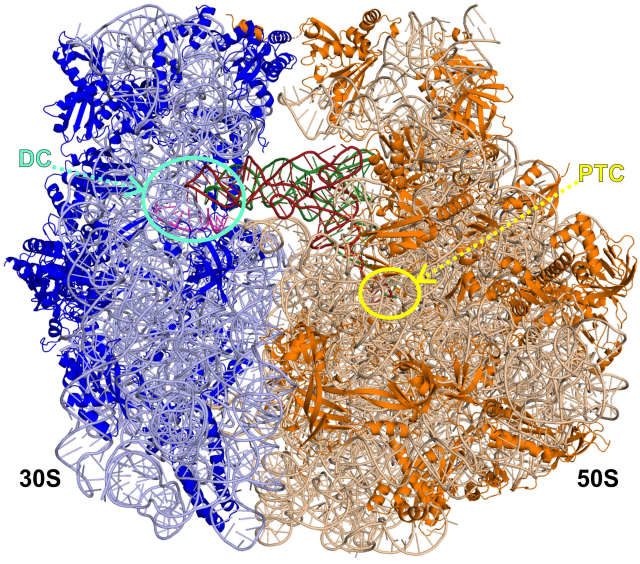

Figure 1 – Three-dimensional architecture of the 70S ribosome from *Escherichia coli*. Side view of 70S ribosome. To aid in visual clarity and distinction, the 30S is depicted using a range of blue hues, while the 50S is illustrated with varying shades of orange. More specifically, r-proteins are color-coded using dark blue and orange, while rRNA molecules are colored light blue and light orange. Additional elements are color-coded: mRNA – magenta, transfer RNA (tRNA) in ribosome A-site – red and tRNA in ribosome P-site – green. The figure is based on a cryogenic electron microscopy structure from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using structure (7k00) and visualized by Pymol software.

1.1.1 Large ribosomal subunit

The large ribosomal subunit of *Escherichia coli* is a complex assembly consisting of two distinct ribosomal RNA (rRNA) molecules: 5S rRNA (120 nt) and 23S rRNA (2304 nt) (Melnikov, Ben-Shem et al. 2012, Dunkle and Cate 2013). Complementing the rRNAs are 33 unique ribosomal proteins (Melnikov, Ben-Shem et al. 2012, Dunkle and Cate 2013), which collectively contribute to the ribosome intricate functionality. The 23S rRNA can be further partitioned into six domains, with the additional presence of 5S rRNA serving as a supplementary seventh domain (Petrov, Bernier et al. 2013).

Within the architecture of the LSU, discernible structural elements encompass the L1 stalk, the L11 stalk and the central protuberance (CP) (Figure 2A). The L1 stalk is proximal to the deacetylated tRNA exit site (E-site) (figure 2A). Meanwhile, the CP is aptly positioned at the core of the LSU, directly overlaying the PTC, a critical catalytic domain (Figure 2A). Conversely, the L11 stalk, operating as a factor binding site, presents itself in opposition to the L1 stalk concerning the CP (Figure 2A).

In the context of translation activity, it is noteworthy that the 50S subunit does not adhere to strict compartmentalization based on structural regions or 23S rRNA domains. Instead, functional sites are distributed across diverse structural regions, thereby presenting challenges in research endeavors. The PTC, located at the heart of the 50S subunit, serves as the central functional site. Here, the essential process of peptide bond formation transpires, involving the elongating peptide chain, the peptidyl-tRNA (P-site tRNA) and the amino-acetylated tRNA occupying the A-site. Emphasizing the significance of RNA in catalysis of peptide bond, it is the predominant component constituting the PTC. Concurrently, ribosomal proteins, while devoid of catalytic activity in peptide bond formation, are indispensable in preserving the correct structural conformation of the PTC.

Another significant feature of the LSU is the nascent peptide exit tunnel (NPET), facilitating the egress of the formed peptide chain from the PTC and its subsequent release from the ribosome. The NPET, composed of both RNA and ribosomal proteins, engages in interactions with the nascent peptide chain, thereby influencing the ribosome elongation rate (Petrone, Snow et al. 2008). These regions, particularly the PTC and NPET, are of substantial interest as they harbor multiple binding sites for antibiotics known to inhibit protein synthesis.

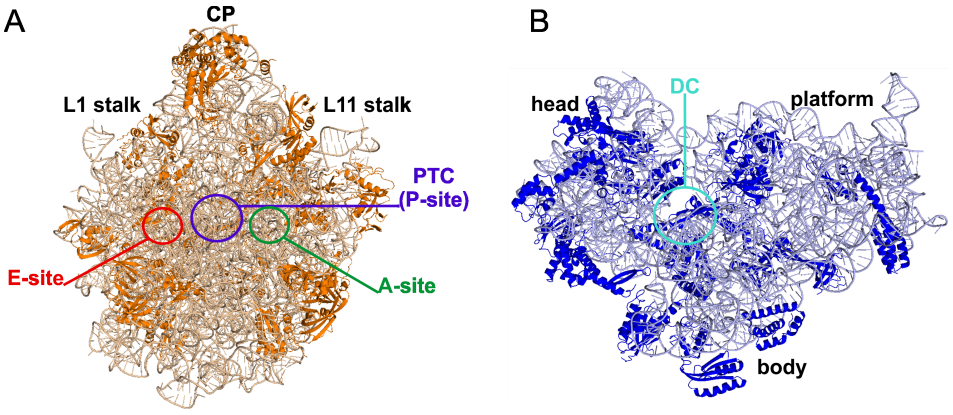

Figure 2 – Ribosomal subunit structural elements.
Notable structural elements and functional sites of ribosome subunits are shown. (A) view of the LSU from an intersubunit plane: A-site (cyan), P-site and the encompassing PTC (lilac), E-site (red), L1 and L11 stalk and CP. R-proteins are colored dark orange and rRNA molecules as light orange. (B) view of the SSU from the side. Notable structural elements and functional sites are shown: head, body, platform and DC (red). R-proteins are colored dark blue and rRNA molecules as light blue. The figure is based on a cryogenic electron microscopy structure from RCSB PDB, using structure (7k00) and visualized by Pymol software.

1.1.2 Small ribosomal subunit

The small ribosomal subunit is composed of a 16S (1543 nt) rRNA molecule and 21 distinct r-proteins (Melnikov, Ben-Shem et al. 2012, Dunkle and Cate 2013). The structural configuration of the 30S subunit exhibits a remarkable sense of organization, wherein the 16S rRNA domains assume a sequential arrangement, forming distinct and autonomous regions. The three primary structural divisions discerned within the SSU are the body, platform and head (Figure 2B).

Regarding the translation apparatus, akin to the PTC reigning as a paramount constituent within the 50S subunit, the DC assumes an equivalent significance for the 30S subunit (Figure 2B). Operating within the DC, tRNA molecules, harboring specific amino acids covalently linked at their 3' ends, feature a 3-nucleotide long anticodon that complements a corresponding mRNA codon. The DC pivotal role lies in scrutinizing and verifying the accuracy of codon-anticodon interactions, thereby guaranteeing the complementarity of the nascent peptide chain to the mRNA sequence.

In a manner reminiscent of the 50S subunit NPET, the 30S subunit exhibits a comparable structural feature identified as the mRNA tunnel. Serving as the binding site for mRNA during translation initiation, this tunnel assumes a crucial role. As the polypeptide chain journeys through the PET during the translation process, the mRNA must similarly traverse the tunnel, during the process termed “translocation”. Consequentially, translocation within the 30S subunit culminates in the precise positioning of the subsequent amino acid-coding codon within the ribosome A site, perpetuating the ongoing translation cycle.

1.2 Ribosome synthesis and degradation

One of the most significant factors affecting translation activity in bacteria is ribosome concentration. While ribosome biogenesis plays a crucial role in increasing the ribosome count during the exponential growth phase, it is equally important to reduce the number of ribosomes through ribosome degradation during stationary phase or stress. In this study, ribosome degradation takes precedence over ribosome biogenesis, hence the latter is briefly summarized, while the former is explored in greater detail.

The assembly and maturation of ribosomes encompass a complex mechanism involving a series of events, including RNA processing and modification, ordered binding of ribosomal proteins (r-proteins) and sequential conformational changes in secondary and tertiary structures (Kaczanowska and Rydén-Aulin 2007). The completion of ribosome assembly is estimated to take approximately 2 minutes at 37 °C *in vivo* (Lindahl 1975). This process utilizes approximately 40% of the cell's energy production and relies on the cooperation of numerous assembly and maturation factors (Kaczanowska and Rydén-Aulin 2007, Bremer and Dennis 2008).

The assembly of ribosome subunits commences with the transcription of a long precursor RNA containing all three major RNA molecules: 5S, 16S and 23S rRNA in a single transcript (Kaczanowska and Rydén-Aulin 2007). Subsequently, these RNA molecules are cleaved from the extended transcript. While 23S and 5S rRNA molecules continue along the large subunit assembly pathway, the 16S rRNA follows the small subunit assembly pathway. Both rRNA and r-proteins undergo modifications during the assembly process (Kaczanowska and Rydén-Aulin 2007). Some modifications occur during the early stages of biogenesis, such as rRNA modifications catalyzed by RlmA and RlmM (Blanchard and Puglisi 2001, Siibak and Remme 2010), while others (catalyzed by RlmH) take place during the first translation cycle in final maturation steps (Ero, Peil et al. 2008, Siibak and Remme 2010). *In vitro* analysis of r-protein binding during subunit reconstitution, along with subsequent experiments conducted under *in vivo* conditions, has demonstrated that r-protein binding to rRNA during assembly is not random but rather follows an ordered pattern. Both subunits possess their distinct r-protein assembly maps, categorizing r-proteins into early, intermediate and late binders (Held, Ballou et al. 1974, Herold and Nierhaus 1987, Kaczanowska and Rydén-Aulin 2007). In vivo studies have shown that 30S subunit assembly does not follow a single pathway, but multiple parallel pathways that converge on the fully assembled complex (Earnest, Lai et al. 2015, Davis and Williamson 2017).

The production of new ribosomes is subject to fluctuations and is not constant. The cellular ribosome pool faces both theoretical and practical limitations in terms of size (Hui, Silverman et al. 2015, Dai, Zhu et al. 2016). Consequently, ribosome biogenesis necessitates tight regulation and a balancing mechanism – ribosome degradation. As ribosomes consist of multiple RNA and protein molecules, it is appropriate to refer to the process as the disassembly of ribosomes and

the degradation of ribosome constituents. Ribosome degradation has been demonstrated to occur in response to nutrient deprivation, such as nitrogen (Ben-Hamida and Schlessinger 1966), carbon (Jacobson and Gillespie 1968) and phosphate (Maruyama and Mizuno 1970) starvation.

The two most common reasons for ribosome degradation are the quality control of ribosome biogenesis intermediates and adverse environmental conditions leading to slowed growth and protein synthesis. The first mechanism is essential for eliminating misassembled ribosomes resulting from errors in biogenesis. These incorrectly assembled ribosomes have the potential to disrupt the function of their normal counterparts, making it imperative to have a mechanism for their removal. Two known enzymes, RNase R and PNPase (Polyribonucleotide nucleotidyltransferase), have been shown to influence the accumulation of rRNA fragments in cells (Cheng and Deutscher 2003). Deletion of both proteins leads to cell death, but single deletion strains remain viable. Cells are known to adjust their ribosome content in response to the growth rate (Bremer and Dennis 2008), thus highlighting the second mechanism: ribosome degradation in response to nutrient deprivation and the onset of the stationary phase. Interestingly, this mechanism is likely to utilize the same enzymes – PNPase and RNase R – to degrade ribosomes in response to changing growth conditions. The use of the same proteins in both pathways suggests that these proteins are either nonspecific or that both pathways share a similar approach to ribosome degradation.

Ribosome degradation has been extensively investigated, with a particular focus on one of its principal constituents – rRNA. Correct processing of rRNA precursors and the removal of harmful rRNA fragments during ribosome assembly necessitate the presence of RNase R and RNA chaperone Hfq (Dos Santos, Andrade et al. 2020). Additionally, the quality control of defective rRNAs is mediated by RNase R and polynucleotide phosphorylase (Cheng and Deutscher 2003). Understanding the stability and activity of ribosome degradation throughout the cell's life cycle becomes particularly important as this process is typically initiated under growth-limiting conditions/during the onset of the stationary phase.

Notably, RNase R exhibits distinct stability characteristics during different phases of cell culture growth. It is highly unstable during the exponential phase but becomes stable during the stationary phase. This can be attributed to the acetylation of RNase R by Pka (peptidyl-lysine N-acetyltransferase), a protein exclusively present during the exponential phase (Liang, Malhotra et al. 2011). Consequently, during the stationary phase, Pka levels are insufficient, resulting in unacetylated RNase R (Liang, Malhotra et al. 2011). RNase R is proficient in degrading both single- and double-stranded RNA, albeit requiring at least a 10 nt long 3' overhang for optimal binding and catalysis (Vincent and Deutscher 2006). Additionally, RNase R possesses intrinsic RNA helicase activity, crucial for efficient nuclease action against double-stranded substrates (Hossain, Malhotra et al. 2015). While RNase R plays a prominent role in ribosome degradation, it is important to acknowledge that this enzyme is but one component of the broader system required to dismantle the intricate and modular ribosomes.

1.3 Ribosomal proteins

R-proteins constitute up to 23% of the total proteome in *Escherichia coli* (Bremer and Dennis 2008). Their sizes range from 4.4 kDa (bL36A) to 61.1 kDa (bS1). The large subunit comprises 33 unique r-proteins, while the small subunit consists of 21 different r-proteins. Notably, most r-proteins exhibit a positively charged surface towards RNA and a negatively charged surface facing outwards from the ribosome (Klein, Moore et al. 2004). Furthermore, a majority of r-proteins possess two domains: a globular domain on the surface of the ribosome and a cooperatively structured extension between rRNA within the ribosome. The amino acid composition of r-proteins also reflects their role in stabilizing rRNA secondary and tertiary structural elements. The extensions are enriched in arginine (15.9%), glycine (13.7%) and lysine (12.7%), with minimal acidic residue content (7%) (Wilson and Nierhaus 2005). On the other hand, the most abundant amino acid residues in the globular protein domains are alanine (9.8%), aspartate (8.6%), glutamate (9.9%) and valine (9.5%), while arginine and lysine together constitute up to 12% of the total (Wilson and Nierhaus 2005). From the perspective of ribosome biosynthesis, the r-proteins are believed to stabilize rRNA conformations both in mature ribosomes and biosynthesis intermediates. Moreover, r-proteins can be categorized based on their binding order during ribosome assembly. *In vitro* reconstitution of ribosome subunits has demonstrated a certain order in which r-proteins bind to the rRNA (Held, Ballou et al. 1974, Herold and Nierhaus 1987, Kaczanowska and Rydén-Aulin 2007). Although this is not a direct observation under *in vivo* conditions, it aligns well with research conducted on ribosome biogenesis under *in vivo* conditions (Earnest, Lai et al. 2015, Davis and Williamson 2017). The r-protein binding order of the large and small subunits also reflects the overall complexity of their structures, with the small subunit exhibiting clearly defined and ordered segments, while the binding order of large subunit appears more cooperative and somewhat chaotic compared to the small subunit. In the assembly of the large subunit, r-proteins uL4, uL13, bL20, uL22 and uL24 are among the initial binders to rRNA (Kaczanowska and Rydén-Aulin 2007). While uL24 is a non-essential r-protein, uL4 is essential for cell culture growth (Shoji, Dambacher et al. 2011). *In vitro* reconstitution of 50S ribosomes has revealed that uL24 is crucial for ribosome assembly, but not for ribosome translation activity (Spillmann and Nierhaus 1978). On the other hand, proteins L5, L18 and L25 facilitate the primary interactions between the 5S and 23S rRNA, playing a key role in the proper formation of the CP (Korepanov, Gongadze et al. 2007).

Based on the assembly map, the r-proteins of the small subunit can be categorized into three groups known as primary, secondary and tertiary binding proteins (Held, Ballou et al. 1974). Primary proteins (uS4, uS7, uS8, uS15, uS17, bS20) bind directly to the rRNA, initiating the nucleation of 30S domains (Brodersen, Clemons et al. 2002). Secondary proteins (uS5, bS6, uS9, uS11, uS12, uS13, bS16, bS18, uS19) require the interaction of rRNA with primary proteins, while tertiary proteins (uS2, uS3, uS10, uS14, bS21) need at least one

primary and one secondary protein for correct association. These findings align well with *in vivo* studies (Chen and Williamson 2013).

The PTC can be regarded as one of the most crucial sites in living organisms. Although its structure remains conserved across all known domains of life and is primarily composed of rRNA, the conformation of rRNA is significantly influenced by r-proteins. Consequently, the modern-day ribosome PTC is postulated to be cooperatively formed from both RNA and r-proteins. As the PTC takes its final conformation during the later stages of LSU biogenesis, it becomes challenging to determine the direct impact of different r-proteins on its formation. However, considering the r-proteins close to the PTC in the mature ribosome structure, uL27 stands out as one of the closest. The C-terminal end of uL27 can interact with P-site tRNA and based on positioning and experimental data, uL27 is likely involved in the correct placement of the P-site tRNA (Maguire et al. 2003).

Another r-protein in close proximity to the PTC is uL16. Arginine 81 of uL16 interacts with the 3' end of the P-site tRNA and is also subject to modification by RoxA (hydroxylation). Although the modification is non-essential, its absence leads to a 3 to 4 times lower global translation rate under low nutrient conditions (Ge, Wolf et al. 2012). Ribosomes reconstituted without uL16 exhibit peptidyl transferase activity, but the reaction time is significantly increased (Franceschi and Nierhaus 1990). Given that the PTC forms during late assembly stages, it is unsurprising that uL16 is also one of the late assembly proteins, playing a critical role in the proper placement of tRNA in the A and/or P-site of the ribosome.

The newly synthesized polypeptide chain initiates its journey from the PTC and eventually emerges from the ribosome, passing through the NPET within the LSU. The NPET surface is lined with specific ribosomal proteins that interact with the growing polypeptide chain. Notably, uL4, uL22, uL23, uL24 and uL29 are involved in these interactions. uL4 and uL22 are located on the NPET surface in one of its narrower regions and mutations in these proteins can confer resistance to macrolide antibiotics that bind to NPET. Additionally, uL23 and uL29 play a crucial role in binding to the sec61 complex, which facilitates the transport of proteins outside the cell or, in the case of *E. coli*, into the periplasmic space (Ferbitz, Maier et al. 2004).

mRNA carries a nucleotide sequence that is "translated" into an amino acid sequence during polypeptide synthesis. This decoding process requires precision to avoid the synthesis of faulty proteins; however, accuracy is balanced with time, as a longer time taken to bind tRNA to the A-site of the ribosome results in a slower elongation rate. For example, the antibiotic streptomycin induces errors in decoding, leading to the production of faulty proteins and overloading the cell with them (Piepersberg, Noseda et al. 1979). Mutations that confer resistance to streptomycin are associated with increased accuracy and a slower elongation rate. One well-known mechanism of streptomycin resistance involves mutations in the SSU r-protein uS12. Interestingly, mutations in uS12 can lead to dependence on streptomycin, as ribosomes become excessively precise, resulting in a decreased elongation rate (Piepersberg, Noseda et al. 1979, Wilson and Nierhaus 2005). *In vitro* reconstitution of ribosomes without uS12 also exhibits increased accuracy,

suggesting that uS12 may enhance translation rate at the expense of accuracy (Piepersberg, Noseda et al. 1979, Wilson and Nierhaus 2005).

The studies of hyperaccuracy in mutation variants of uS12 have led to the discovery of revertant mutations known as "ram" (ribosome ambiguity mutations) mutations. The ram phenotype involves mutations in the r-proteins uS4 and uS5 (Deusser, Stöffler et al. 1970, Cabezón, Herzog et al. 1976). These mutations disrupt the interactions between uS4 and uS5 near the DC. As a result, when the EF-Tu::tRNA complex interacts with the ribosome A-site for codon-anticodon matching, the mutated uS4 and uS5 allow "locking" of tRNA into the A-site even in near-cognate matches, thereby decreasing the overall accuracy of the translation. Also, mutations in uS4 are associated with ribosome assembly defects (Mayerle and Woodson 2013).

In *E. coli*, bS1 is the largest r-protein that is not universally conserved but is found in all gram-negative and some gram-positive bacteria (Karlin, Theriot et al. 2004). bS1 is positioned between the platform, head and body of the SSU and is surrounded by r-proteins uS2, bS6, uS9, uS11 and bS18. Although bS1 is not an essential r-protein for cell culture growth in *E. coli*, it plays a vital role during the initiation of translation. bS1 assists in the recruitment of mRNA to the SSU and ensures the correct placement of mRNA into the DC. mRNAs with a Shine-Dalgarno sequence are dependent on bS1 to bind to the SSU, while leaderless mRNA does not require bS1 (Moll, Grill et al. 2002).

bL7/bL12 and uL10 are integral components of the L11 stalk located within the 50S subunit. The bL7/bL12 complex forms two dimers, each consisting of one bL7 and bL12 and interacts with uL10. This interaction is responsible for binding GTP-activated factors to the ribosome, with specific factors like EF-Tu, EF-G and IF-2 shown to bind to the bL7/bL12 tetramer in *E. coli* (Brot and Weissbach 1981, Wahl and Möller 2002, Christodoulou, Larsson et al. 2004, Mulder, Bouakaz et al. 2004). Interestingly, the binding of bL7/bL12 to GTP-activated factors is unique, as hybrid *E. coli* ribosomes containing eukaryotic counterparts of bL7 and bL12 required eukaryote-specific factors EF-2 and EF-1α for translation activity (Uchiumi, Honma et al. 2002). This means that bL7/bL12 binding to GTP-activated factors is specific and also important for factor recognition and GTPase activity stimulation.

In *E. coli*, there are two r-proteins, bL31 and bL36, each having two paralogs (Makarova, Ponomarev et al. 2001). Both proteins are bacterial-specific and non-essential (Baba, Ara et al. 2006). The paralogs of bL31 (bL31A and bL31B) are encoded by *rpmE* and *ykgM* genes, respectively, while the paralogs of bL36 (bL36A and bL36B) are encoded by *rpmJ* and *ykgO* genes (Dabbs 1981, Wada and Sako 1987, Blattner, Plunkett et al. 1997, Hensley, Gunasekera et al. 2012). Interestingly, *ykgM* (bL31B) and *ykgO* (bL36B) genes are encoded by the same ykgMO operon (Makarova, Ponomarev et al. 2001), while *rpmE* (bL31A) and *rpmJ* (bL36A) genes are located in different operons (Aseev and Boni 2011). bL31A is situated in the CP of the large subunit, with its N-terminus interacting with the r-protein uL5 and its C-terminus forming intersubunit contacts with r-proteins uS13 and uS19 (Jenner, Demeshkina et al. 2010). On the other hand,

bL36A is located near the L7/L12 stalk and binds to 23S rRNA (Schuwirth, Borovinskaya et al. 2005). As is common among r-protein paralogs, bL31A and bL36A contain a zinc-binding motif, while their B paralogs do not, resulting in differential zinc binding (Härd, Rak et al. 2000, Makarova, Ponomarev et al. 2001, Hensley, Gunasekera et al. 2012).

These specific examples underscore the importance of r-proteins in ribosome assembly, translation initiation efficiency, decoding accuracy and translation efficiency. However, considering the cooperative nature of the ribosome, all r-proteins play an important role in various aspects of ribosome functionality.

1.4 R-protein synthesis

Since ribosomal proteins (r-proteins) constitute a significant proportion (approximately 23% in *E. coli*) of the proteome and play consequential roles in ribosome assembly, their synthesis must be tightly regulated. In *E. coli*, each r-protein is encoded by one gene per genome, organized into operons of varying sizes (see Table 1) (Nomura, Gourse et al. 1984). In contrast, there are seven copies of rRNA genes per genome (Blattner, Plunkett et al. 1997). Ribosome biosynthesis requires a stoichiometric balance among its components (Chen, Sperling et al. 2012). Thus, the regulation of r-protein expression and rRNA synthesis must be coordinated. The rRNA genes possess one of the strongest promoter regions known in *E. coli* (Bremer and Dennis 2008). However, r-protein mRNAs can be simultaneously translated by multiple ribosomes, resulting in a higher number of protein copies per transcribed mRNA. During the mid-log phase, most r-proteins are rapidly bound to ribosomes during assembly, implying that they do not exist in large free pools (Chen, Sperling et al. 2012). It is important to note that this holds true only when different operons are transcribed and translated in equal proportions. R-proteins are known to autoregulate their own operons and polycistronic mRNA at both the transcriptional and translational levels (Lindahl 1975, Nomura, Gourse et al. 1984). However, prevailing theories suggest that as cells transition into the stationary phase, ribosome assembly slows down, leading to larger free pools of r-proteins, which then become more influential in regulating their own expression. It is also pertinent to mention that not all operons are regulated via r-protein-mediated mechanisms. For example, the ykgMO operon (transcribing bL31B and bL36B proteins) is under ZUR (zinc uptake regulator) repressor regulation (Shin and Helmann 2016).

R-proteins bS1, uS2, uS4, uS7, uS8, uS15, bS20, uL1, uL4, uL10, bL12 and bL20 are involved in the translational regulation of their respective operons or polycistronic mRNA (Nomura, Gourse et al. 1984). Now, let’s view a couple of different operons and their regulation mechanisms in detail.

Table 1 – R-protein operons

Operon	Genes in the operon (Gene) Protein	Operon	Genes in the operon (Gene) Protein
rpoBC	(*rplK*) uL11 (*rplA*) **uL1** (*rplJ*) uL10 (*rplL*) bL7/bL12 (*rpoB*) RNA polymerase subunit beta (*rpoC*) RNA polymerase subunit beta'	L32	(*rpmF*) bL32 (*plsX*) Phosphate acyltransferase (*fabH*) Beta-ketoacyl-ACP synthase III (*fabD*) MCT (*fabG*) Beta-ketoacyl-ACP reductase
Str	(*rpsL*) uS12 (*rpsG*) **uS7** (*fusA*) EF-G, elongation factor G (*tufA*) EF-Tu, elongation factor Tu	Alpha	(*rpsM*) uS13 (*rpsK*) uS11 (*rpsD*) **uS4** (*rpoA*) RNA polymerase subunit alpha
S10	(*rpsJ*) uS10 (*rplC*) uL3 (*rplD*) **uL4** (*rplW*) uL23 (*rplB*) uL2 (*rpsS*) uS19 (*rplY*) uL22 (*rpsC*) uS3 (*rplP*) uL16 (*rpmC*) uL29 (*rpsQ*) uS17	Spc	(*rplN*) uL14 (*rplX*) uL24 (*rplE*) uL5 (*rpsN*) uS14 (*rpsH*) **uS8** (*rplF*) uL6 (*rplR*) uL18 (*rpsE*) uS5 (*rpmD*) uL30 (*rplO*) uL15 (*rpmJ*) bL36A
S1	(*cmk*) Cytidylate kinase (*rpsA*) **bS1**	S2	(*rpsB*) **uS2** (*tsf*) EF-Ts, elongation factor Ts
S6	(*rpsF*) bS6 (*rpsR*) bS18 (*rplI*) bL9	MMS	(*rpsU*) bS21 (*dnaG*) DNA primase (*rpoD*) RNA polymerase sigma factor
L20	(*rpmI*) bL35 (*rplT*) **bL20**	S15	(*rpsO*) **uS15** (*pnp*) Polyribonucleotide nucleotidyltransferase
L13/S9	(*rplM*) uL13 (*rpsI*) uS9	L21/L27	(*rplU*) bL21 (*rpmA*) bL27
L28/L33	(*rpmB*) bL28 (*rpmG*) bL33	ykgMO	(*ykgM*) bL31B (*ykgO*) bL36B
S20	(*rpsT*) **bS20**	L31	(*rpmE*) bL31A
L25	(*rplY*) bL25	L34	(*rpmH*) bL34
		trmD	(*rpsP*) bS16 (*rimM*) ribosome maturation factor RimM (*trmD*) tRNA (guanine-N(1)-)-methyl-transferase (*rplS*) bL19

Highlighted proteins take part in transcriptional and/or translational regulation of their own expression.

The S10 operon in *E. coli* contains 11 r-protein genes and its transcription and translation are controlled by the r-protein uL4 (Zengel, Mueckl et al. 1980, Lindahl, Archer et al. 1983, Freedman, Zengel et al. 1987). The C-terminal part of uL4 is necessary for binding its mRNA and the central domain is required for embedding in the ribosome (Li, Lindahl et al. 1996).

R-protein uL4 binds to the 5'-untranslated region (5'-UTR) of the first gene (*rpsJ*, uS10) in the operon. Interestingly, the mRNA regions responsible for transcription termination and translation inhibition partially overlap but are not identical (Freedman, Zengel et al. 1987, Zengel and Lindahl 1990). The regulation of transcription of the S10 operon involves interactions between mRNA, uL4, the NusA factor and RNAP (Zengel and Lindahl 1990, Zengel and Lindahl 1991). However, the details of transcriptional and translational regulation of r-protein operons may vary in different organisms (Mikhaylina, Kostareva et al. 2014). For instance, the S10-like operon in the archaea *M. jannaschii* encodes not 11, but 5 r-proteins (uL3, uL4, uL23, uL2 and uS19) (Mikhaylina, Kostareva et al. 2014). Moreover, the first gene in this operon is *rplC* (uL3) instead of *rpsJ* (uS10) and the uL4-binding site includes the 5'-UTR and the beginning of the coding part of the uL3 mRNA (Williams 2008).

The gene *rpsA*, which encodes r-protein bS1, is part of an operon that is regulated at the translation level by bS1 itself (Boni, Artamonova et al. 2000, Boni, Artamonova et al. 2001). R-protein bS1 is known to be necessary for the translation of many mRNAs, including its own (Sørensen, Fricke et al. 1998, Boni, Artamonova et al. 2001). The S1 operon exhibits one of the strongest effective negative autogenic controls in *E. coli* (Boni, Artamonova et al. 2000). The regulation of bS1 synthesis occurs competitively with the formation of the 30S preinitiation complex. *In vitro*, 30S ribosomal subunits lacking bS1 are unable to form a preinitiation complex with bS1 mRNA. When bS1 is added to 30S subunits without bS1 in a 1:1 molar ratio, it restores the ability of the 30S subunit to bind *rpsA* mRNA, while an excess of bS1 inhibits this binding. The S1 protein interacts with single-stranded regions of *rpsA* mRNA, leading to changes in the mRNA structure and preventing the formation of a preinitiation complex with the 30S subunit (Boni, Artamonova et al. 2001).

The S2 operon of bacteria encodes r-protein uS2 and the elongation factor Ts. In *E. coli* cells this operon is regulated at the translation level by the uS2 (Aseev, Levandovskaya et al. 2008). The regulatory site that the uS2 protein interacts with is located in the 5'-UTR of *rpsB* mRNA. Interestingly, the uS2 protein regulates *rpsB–lacZ* expression more effectively in the presence of the S1 protein (Aseev, Levandovskaya et al. 2008). The activity of the S2 operon promoter decreases with amino acid starvation *in vivo* or with an increase in the concentration of the (p)ppGpp alarmone *in vitro* (Aseev, Koledinskaya et al. 2014). Thus, on the translational level, the synthesis of uS2 and EF-Ts is regulated by uS2, while on the transcriptional level, it is regulated by the alarmone (p)ppGpp. The role of the alarmone (p)ppGpp in ribosome synthesis and other cellular processes is further elucidated in the next chapter.

1.5 Onset of the stationary phase in *Escherichia coli*

1.5.1 Stringent response

Stringent response was first discovered in *E. coli* via amino acid starvation (Cashel 1969). However, the stringent response is also triggered in response to antibiotics, iron limitation, immature tRNAs and fatty acid limitation (Xiao, Kalman et al. 1991, Vinella, Albrecht et al. 2005, Battesti and Bouveret 2006, Abranches, Martinez et al. 2009). The main carrier of this response is an alarmone (p)ppGpp, which is synthesized in *E. coli* by RelA and SpoT (Wendrich, Blaha et al. 2002, Irving, Choudhury et al. 2021). During amino acid deprivation, RelA binds to stalled ribosomes and catalyzes the synthesis of (p)ppGpp (Wendrich, Blaha et al. 2002). (p)ppGpp-mediated stringent response has been shown to affect cellular systems on a global scale. From the viewpoint of ribosomes, the stringent response is a strong suppressor of both ribosome biogenesis via inhibition of transcription of ribosomal components and translation via binding to GTP activated factors necessary for translation.

Rel and RelA (p)ppGpp synthesis is regulated via binding to stalled ribosomes (Wendrich, Blaha et al. 2002). Upon ribosome binding, RelA transitions into open conformation that favors (p)ppGpp synthesis (Arenz, Abdelshahid et al. 2016), however, when not bound to the ribosome, RelA and Rel adopt a closed conformation that favors (p)ppGpp hydrolysis (Gratani, Horvatek et al. 2018, Irving, Choudhury et al. 2021). Uncharged tRNA is not required for this initial RelA or Rel binding event, but it does stabilize the interaction and promotes synthesis (Kudrin, Dzhygyr et al. 2018, Takada, Roghanian et al. 2021). (p)ppGpp can also allosterically bind to the N-terminal domain of RelA and Rel and positively influence its own synthesis (Shyp, Tankov et al. 2012). This means that Rel and RelA have the strongest effect during amino acid starvation.

(p)ppGpp accumulation during the stringent response leads to changes in the transcriptional profile. Repression of rRNA synthesis and genes involved in the metabolism of DNA and phospholipids, alongside a concurrent increase in expression of amino acid biosynthesis enzymes to overcome nutrient limitations are some of the more common expressions during stringent response. These transcriptional changes are noteworthy because they lead to the slow-growing phenotype distinctive to this response. In *E. coli*, (p)ppGpp binds directly to RNAP to alter the transcription of genes both positively and negatively, with (p)ppGpp being a more potent effector nucleotide than (p)ppGpp (Mechold, Potrykus et al. 2013). There are two (p)ppGpp binding sites on RNAP: site 1 is found at the interface between the ω and β′ subunits (Ross, Vrentas et al. 2013) and site 2 is located at the interface between the β′-subunit and the transcription factor DksA (Ross, Sanchez-Vazquez et al. 2016). In *E. coli*, when (p)ppGpp binds to site 1, transcription is inhibited approximately twofold, whereas when both sites are bound, together with DksA, there is 20-fold inhibition (Paul, Berkmen et al. 2005, Ross, Sanchez-Vazquez et al. 2016).

A recent study has revealed the extent to which transcription is modulated by activation of the stringent response in *E. coli*: expression of RelA from a plasmid, altered expression of 757 genes after 5 minutes and 1,224 genes after 10 minutes (Sanchez-Vazquez, Dewey et al. 2019). In the *E. coli* strain expressing a mutant RNAP that cannot bind to (p)ppGpp, there were a few changes in transcription following (p)ppGpp accumulation (Sanchez-Vazquez, Dewey et al. 2019). This means that in *E. coli*, some genes are regulated by (p)ppGpp in an RNAP-independent manner. Comparing the transcriptional profiles through the accumulation of (p)ppGpp by recombinant expression of RelA (Sanchez-Vazquez, Dewey et al. 2019) versus nutritional limitation (Durfee, Hansen et al. 2008), showed large differences in them. 75% of the genes that are differentially expressed through a recombinant expression of RelA are different from those seen on stringent induction by serine hydroxamate (Sanchez-Vazquez, Dewey et al. 2019).

One of the strongest inhibitors of both rRNA and r-protein expression is alarmone (p)ppGpp coordinated stringent response. The inhibitory action of (p)ppGpp on several key enzymes on stringent response induction, is a generally accepted form of translation inhibition in mature ribosomes. However, translation of a subset of proteins is still required even during the stringent response. For instance, the initiation factor IF2 is inhibited by (p)ppGpp, preventing the formation of the 30S initiation complex (30S IC) and thus reducing translation (Legault, Jeantet et al. 1972, Vinogradova, Zegarra et al. 2020). Recent work indicates that 30S-bound IF2 has different tolerances for (p)ppGpp depending on the mRNA present in the 30S pre-IC (Vinogradova, Zegarra et al. 2020). Two consecutive hairpins known as a structured enhancer of translation initiation (SETI) next to the translation initiation region mediate (p)ppGpp tolerance for *tufA* (encoding elongation factor EF-Tu) and *rnr* (encoding RNase R) mRNA in *E. coli* (Vinogradova, Zegarra et al. 2020). Alarmone (p)ppGpp regulates translation differently when bound to IF2, permitting 30S IC formation in *E. coli*, but this requires higher concentrations of IF2 for the 30S IC to form (Vinogradova, Zegarra et al. 2020). In addition to IF2, (p)ppGpp also regulates translation through the inhibition of the elongation factors EF-Tu and EF-G. EF-Tu and EF-G are required for charged tRNA delivery and translocation of the peptide chain during translation (Rojas, Ehrenberg et al. 1984, Mitkevich, Ermakov et al. 2010, Zhang, Zborníková et al. 2018). Finally, release factor RF3, which is required for recycling RF1 and RF2 from the ribosome, is inhibited by (p)ppGpp (Zhang, Zborníková et al. 2018), whereas the inhibition of EF-G by (p)ppGpp could also affect recycling of the post-termination complex. Alarmone (p)ppGpp has been shown to inhibit the formation of mature 50S and 30S by inhibiting small GTPases involved in ribosome maturation (Feng, Mandava et al. 2014, Corrigan, Bellows et al. 2016). (p)ppGpp binds to the GTPases RsgA, RbgA, Era and Obg in several species (Persky, Ferullo et al. 2009, Corrigan, Bellows et al. 2016, Zhang, Zborníková et al. 2018, Yang, Anderson et al. 2021) and inhibits their GTPase activities (Persky, Ferullo et al. 2009, Corrigan, Bellows et al. 2016) to reduce the 70S ribosome pool.

In addition, (p)ppGpp can promote the hibernation of ribosomes. (p)ppGpp induces the transcription of *hpf* (encoding hibernation-promoting factor), *rmf*

(encoding ribosome modulation factor) and *raiA* (encoding ribosome-associated inhibitor A), which are important for ribosome inactivation and dimerization in *E. coli* (Izutsu, Wada et al. 2001, Prossliner, Skovbo Winther et al. 2018). In *B. subtilis*, overexpression of the synthetase RelP results in a strain that does not produce (p)ppGpp and leads to the formation of inactive 70S ribosome dimers termed "100S ribosomes" in a manner dependent on the hibernation-promoting factor (Hpf) (Tagami, Nanamiya et al. 2012). Furthermore, in S. aureus the GTPase HflX can dissociate 100S ribosomes in a GTPase-dependent manner, however, HflX activity is inhibited by (p)ppGpp (Basu and Yap 2017). Thus, (p)ppGpp can halt protein production at several key stages, ensuring suppressed translation under stress.

1.5.2 Ribosome hibernation

Ribosome hibernation-promoting factors are highly conserved in bacteria (Ueta, Ohniwa et al. 2008). In *E. coli*, the occurrence of hibernating 100S and 70S ribosomes has been associated with three factors: ribosome modulation factor (Rmf), hibernation-promoting factor (Hpf) and ribosome-associated inhibitor A (RaiA). Rmf is a small, basic protein of 55 amino acids, that is essential for 100S formation (Wada, Yamazaki et al. 1990). The protein binds exclusively with 100S dimers and is necessary for the dimerization mechanism – deletion of *rmf* gene results in abolishment of 100S formation (Yamagishi, Matsushima et al. 1993, Wada, Mikkola et al. 2000), while *in vitro*, Rmf can dimerize 70S ribosomes to a 90S dimer complex (Ueta, Yoshida et al. 2005, Ueta, Ohniwa et al. 2008). Next up is Hpf which is capable of converting intermediate 90S dimers into stable 100S dimers (Wada, Igarashi et al. 1995, Maki, Yoshida et al. 2000). Hpf alone is not capable of dimerizing ribosomes on its own *in vitro* (Ueta, Ohniwa et al. 2008), however, *E. coli hpf* mutant is deficient in forming 100S dimers *in vivo* (Ueta, Yoshida et al. 2005). One of the reasons might be RaiA, which has been shown to stabilize 70S ribosomes in an inactive state (Agafonov, Kolb et al. 1999, Vila-Sanjurjo, Schuwirth et al. 2004). Also, the fact that RaiA and Hpf share a binding site on the ribosome (Ueta, Yoshida et al. 2005, Prossliner, Skovbo Winther et al. 2018) implies that in *E. coli,* there are two distinct competitive populations of hibernating ribosomes: Rmf-Hpf-100S dimers and RaiA-70S monomers (Maki, Yoshida et al. 2000, Ueta, Yoshida et al. 2005).

The expression of hibernation factors is regulated by various mechanisms. In mid-log phase *E. coli* cells, *rmf* mRNA becomes detectable upon transition into the stationary phase alongside the appearance of 100S ribosomes (Yamagishi, Matsushima et al. 1993, Shimada, Makinoshima et al. 2004, Aiso, Yoshida et al. 2005). Transcription of *rmf* is induced by a variety of stresses: amino acid starvation, heat and cold shock, ethanol and ethidium bromide treatment, changes in pH, osmotic stress and envelope stress (Yamagishi, Matsushima et al. 1993, Garay-Arroyo, Colmenero-Flores et al. 2000, Izutsu, Wada et al. 2001, el-Sharoud and Niven 2005, Moen, Janbu et al. 2009, Raivio, Leblanc et al. 2013). The *rmf* expression level in stationary phase cultures of *E. coli* is not maintained but

downregulated during prolonged stationary phase in LB medium (Arunasri, Adil et al. 2014). The main mediators of *rmf* transcription are (p)ppGpp and cAMP (Izutsu, Wada et al. 2001). Differential sigma factor 54 may also contribute to transcriptional regulation of *rmf* expression (Bonocora, Smith et al. 2015). Similarly to the *rmf* transcript, the Rmf protein is also detected preferentially during the stationary phase (Wada, Yamazaki et al. 1990). The disappearance of the Rmf protein precedes the loss of *rmf* mRNA, which implies the existence of a mechanism for Rmf degradation (Aiso, Yoshida et al. 2005). Like *rmf*, *hpf* and *raiA* are induced by (p)ppGpp (Durfee, Hansen et al. 2008, Traxler, Summers et al. 2008).

The expression of Hpf is strongly correlated with the expression of Rmf through the formation of 100S dimers. Hpf is scarcely detected during the mid-log phase, becomes more abundant alongside Rmf in the stationary phase and finally disappears within 30 minutes, as starved cells are transferred to fresh medium (Maki, Yoshida et al. 2000).

Expression of RaiA is induced by cAMP–CRP (Shimada, Yoshida et al. 2013), during the Cpx envelope stress response in *E. coli* (Raivio, Leblanc et al. 2013) and in the production of *P. aeruginosa* biofilms (Williamson, Richards et al. 2012). Intriguingly *raiA* promoter activity is also high during the mid-log phase in *E. coli* (Shimada, Yoshida et al. 2013). In correlation, RaiA protein can be detected in exponentially growing cells and its levels do increase upon transition to stationary phase or during cold shock-induced growth arrest (Agafonov, Kolb et al. 1999, Maki, Yoshida et al. 2000, Agafonov, Kolb et al. 2001).

The formation of hibernating 100S dimers by Rmf+Hpf and 70S ribosomes by RaiA, is confined mostly to gammaproteobacteria (Ueta, Wada et al. 2013, Yoshida and Wada 2014). Other bacteria do not have Rmf and RaiA, however, they have a Hpf homolog that is necessary and sufficient for the formation of 100S ribosome dimers (Ueta, Wada et al. 2013, Puri, Eckhardt et al. 2014, Kline, McKay et al. 2015, Akanuma, Kazo et al. 2016). Long Hpf (lHpf) contains a C-terminal extension and mediates dimerization by direct interaction between the two lHpf molecules in the complex. Interestingly, lHpf–100S dimers have also been detected in exponentially growing cells, albeit at lower levels than in the stationary phase (Ueta, Wada et al. 2010, Ueta, Wada et al. 2013, Puri, Eckhardt et al. 2014, Kline, McKay et al. 2015, Akanuma, Kazo et al. 2016). In contrast to Rmf and short Hpf, lHpf is present not only in the 100S fraction of the ribosome pool but also in the 70S fraction (Ueta, Wada et al. 2010, Ueta, Wada et al. 2013, Kline, McKay et al. 2015, Basu and Yap 2016). This occurrence might be reminiscent of the RaiA-stabilized 70S monosomes in *E. coli*.

2. AIMS OF THE THESIS

The primary objective of this thesis is to illuminate the translation apparatus and its adaptation during a prolonged stationary phase in *E. coli*, with a focus on ribosomal proteins. In bacteria, the ribosome r-protein composition has been shown to undergo changes both *in vivo* and *in vitro* (Robertson, Dowsett et al. 1977, Subramanian and van Duin 1977, Pulk, Liiv et al. 2010). These variations in ribosome r-protein composition have been hypothesized to act as a possible mechanism for repair or fine-tuning of translation regulation via ribosome specialization (Pulk, Liiv et al. 2010). In this work, the goal was to understand how ribosomes and their constituents: r-proteins, change in response to prolonged stationary phase.

To achieve this, we have formulated several key questions concerning ribosomes during the stationary phase:

- How does the ribosome content in cells change during the stationary phase?
- How does the r-protein content of ribosomes and proteome change in response to the stationary phase?
- How does the ribosome-associated protein (RAP) content change during the stationary phase?
- Does the stability of r-proteins remain unified during the stationary phase?

To answer these questions, the following aims were set forth:

- To determine ribosome and its subunit partitions and the ribosome content in cells
- To quantify r-proteins and RAPs in *E. coli* ribosomes and proteome during the early and prolonged stationary phase using quantitative mass-spectrometry
- To assess r-protein turnover during the stationary phase using data obtained from mass-spectrometry

By pursuing these research aims, we aim to advance our understanding of ribosome behavior and adaptation during prolonged stationary phase, shedding light on the role of ribosomal proteins in this process.

3. MATERIALS AND METHODS

3.1 Quantitative mass-spectrometry

Stable isotope-labeled amino acids in cell culture (SILAC) based mass spectrometry was selected for quantifying proteins in isolated ribosomes and whole proteomes. To use SILAC for quantification effectively, a strain with disrupted arginine and lysine *de novo* synthesis was required. In this work, a laboratory strain was used where arginine and lysine metabolism were disrupted by deleting *argA* and *lysA* genes from the genome. The resulting strain *MG1655-SILAC (F-, λ-, rph-1, ΔargA, ΔlysA)* had no detectable differences in growth speed and viability in comparison to the wildtype MG1655 strain.

The experimental setup had two distinct nuances to it from the viewpoint of SILAC. First, the cells were uniformly labeled with "heavy" labeled arginine and lysine and grown to the mid-log phase. Then culture was chased with unlabeled arginine and lysine and grown to the stationary phase. This in itself created two subpopulations of proteins in the cells separated by time: older, before chase synthesized proteins and newer, after the chase synthesized proteins. The second nuance was the integration of internal standard into the mix. In short: The *E. coli* MG1655 SILAC strain proteome was uniformly labeled with "medium-heavy" arginine and lysine and grown to the mid-log phase. Either the isolated ribosomes or the lysate was mixed in a 1:1 ratio with the samples and analyzed with quantitative mass-spectrometry. The addition of internal standard allowed for comparative analysis of samples from different timepoints, while the two subpopulations granted the ability to distinguish protein synthesis, stability and degradation in the samples.

3.2 Cell culture growth

E. coli MG1655-SILAC (F-, λ-, rph-1, ΔlysA, ΔargA) strain was grown in MOPS medium (Neidhardt, Bloch et al. 1974), supplemented with 0.1 mg/ml "heavy" arginine (Arg10 – $[^{13}C]_6H_{14}[^{15}N]_4O_2$) and lysine (Lys8 – $[^{13}C]_6H_{14}[^{15}N]_2O_2$) (SILANTES, Germany). At mid-log ($A_{600} \approx 1$), 2 mg/ml of unlabeled arginine (Arg0) and lysine (Lys0) were added to the culture. Cell culture was divided into 8 separate batches and growth was continued for a maximum of 14 days. Cells were harvested by low-speed centrifugation (4500 g/15 min) after 24 and 48 hours (from here on referred to as day one and day two) of growth and subsequently on days 4, 6, 8, 10, 12 and 14.

As an internal reference, *E. coli* MG1655-SILAC cells were grown in MOPS medium supplemented with 0.1 mg/ml medium-heavy arginine (Arg6 – $[^{13}C]_6H_{14}N_4O_2$) and lysine (Lys4 – $C_6H_{10}[^{2}H]_4N_2O_2$) (SILANTES, Germany). Cells were grown to mid-log ($A_{600} \approx 1$) and harvested by low-speed centrifugation (4500 g/15 min).

3.3 Total RNA analysis

Total RNA was extracted from 2 mg of wet cell mass (from the stationary phase) using hot-phenol extraction. Cells were suspended in 200 µl of buffer A (0.5% SDS and 10 mM EDTA). 200 µl of phenol/H_2O (pH 5.5) was added to the cell suspensions, mixed and incubated at 65 ºC for 30 minutes. Samples were centrifuged (16000 g/10 min) and the water phase was transferred to a new tube. buffer A was added to the remaining phenol mixture in a 1:1 ratio, mixed and centrifuged (16000 g/10 min). The water phase was again moved to a new tube. Then, chloroform was added in a 1:1 ratio, mixed and phases were separated by centrifugation (16000 g/10 min). The water phase was transferred to a new tube and RNA was sedimented by adding 5 volumes of 96% ethanol and 0.3M of sodium acetate (pH 5.5) and incubating at –20 ºC overnight. Precipitation was collected by centrifugation (16000 g/10 min), washed 2 times with 96% ethanol and dried at 37 ºC for 5 min. RNA precipitate was dissolved in water and absorbance at 260 nm (A_{260}) was measured. RNA concentrations (A_{260}) were normalized by dividing with the day one A_{260} value (day n / day one). Total RNA concentration values were analyzed across all time points using the two-way analysis of variance (ANOVA) statistical test in the software GraphPad 7.0. Total RNA concentration values were also fitted into a one-phase decay model in the software GraphPad 7.0. One-phase decay model: Y=(Y0 – Plateau)*exp(-K*X) + Plateau, where X is time, Y starts at Y0 and then decays down to plateau with one phase and K is the rate constant in units that are reciprocal of the X-axis units. For our data, plateau and K-values were restricted to being larger than 0.

To calculate the number of ribosomes per cell value, RNA concentrations (A_{260}) were normalized by dividing with the mid-log phase A_{260} value (day n / mid-log). Log phase generation time and the number of ribosomes per cell from (Bremer and Dennis 2008) were used for the creation of the equation: Y=28371x-12289, where Y is the number of ribosomes per cell and X is the generation time (hours). Using the generation time of the *E. coli MG1655-SILAC* strain used in this work (average 35 minutes), the NRC in the mid-log phase was calculated. Multiplying the day n/mid-log value in the stationary phase with NRC in the mid-log phase allowed the estimation of NRC in stationary phase cells.

3.4 Sucrose gradient centrifugation

Cell pellets were suspended in lysis buffer [20 mM Tris (pH 7.5), 100 mM NH_4Cl, 10 mM Mg-acetate and 6 mM β-mercaptoethanol]. After the addition of DNase I (40 units/ml), the cells were disrupted with glass beads using Precellys 24 homogenizer (6000 rpm, 4 °C, 3×1 min, pause 1 min). Lysate was cleared of cell debris by centrifugation (16000 g, 20 min at 4 °C). A maximum of 100 A_{260} units (for precise amounts look Ref. II, table S8) of supernatant was loaded onto a 15–25% sucrose gradient in OV-10 buffer [20 mM Tris (pH 7.5), 100 mM NH_4Cl, 0.25 mM EDTA (ethylenediaminetetraacetic acid) and 6 mM β-mercaptoethanol]

supplemented with 10 mM Mg-acetate and centrifuged at 56000 g for 16 h in a Beckman SW-28 rotor. Ribosome profiles were recorded at 260 nm. Areas under the 70S, 50S and 30S peaks were quantified by ImageJ and corresponding ratios were calculated. Subunit ratios were analyzed across all time points using the two-way analysis of variance (ANOVA) statistical test in the software GraphPad 7.0. Fractions containing 70S were collected for further analysis via liquid-chromatography mass spectrometry (LC-MS/MS).

3.5 Ribosome r-protein content analysis

70S ribosomes from stationary phase and reference cells were mixed in a 1:1 molar ratio and precipitated with 10% trichloroacetic acid (TCA) overnight at 4 ºC. Precipitated proteins were pelleted by centrifugation (16000 g for 60 min) at 4 ºC, washed twice with 80% ice-cold acetone and air-dried at 37 ºC for 5 minutes. All subsequent sample preparations were conducted at room temperature. Proteins were dissolved in 50 µL of 8M urea/2M thiourea solution, reduced for 1 h at 56 ºC by adding 1 mM dithiothreitol (DTT) and carbamidomethylated with 5 mM chloroacetamide for 1 h in the dark. Proteins were digested with endoproteinase Lys-C (Wako) at a 1:50 enzyme-to-protein ratio for 4 h. Urea concentration in the solution was reduced by adding 4 vol of 100 mM ammonium bicarbonate (ABC) and peptides were further digested using mass spectrometry grade trypsin (enzyme to protein ratio 1:50) overnight. Enzymes were inactivated by the addition of trifluoroacetic acid (TFA) to 1% final concentration. For LC-MS/MS analysis, peptides were desalted on self-made reverse-phase C_{18} StageTips columns and analyzed by LC-MS/MS using LTQ-Orbitrap XL (Thermo Scientific) coupled with an Agilent 1200 nanoflow LC via nanoelectrospray ion source (Proxeon). 1 mg of purified peptides were injected at a flow rate of 700 nl/min into 75 mm × 150 mm fused silica emitter (Proxeon), packed in-house with Reprosil-Pur 120C18-AQ, 3 mm stationary phase beads (Dr. Maisch GmbH) and eluted over 120 min using a linear gradient of 3% to 40% of solvent B (80% acetonitrile and 0.5% acetic acid) in solvent A (0.5% acetic acid) at a flow rate of 250 nl/min. The LTQ-Orbitrap was operated in a data-dependent mode and a "lock mass" option was enabled for m/z 445.120030 to improve mass accuracy. Precursor ion full scan spectra (m/z 300 to 1800) were acquired in the Orbitrap in profile with a resolution of 60000 at m/z 400 (target value of 1000 000 ions and maximum injection time 500 msec). The five most intense ions were fragmented in linear ion trap by collision-induced dissociation (normalized collision energy 35.0%) and spectra were acquired in centroid (target value of 5000 ions and maximum injection time 150 msec). Dynamic exclusion option was enabled (exclusion duration 120 s) and ions with unassigned charge state as well as singly charged ions were rejected.

3.6 Total proteome analysis

Cells were suspended in 10 volumes of 4% SDS (sodium dodecyl sulfate), 100 mM Tris-HCl pH 7.5 and 100 mM DTT containing lysis buffer. Cell suspensions were heated at 95 ºC for 5 min and lysed by sonication (Bandelin) (60 × 1-sec pulses at 50% intensity). Cell debris was removed by centrifugation at 14 000 g for 10 min. Protein concentration was determined at A_{280} using bovine serum albumin (BSA) as a standard. 7.5 µg of total protein from stationary phase cell lysates was mixed in a 1:1 ratio with total protein from reference cell lysates. For r-protein stoichiometry in the early stationary phase total proteome, 18 µg of total protein from stationary phase cell lysates were mixed in a 24:1 ratio with 70S ribosomes (0.763 µg). Samples were precipitated with 2:1:3 volume methanol: chloroform:water. Protein pellets were suspended in 25 µl of 7 M urea and 2 M thiourea, followed by disulfide reduction with 5 mM DTT for 30 min and cysteine alkylation with 10 mM chloroacetamide for 30 min at room temperature. Proteins were digested with endoproteinase Lys-C (Wako) at a 1:50 enzyme-to-protein ratio for 4 h. Urea concentration in the solution was reduced by adding 4 vol of 100 mM ABC and peptides were further digested using mass spectrometry grade trypsin (enzyme to protein ratio 1:50) overnight. Enzymes were inactivated by the addition of trifluoroacetic acid (TFA) to 1% final concentration. Peptides were desalted with self-made reverse-phase C18 StageTips columns. The resulting peptides were fractionated and analyzed by LC-MS/MS (Mets, Kasvandik et al. 2019).

3.7 Mass spectrometry data analysis

Data analysis was performed using Maxquant (v1.5.6.0) with default settings (Cox and Mann 2008), except that the minimal peptide length for the specific and non-specific search was 5 amino acids. Unique peptides were used for quantification, the main search peptide tolerance was 8 ppm and variable modification was used for quantitation of oxidation (methionine). The peptide identification search was carried out against the *E. coli K-12 MG1655* protein sequence database from UniProtKB (as of Oct. 2019). The search results were filtered and transformed using Perseus (v1.6.14.0) (Tyanova, Temu et al. 2016). For proteins bL20, bL33, bL34, bS20 and bS21 MS data analysis was done using the Mascot search engine and Skyline as described in (Lilleorg, Reier et al. 2019). Each protein was quantified through SILAC ratios H/M, L/M and/or (L+H)/M, comparing unlabeled (L) and/or "heavy"-labeled (H) relative quantities against medium-heavy labeled (M) internal reference. Protein bL35 could not be quantified reproducibly in our datasets due to a small number of unique peptides. H/M, L/M and (L+H)/M values were analyzed across all time points using the two-way analysis of variance (ANOVA) statistical test in the software GraphPad 7.0.

3.8 R-protein stability modeling in total proteome datasets

Individual r-protein degradation dynamics were analyzed using non-linear regression in the software GraphPad 7.0. Data were fitted into plateau followed by one phase decay model: IF X<X0,Y=Y0 ELSE Y= Plateau+(Y0-Plateau)* exp(-K*(X-X0)), where X is time, Y is Y0 until X<X0 and then decays down to plateau with one phase and K is the rate constant in units that are reciprocal of the X-axis units. For our data, plateau and K-values were restricted to being larger than 0. The alternative model was a straight line: Y= YIntercept + Slope*X. Data were weighted by $1/Y^2$, this minimizes the sum of the squares of the relative distance of points from the curve. The fit of models was compared using Akaike information criteria (Sugiura 1978).

3.9 Measurement of translation activity during the stationary phase

E. coli MG1655-SILAC (F-, λ-, rph-1, ΔlysA, ΔargA) strain, which is incapable of *de novo* synthesis of arginine and lysine, was grown in MOPS medium (Neidhardt, Bloch et al. 1974). At mid-log ($A_{600} \approx 1$), 2 mg/ml of unlabeled arginine (Arg0) and lysine (Lys0) were added to the culture. Cell culture was grown for a maximum of 14 days. A portion of the cells were collected in the mid-log phase ($A_{600} \approx 1$) and on days 1, 2, 4, 6, 8, 10, 12 and 14 after the start of the experiment. Cells were pulse-labeled with 0.5 mCi of [^{14}C]-labeled amino acids (L-Amino acid mixture; Hartmann Analytic) and incubated at 37 ^{o}C on a shaker for 3 hours. Samples were collected every 30 minutes and precipitated with TCA (trichloroacetic acid). Radioactivity in the precipitate was determined using an Optiphase HiSafe III scintillator (PerkinElmer). Disintegrations per minute (Dpm) values were normalized to the A_{600} of the cell culture at respective timepoint.

3.10 Quantification of ribosome-associated proteins

Data analysis was performed using Maxquant (version 2.14) with default settings (Cox and Mann 2008), except that the minimal peptide length for the specific and nonspecific search was 5 amino acids. Unique peptides were used for quantification, the main search peptide tolerance was 8 ppm and variable modification was used for the quantitation of oxidation (methionine). The peptide identification search was carried out against the *E. coli K-12 MG1655* protein sequence database from UniProtKB (as of Mar. 2021). The search results were filtered and transformed using Perseus (v1.6.14.0) (Tyanova, Temu et al. 2016). Calculations with data are as listed: First, r-protein medium-heavy intensities were normalized to day 1 values and an average was calculated for each sample (=R). Next, the

light (unlabeled) and heavy intensities of proteins of interest in samples were added together (L+H) and normalized to day 1 values (L+H fraction). Finally, the (L+H) fraction for proteins of interest was divided with the R to get a normalized (L+H) fraction.

3.11 Quantification of r-protein bL31 and bL36 paralogs in ribosomes

Data analysis was performed using Maxquant (version 2.14) with default settings, except that the minimal peptide length for the specific and nonspecific search was 5 amino acids. Unique peptides were used for quantification, the main search peptide tolerance was 8 ppm and variable modification was used for the quantitation of oxidation (methionine). The peptide identification search was carried out against the *E. coli K-12 MG1655* protein sequence database from UniProtKB (as of Mar. 2021). Next, Maxquant results were imported to Skyline (version 20.1.0.31) and peptides originating from r-proteins bL31A, bL31B, bL36A and bL36B (Table S1) were quantified and (L+H)/M ratio (L+H = sample; M = reference) were calculated for respective proteins. The r-protein bL36A had only one peptide for quantification. The reference ribosomes were heterogeneous concerning bL31 and bL36 paralogs (Lilleorg, Reier et al. 2019), however, an assumption that all ribosomes in the exponential growth phase contain at least one copy of bL31A or bL31B and bL36A or bL36B, allows quantification of A and B paralogs for both bL31 and bL36 r-proteins via single reference. The (L+H)/M ratios of bL31A and bL31B alongside bL36A and bL36B were normalized against the fraction of bL31A (0,82), bL31B (0,18), bL36A (0,88) and bL36B (0,12) in the reference ribosomes (Lilleorg, Reier et al. 2019).

4. RESULTS AND DISCUSSION

4.1 The fate of ribosomes during the stationary phase

In order to study r-proteins in the stationary phase, first requires knowledge about the dynamics of ribosome population in the same timescale. Analysis of ribosome population in the culture via particle partitions (30S, 50S, 70S, 100S and polysomes) is a simple method that gives insight into ribosome stability and particle distribution in the culture. To determine how ribosome population changes during the stationary phase, ribosome particle partitions were analyzed via sucrose gradient centrifugation over the course of 14 days. The same volume of culture was taken from each timepoint. Ribosome gradient profiles were plotted and ribosome particle partitions were evaluated (Figure 3).

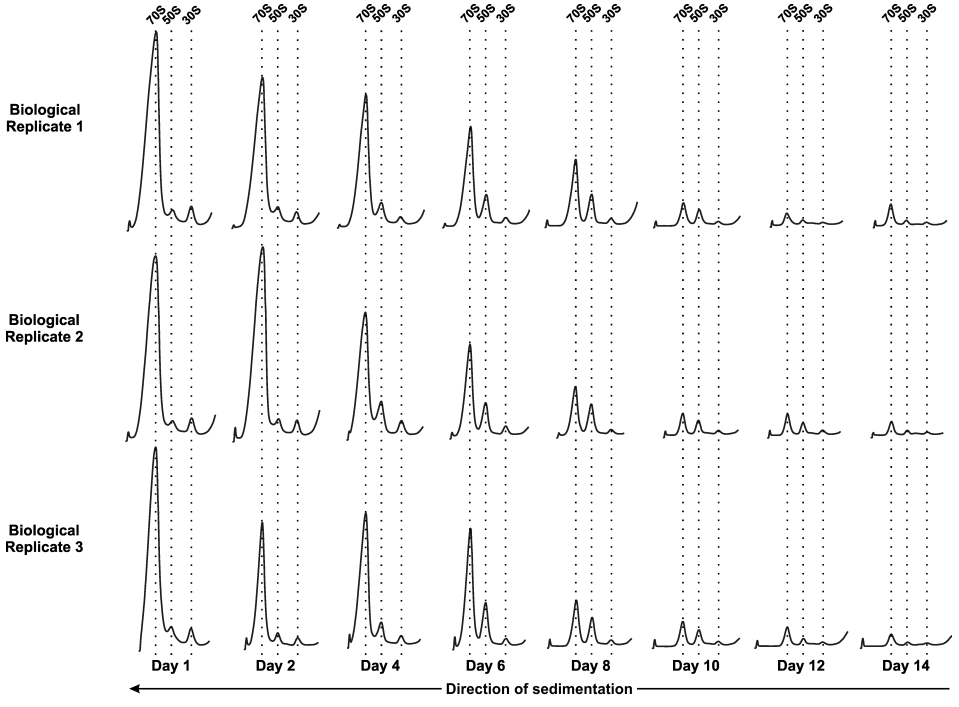

Figure 3 – Ribosome gradient profile analysis from stationary phase cells using sucrose gradient centrifugation.
Cells were harvested from the stationary phase cultures over the course of 14 days, lysed and analyzed in a sucrose gradient via centrifugation (Ref. II). The volume of culture taken for analysis was the same for each timepoint. The direction of sedimentation is from right to left. Location of fractions corresponding to 70S, 50S and 30S are shown by dashed line. This figure is based on Figure S2 in Ref II without any normalization.

Upon initial observation, a decrease in the overall ribosome particles was observed over the 14 days, particularly in the 70S fraction, as illustrated in Figure 3. This decrease in ribosome particle fractions suggests ribosome degradation. Further examination of the gradient profiles indicated an increase in free 50S

particles during the first ten days, followed by a gradual decrease until day 14 (see Figure 2B in ref. II). However, no accumulation of free 30S particles was observed throughout the 14 days (see Figure 2B in ref. II). This discrepancy between the free 50S and 30S subunit populations provides clear evidence of differing subunit stabilities during the stationary phase. Moreover, during the first 8 days, the 70S peak exhibited a significant shoulder, which upon further analysis was identified as 100S particles (see Figure S2 and S3 in ref. II). The number of 100S particles was highest on day 1 and gradually decreased over the course of 8 days (see Figure S3 in ref. II). In summary, the overall ribosome levels decreased over the 14 days, the free 50S and 30S subunits exhibited different stabilities and 100S particles were present in the cells during the early stationary phase and were gradually lost as the culture transitioned into prolonged stationary phase.

While ribosome gradient profiles are excellent for general observations, they usually lack details to make more scilicet conclusions. To specify the dynamics of ribosome degradation in stationary phase, the total RNA levels were determined over the course of 14 days (Figure 4). Previous reports have shown that rRNA constitutes 80% of the total RNA in cells (Bremer and Dennis, 2008; Ehrenberg, Bremer et al., 2013). Thus, the rRNA content could be evaluated by quantifying the total RNA in cells (see Figure 3 and S4 in ref. II). Total RNA concentration started to decrease at late-log phase and continued to decrease until an apparent plateau on day 6. On day 6, total RNA levels had dropped approximately 65% compared to day 1 (See Figure 3 in ref II). Total RNA was further analyzed via dot-plot assay with 23S and 16S rRNA-specific sequences (see Figure S5 in ref. II). Dot-plot assay confirmed that the decrease of total RNA levels happened in conjunction with rRNA decrease. Hence, the decrease of total RNA was attributed to the degradation of ribosomes during stationary phase.

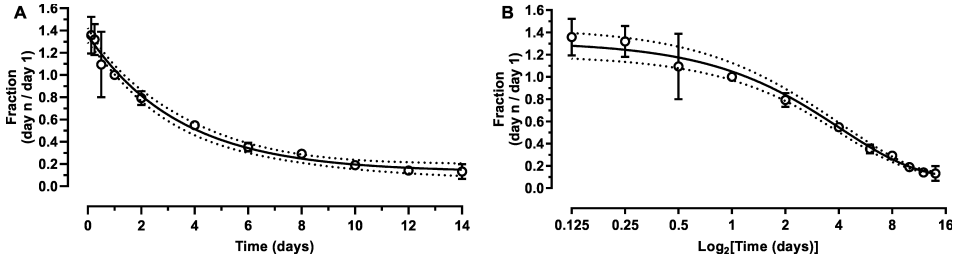

Figure 4 – Total RNA content in cells decreases during the stationary phase.
Samples were collected over the course of 14 days. Total RNA was extracted from cells using hot-phenol extraction and ethanol precipitation. RNA concentration was determined by measuring absorbance at 260 nm and normalized to the corresponding value on day one (Y-axis). Data were fitted into a one-phase decay model, where a black solid line represents a mean and a dotted line 95% confidence interval. Panel (A) has a linear time scale, while panel (B) presents the same data on a binary logarithmic time scale for a detailed view of the late-log and early stationary phase period. Values shown in the figure are the mean of three independent biological experiments with standard deviation (n=3; mean ± SD). This figure is based on Fig.3 and Fig.S4 in Ref. II.

While determining the quantity of ribosomes in cells is an important aspect of characterizing ribosome populations, translation activity is another crucial element that remains unaccounted for. To assess changes in translation activity, we measured the synthesis of new proteins during stationary phase. Translation activity was determined via radioactively labeled amino acids as described in the methods.

Our results showed that newly synthesized protein quantity dropped by approximately 40-fold as the culture transitioned from mid-log to early stationary phase (Day one) (Figure 5A, see Figure 1A in ref. III). However, as the culture progressed towards prolonged stationary phase, the newly synthesized protein amount increased by approximately 2-fold compared to early stationary phase (Day two) (Figure 5A). This indicates that there is a significant inhibition of translation activity during the early stationary phase, followed by a minor increase during the transition into the prolonged stationary phase. By comparing translation activity to the number of ribosomes per cell (Figure 5B), we observed a decrease in ribosome-specific activity in the early stationary phase and its gradual increase in prolonged stationary phase. Based on this, ribosomes during the onset of stationary phase are strongly inhibited and as the culture transitions into prolonged stationary phase, the ribosome translation activity per ribosome is partially restored.

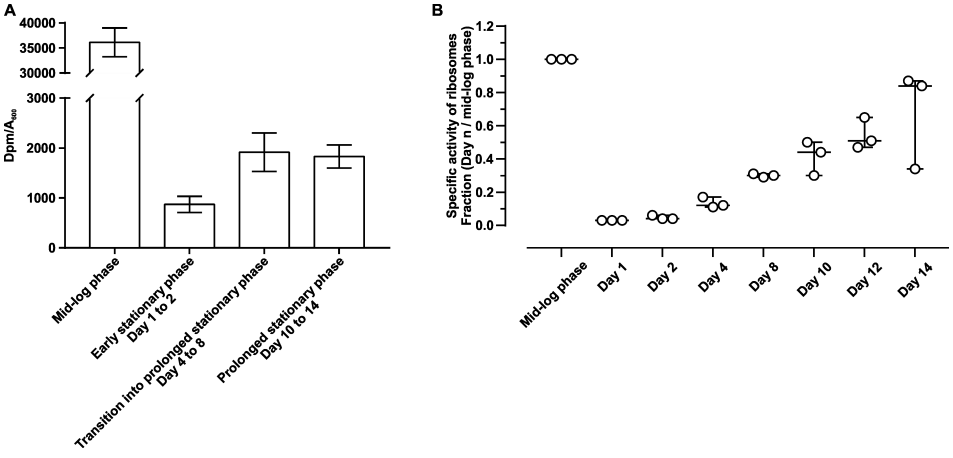

Figure 5 – Global translation activity in the stationary phase.
E. coli culture was grown for 14 days. Translation activity was determined via radioactively labeled amino acids as described in the methods. (A) Obtained values of disintegrations per minute (Dpm) were normalized against A_{600} and plotted (Y-axis). Values from day 1 to 2, day 4 to 8 and day 10 to 14 were grouped. (B) The specific activity of ribosomes during stationary phase. The ratio between Dpm/A_{600} and ribosomes per cell was calculated and normalized against mid-log phase values. This figure is based on Fig.1 in Ref.III, with the addition of timepoint groupings and calculated specific activity of ribosomes during stationary phase.

The overall decrease in ribosome particles in gradient profiles implied ribosome degradation (Figure 3). This was further verified by decrease in total RNA concentrations during stationary phase (Figure 4) and consistency of number of viable cells in the culture (see figure S1 in ref. II). Ribosome degradation has been shown to occur in response to nutrient deprivation, for example, nitrogen (Ben-Hamida and Schlessinger 1966), carbon (Jacobson and Gillespie 1968) and phosphate (Maruyama and Mizuno 1970) starvation. In laboratory strains, these stress conditions emerge in late-log phase or during the onset of stationary phase. Previous studies conducted in this time period have shown that rRNA degradation begins in late log phase and continues as culture transitions into stationary phase (Piir, Paier et al. 2011). In this study, similar beginning was observed: RNA concentration started to decrease in late log phase (Figure 4B), continued into stationary phase and kept going after reaching stationary phase. In our experimental system, the observable ribosome degradation comes to an end by day 6 as after that, no statistically significant difference was seen. Data in this study allowed to comprehensively conclude the magnitude and extent of ribosome degradation in the prolonged stationary phase, amending previous experiments observing ribosome degradation in the early stationary phase.

The ribosome gradient profiles revealed the differential stabilities of free 50S and 30S subunits. The accumulation of free 50S particles but no increase in free 30S fraction implies that the 30S is degraded more quickly than the 50S during the early stationary phase. However, free 50S does not accumulate indefinitely, eventually after day 10 we see a decrease in free 50S fraction as well, meaning it is also likely degraded. In all likelihood, this difference is not an example of preferred degradation of 30S, but merely an inherent disparity in ribosome subunit stabilities. This early prioritization to removing free 30S from the cells is logical from the perspective of translation initiation: free 30S subunits can bind mRNAs and initiation factors to form initiation complexes and if these complexes cannot continue translation due to limited resources, it can lead to translational arrest and eventual cell death. This means that free 30S (in)stability itself could be considered as a translation regulation mechanism during stationary phase.

The analysis of gradient profiles during the stationary phase revealed translationally inactive 100S particles during the early stationary phase, but they were lost as the culture transitioned into the prolonged stationary phase (see Figure S2 and S3 in ref. II). The presence and eventual loss of 100S peaks in the early stages of the stationary phase correlates well with previous studies on 100S particles, which have shown their presence in the early stationary phase and eventual loss as the cell culture transitions into the prolonged stationary phase (Prossliner, Gerdes et al. 2021). The formation of 100S particles is a known mechanism, catalyzed in *E. coli* by two hibernation factors Rmf and Hpf (Ueta, Ohniwa et al. 2008), which have been comprehensively studied in both their function and structure. The 100S particles have been contributed with at least two functions in biology: First, 100S particles are formed to stabilize ribosomes and protect them from degradation and second, formed 100S particles are translationally inactive, hence they lead to inhibition of translation (Prossliner, Skovbo Winther et al. 2018).

Previous studies have shown that strains unable to form 100S particles have decreased fitness in early stationary phase (Prossliner, Skovbo Winther et al. 2018) and longer lag phase during resuscitation of growth (Akiyama, Williamson et al. 2017). In this study, both the stability and translation activity of the ribosomes during stationary phase were investigated. The initial influx of 100S particles in early stationary phase suggests high translation activity inhibition and protection of ribosomes. This was further confirmed by translation activity assay during stationary phase (Figure 5). However, the gradual decrease of 100S particles could mean that the ribosome stability decreases and/or their activity increases as the culture transitions into prolonged stationary phase. In case of ribosome stability, a decrease in total RNA showed that ribosomes are apparently degraded alongside with disappearance with 100S particles. Comparison of 100S particles and translation activity in stationary phase revealed that translation activity in the cells decreases with the appearance of 100S particles and vice versa, as 100S start to disappear from the culture the translation activity increases.

By comparing the number of ribosomes per cell with translation activity during stationary phase we get translation activity per ribosome. This value decreased as the culture entered stationary phase and then gradually increased as culture transitioned prolonged stationary phase. This further confirmed that 100S formation and concurrent translation inhibition is a temporary measure at the onset of stationary phase. Also, it revealed that ribosomes in prolonged stationary phase have high translation activity, almost as high as ribosomes in mid-log phase. This means that translational machinery is kind of "put on hold" as the cells adapt their metabolism to new challenges and limitations, but it continues with similar vigor when adaptations are done.

To summarize: the keywords that characterize ribosomes during stationary phase are degradation, hibernation and changes in translation activity. Ribosome levels in stationary phase diminish considerably, differences in ribosome subunit stabilities are also accentuated, the 100S particles both stabilize and inhibit a part of the ribosome population in the early stationary phase and in prolonged stationary phase specific activity of the ribosomes is partially restored.

4.2 R-proteins and RAPs in *E. coli* proteome during prolonged stationary phase

Previous chapter gave a general overview of the ribosomes during stationary phase and how they change as a whole. These details are important when trying to understand the main targets of this thesis – r-proteins and their importance both in ribosome and the whole proteome during stationary phase. Previous studies have shown that r-proteins are present in proteome in nearly stoichiometric amounts during steady state growth in mid-log phase (Chen et al 2012). However, same statement has not been proven in stationary phase. To shed light on the stoichiometry and stability of r-proteins during prolonged stationary phase, the r-protein quantities were first determined in the early stationary phase proteome

(24h after start of growth). The stoichiometry of individual r-proteins in the total proteome on day one (L+H) was compared to that of r-proteins in the reference 70S ribosomes isolated from exponential phase cells (M) (see Figure 4A in ref. II).

49 out of 51 detected r-proteins exhibited a (L+H)/M ratio of approximately 1 (±10%). This meant that majority of r-proteins are present in the same stoichiometry in proteome, as in 70S ribosomes isolated from mid-log phase. The exceptions were r-proteins bL7/bL12 and bS1 which had the (L+H)/M ratio of 1.3 and 2.0 respectively. This meant there were 1.3 and 2 times more bL7/bL12 and bS1 in proteome than in 70S ribosomes and that these r-proteins had a free pool in the early stationary phase proteome.

Next, using this experiment as a foundation, the r-protein quantities were determined in whole proteome during prolonged stationary phase. The experiment was done as described above except: culture was grown for 14 days, total protein from the cells was mixed in 1:1 (A_{280}) ratio with total protein extracted from reference cells grown to mid-log phase in the presence of medium-heavy labeled arginine and lysine. Stoichiometry of individual r-proteins in the total proteome over the course of 14 days (L and H separately) were compared to that of r-proteins in the reference cells grown to mid-log phase (M). Hence two SILAC ratios were calculated for each detected r-protein: L/M and H/M. This experimental design created two subpopulations of proteins in the culture: unlabeled and heavy labeled. These subpopulations were separated by time, meaning older proteins synthesized before the chase were heavy labeled and newer proteins synthesized after the chase were mainly unlabeled. By comparing these subpopulations in ribosomes to the reference (L/M and H/M), it was possible to show changes in protein composition through time.

Changes in the 51 r-protein H/M ratios are summarized on the heatmap on (see Figure 5B in ref. II). For 30 r-proteins (uL1, uL3, uL4, uL5, uL6, bL7/bL12, bL9, uL10, uL11, uL13, uL14, bL19, bL20, bL21, uL22, uL23, bL25, bS1, uS2, uS3, uS4, uS5, bS6, uS7, uS8, uS10, uS11, uS15, uS17 and bS18) the H/M and L/M (see Figure 5B and S8 in ref. II) ratios did not change in the total proteome. On the other hand, for 21 r-proteins (uL2, uL15, uL16, uL17, uL18, uL24, bL27, bL28, uL29, uL30, bL32, bL33, bL34, uS9, uS12, uS13, uS14, bS16, uS19, bS20 and bS21) the H/M and L/M (see Figure 5B and S8 in ref. II) ratio decreased over the next 14 days. The experimental design created a situation where H/M ratio can only stay the same or decrease, while L/M ratio can potentially increase, decrease or stay the same. If both ratios stayed the same then the protein was considered stable in proteome, while if both ratios decreased over time then the protein in question was lost from proteome and considered short-lived. The results show that 30S r-proteins are stable in the proteome over the course of 14 days. However, the 21 r-proteins with decreased ratios were considered short-lived and were degraded in the proteome during prolonged stationary phase.

The results clearly show that r-proteins are degraded in stationary phase, however, synthesis is completely different subject. As mentioned before, The H/M and L/M ratios describe r-protein subpopulations that are synthesized in the mid-log phase (H/M) or the late-log and after the entry to stationary phase (L/M). As

a result, H/M ratio can only remain the same (stability) or decrease (degradation) during the experiment. However, L/M ratio can remain the same, decrease, or increase (synthesis) during stationary phase. Since we see that both H/M and L/M ratios decrease in stationary phase, the first logical conclusion would be that no synthesis of r-proteins takes place in stationary phase. This is an illusion created by excessive degradation that blocks out detection of small-scale synthesis. Luckily there is a way to visualize r-protein synthesis by comparing L/M and H/M ratios. If r-proteins are synthesized in stationary phase, then L/M ratio would decrease more slowly than H/M ratio. When we compared the kinetics of r-protein L/M and H/M ratios, there was only one r-protein in our dataset that showed distinct difference between H/M and L/M ratio – bL34 (Figure 6). This means that while overall levels of bL34 are decreasing in the cell proteome, new copies of bL34 are also actively synthesized. While no other r-protein had this difference, it does not mean that they are not synthesized: merely that using this method, no accumulation of newer r-proteins was determined.

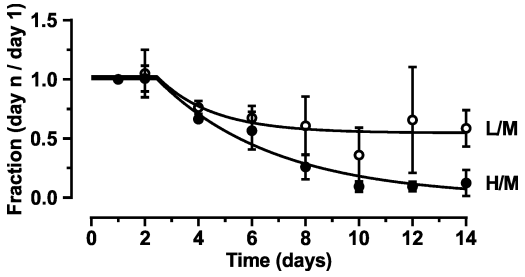

Figure 6 – bL34 synthesis during stationary phase.
Cells were collected over the course of 14 days, lysed and total protein from cells (day 1 to 14) was mixed in 1:1 (A_{280}) ratio with total protein from reference cells containing medium-heavy labeled arginine and lysine. Samples were analyzed using quantitative mass spectrometry. SILAC ratios were calculated for r-protein bL34: older proteins synthesized before the chase were heavy labeled (H/M) and newer proteins synthesized after the chase were mainly unlabeled (L/M). The H/M describes the protein stability and degradation, while the L/M describes the protein stability, degradation and synthesis. Differences between these two ratios characterize synthesis. Values shown in the figure are the mean of three independent biological experiments with standard deviation (n=3; mean ± SD).

R-proteins were the main focus of this work, but the ribosome-associated proteins also piqued our interest after seeing the disparity between r-protein stabilities. Hence RAPs were also quantified in total proteome during stationary phase. However, RAPs had one disadvantage: they were missing from reference cells and could not be quantified via SILAC ratios. Luckily knowing which r-proteins were stable over the course of stationary phase, meant they could be used as an internal reference point to calculate RAPs quantities in proteome. Intensities if individual RAPs were normalized to stable r-proteins average (L+H)/M ratio and presented as a fraction from day 1 (See Figure S3 in ref. III).

Hibernation factors Rmf and Sra were present during the early stationary phase, but their quantity decreased until day 6 (See Figure 2 in ref. III). After day 6, Rmf and Sra were not detected in the samples anymore. Based on these results, the Rmf and Sra are present during the onset of stationary phase, but are degraded as culture transitions into prolonged stationary phase. Hibernation factors Hpf and RaiA are also present during the onset of stationary phase, but as cells transition into prolonged stationary phase their quantity decreased more slowly and on day 14 their quantity in proteome had decreased approximately 50% compared to day 1. The quantity of translation factors (IF-2, EF-G, EF-Tu, EF-Ts), RNA polymerase subunits (RpoA, RpoB and RpoC) and other observed RAPs (Tig – trigger factor) did not decrease or increase over the course of 14 days, hence they were considered as stable in proteome during prolonged stationary phase.

The most intriguing part of these results is the fact that the r-proteins are present in stationary phase cells in non-stochiometric quantities. Previously, no study had observed such a disparity between r-protein stabilities before, hence it created multiple questions of interest both about the r-proteins and their role in the cells. The first question was simple: "Why are the r-proteins degraded non-stoichiometrically during stationary phase?". One of the first things to look at when analyzing the stability of any protein is its composition. N-terminal rule, also known as N-degron pathways, is one of the better-known ways to identify short-lived proteins via their N-terminal amino acid residue (Varshavsky 2019). There were also C-degron pathways analogous to N-degrons (Varshavsky 2019). However, data in this study did not correlate well with known N- or C-degron pathways, indicating that the disparity between r-protein stabilities in stationary phase are not explained by only N or C-degron pathways (Table 2).

The r-protein sequences were further analyzed for motifs and patterns for correlation with their observed stability. The analysis did not find any sequence-founded correlation with protein stability. Sadly, our preliminary analysis revealed no concrete answer on why r-proteins stabilities differ in stationary phase. This in itself indicates that the r-protein degradation is not only determined via N- or C-degron rules alone. Furthermore, these results lead to an intriguing question: if the amino acidic composition and sequence does not correspond to r-proteins stabilities in stationary phase, then perhaps it is their role in some structure or function outside the ribosome that explains their difference in stabilities? Some r-proteins have known extra ribosomal functions, for example, proteins bS1, uS2, uS4, uS7, uS8, uS15, bS20, uL1, uL4, uL10, bL12 and bL20 take part in translational autoregulation of their own operon. Alas, this specific function does not correlate well with their stabilities. However, there was a simple correlation between r-protein size and stability: larger r-proteins tend to be more stable than smaller ones (See Figure 7 in ref. II).

Table 2 – Comparison of N- and C-degrons pathways and r-protein stability

Protein	MW	fMet?[1]	second residue	third residue	Degradation in our datasets?	C-term third residue	C-term second residue	C-term residue
uL1	24730	–	Ala	Lys	–	Ser	Val	Asn
uL2	29860	–	Ala	Val	+	Arg	Ser	Lys
uL3	22244	+	Ile	Gly	–	Val	Lys	Ala
uL4	22087	+	Glu	Leu	–	Met	Leu	Ala
uL5	20302	–	Ala	Lys	–	Phe	Arg	Lys
uL6	18904	–	Ser	Arg	–	Lys	Lys	Lys
bL7	12295	–	Ser	Ile	–	Glu	Val	Lys
bL9	15769	+	Gln	Val	–	Val	Ala	Glu
uL10	17712	–	Ala	Leu	–	Glu	Ala	Ala
uL11	14875	–	Ala	Lys	–	Val	Glu	Asp
uL13	16019	+	Lys	Thr	–	Arg	Leu	Asn
uL14	13541	+	Ile	Gln	–	Glu	Val	Leu
uL15	14980	+	Arg	Leu	+	Ile	Glu	Glu
uL16	15281	+	Leu	Gln	+	Phe	Glu	Gly
bL17	14365	+	Arg	His	+	Ala	Ala	Glu
uL18	12770	+	Asp	Lys	+	Leu	Gln	Lys
bL19	13133	+	Ser	Asn	–	Arg	Leu	Asn
bL20	13497	–	Ala	Arg	–	Ala	Leu	Ala
bL21	11564	+	Tyr	Ala	–	Ile	Ser	Ala
uL22	12226	+	Glu	Thr	–	Ser	Asp	Arg
uL23	11199	+	Ile	Arg	–	Gly	Ala	Glu
uL24	11316	–	Ala	Ala	+	Thr	Ile	Lys
bL25	10693	+	Phe	Thr	–	Val	Arg	Ala
bL27	9124	–	Ala	His	+	Glu	Ala	Glu
bL28	9006	–	Ser	Arg	+	Glu	Lys	Tyr
uL29	7273	+	Lys	Ala	+	Ala	Gly	Ala
uL30	6542	–	Ala	Lys	+	Val	Glu	Glu
bL32	6446	–	Ala	Val	+	Ile	Ala	Lys
bL33	6372	–	Ala	Lys	+	Lys	Ile	Lys
bL34	5380	+	Lys	Arg	+	Val	Ser	Lys
bS1	61158	?	Thr	Glu	–	Lys	Gly	Glu
uS2	26744	–	Ala	Thr	–	Glu	Ala	Glu
uS3	25983	–	Gly	Gln	–	Gly	Arg	Lys
uS4	23469	–	Ala	Arg	–	Tyr	Ser	Lys
uS5	17603	–	Ala	His	–	Leu	Gly	Lys
bS6	15703	+	Arg	His	–	Glu	Glu	Glu
uS7	20019	–	Pro	Arg	–	Tyr	Leu	Asn
uS8	14127	–	Ser	Met	–	Tyr	Val	Ala

Table continued on the next page.

Table 2 – Continuation

Protein	MW	fMet?[1]	second residue	third residue	Degradation in our datasets?	C-term third residue	C-term second residue	C-term residue
uS9	14856	–	Ala	Glu	+	Ser	Lys	Arg
uS10	11736	+	Gln	Asn	–	Ser	Leu	Gly
uS11	13845	–	Ala	Lys	–	Arg	Arg	Val
uS12	13737	–	Ala	Thr	+	Pro	Lys	Ala
uS13	13099	–	Ala	Arg	+	Ile	Lys	Lys
uS14	11580	–	Ala	Lys	+	Ala	Ser	Trp
uS15	10269	–	Ser	Leu	–	Leu	Arg	Arg
bS16	9191	+	Val	Thr	+	Lys	Ala	Ala
uS17	9704	–	Thr	Asp	–	Ala	Val	Leu
uS18	8986	–	Ala	Arg	–	Arg	His	Gln
uS19	10430	–	Pro	Arg	+	Lys	Lys	Lys
bS20	9684	–	Ala	Asn	+	Lys	Leu	Ala
bS21	8500	–	Pro	Val	+	Arg	Leu	Tyr

Amino acids with red color are primary (necessary) residues for N- and C-degrons
Amino acids with yellow color are secondary (amplifiers) residues for N- and C-degrons
1 – Is formyl-methionine removed from N-terminus?

The stationary phase is only a part of the mystery. Another aspect of it is: what happens to these stable r-proteins later, at lag phase during resuscitation of growth? Our current knowledge about ribosome biogenesis says that it starts in lag phase and consistent biogenesis requires stochiometric amounts of ribosome components, including r-proteins. Previous studies have shown that younger cells from early stationary phase have shorter lag phase, while older cells from prolonged stationary phase have longer lag phase during the resurgence of growth (Pin, Rolfe et al. 2009). Also, it has been shown that unbound r-proteins can be reused in ribosome biogenesis as growth conditions improve (Maruta 1970). Our data showing the non-stochiometric presence of r-proteins is like a contradiction or a problem to be solved so that growth can start again. An intriguing possibility is that perhaps in older cells the length of lag phase is dictated largely by r-proteins or their effect on ribosome biogenesis.

The larger picture of ribosome turnover is most likely a complex one. First, the fraction of r-proteins in proteome seems to be mathematically correlated to success of cell culture growth (Dai, Zhu et al. 2016). However, the living conditions of bacteria are everchanging, hence the necessity to understand what happens during stationary phase. We know that in simplified model ribosome biogenesis is inhibited during stationary phase and enchanted during exponential growth phase, but in reality, life is rarely so binary. For instance, bacteria *in situ* conditions are usually characterized by quiescence (Rittershaus, Baek et al. 2013). From this point of view the ribosome stability and translational efficiency are not only constants in life but also relative to the success of cell culture growth. This

leads to a rather intriguing question about free pools of r-proteins: is there a functional aspect to their existence or are they merely a problem to be solved during resurgence of growth?

Considering the resource deficiency in stationary phase, the synthesis of new proteins should be tightly regulated and based on necessity. bL34 is a small r-protein that has been shown to participate in regulation of polyamide biosynthesis pathway (Panagiotidis et al 1995). bL34 is shown also to be important for correct formation of 70S ribosomes in later stages of ribosome assembly (Akanuma, Kobayashi et al. 2014). Synthesis of r-proteins in stationary phase supports the theory of ribosome repair via r-protein exchange, where damaged r-proteins would be exchanged with newly synthesized r-proteins (Pulk, Liiv et al. 2010). However, very little is known about r-protein bL34. It is not essential r-protein for cell culture growth, as the deletion strain of bL34 is viable, but has a strong growth defect (Akanuma, Kobayashi et al. 2014).

In *E. coli*, Rmf is necessary for the formation of the 90S intermediate particles, which are converted into more stable 100S particles via Hpf (Ueta, Ohniwa et al., 2008). The disappearance of Rmf from the proteome and the concurrent loss of 100S particles (See Figure 2 in ref. III), is consistent with previous studies, where the deletion of Rmf from the *E. coli* genome led to an inability to form 100S particles (Wada, Igarashi et al., 1995). The stability of Hpf in the proteome is also intriguing because 100S particles are lost as culture transitions into prolonged stationary phase. Secondary function of Hpf could explain why it is not degraded as 100S are lost. RaiA is a ribosome hibernation factor that acts as an antagonist with Hpf. When RaiA binds to 70S ribosomes, they cannot dimerize into 100S particles (Polikanov, Blaha et al., 2012). Sra is a protein that binds to 30S during the early stationary phase (Izutsu, Wada et al., 2001). The tertiary knockout strain (ΔRmf, ΔHpf, ΔRaiA, ΔSra) has a longer lag phase than the triple knockout (ΔRmf, ΔHpf, ΔRaiA), suggesting that Sra plays a role in 100S formation (Bubunenko, Baker et al., 2007). RaiA and Hpf are relatively stable in both ribosome and proteome fractions, while Rmf and Sra are degraded as cells transition into prolonged stationary phase. The similar dynamics of Sra and Rmf further complement the potential shared functionality of Rmf and Sra in 100S formation.

4.3 Ribosome r-protein composition in stationary phase

The main targets of this thesis are r-proteins and their importance both in ribosome and the whole proteome during stationary phase. In order to see if ribosome r-protein composition undergoes changes during stationary phase, their protein composition was determined via SILAC-based quantitative mass-spectrometry. First, exponential and stationary phase ribosome protein compositions were compared for differences. Cells were grown to mid-log phase in presence of heavy labeled arginine and lysine, then chased by adding unlabeled arginine and lysine

to the culture. Cultures were then grown to early stationary phase, 70S ribosomes were isolated from them, mixed in 1:1 molar ratio with reference ribosomes containing medium-heavy arginine and lysine, and analyzed using quantitative mass spectrometry. This experimental design created two subpopulations of proteins in the culture: unlabeled and heavily labeled. This is the same as in Chapter 4.2, regarding proteome analysis, hence the same points apply in this case – ribosome analysis. These subpopulations were separated by time, meaning older proteins synthesized before the chase were heavy labeled and newer proteins synthesized after the chase were mainly unlabeled. By comparing these subpopulations in ribosomes to the reference (L/M and H/M), it was possible to show changes in ribosome protein composition through time.

Most r-proteins show no change in quantity in ribosomes, as cell culture transitions into stationary phase. However, r-protein bL31A and bL36A quantity in ribosomes decreases, while their genetic paralogs bL31B and bL36B quantity increases in ribosomes as cells transition into stationary phase (Figure 7, see Figure 2 in ref. I). This indicated that bL31A and bL36A are exchanged against bL31B and bL36B during the onset of stationary phase. Alongside with r-proteins, the ribosome-associated proteins (RAP) were also quantified. As the culture enters stationary phase, the quantity of translational factors (EF-Tu, RF-1), chaperons (Trigger factor) and RNA polymerase subunits (RpoA, B and C) bound to ribosomes decreases 4 to 64 times. At the same time, the quantity of hibernation factors (RaiA, Hpf, Rmf and Sra) bound to ribosomes increases up to 1000 times (Figure 7). This is in good correlation with previous observing that translation activity decreases in the cell and ribosomes form 100S particles as cells transition into stationary phase.

The switch from translation factors to hibernation factors bound to ribosomes is in good correlation with the decrease in translation activity (Figure 5). Furthermore, as bL31A and bL36A are exchanged against bL31B and bL36B respectively in the ribosome, so are the hibernation factors detected in ribosome fraction (Figure 7). The observation between r-protein bL31 and 100S particle formation has been documented previously in other studies as well (Ueta, Wada et al. 2017). Based on previous studies and our own results, it seems that r-proteins bL31A and bL36A are present in more translationally active ribosomes, while bL31B and bL36B are switched into composition of ribosomes during suppression of translation activity via hibernation factors (Figure 7). However, it is not clear whether the induction of hibernation factors and the exchange of bL31B and bL36B are directly linked or merely concomitant events as cells transition into stationary phase.

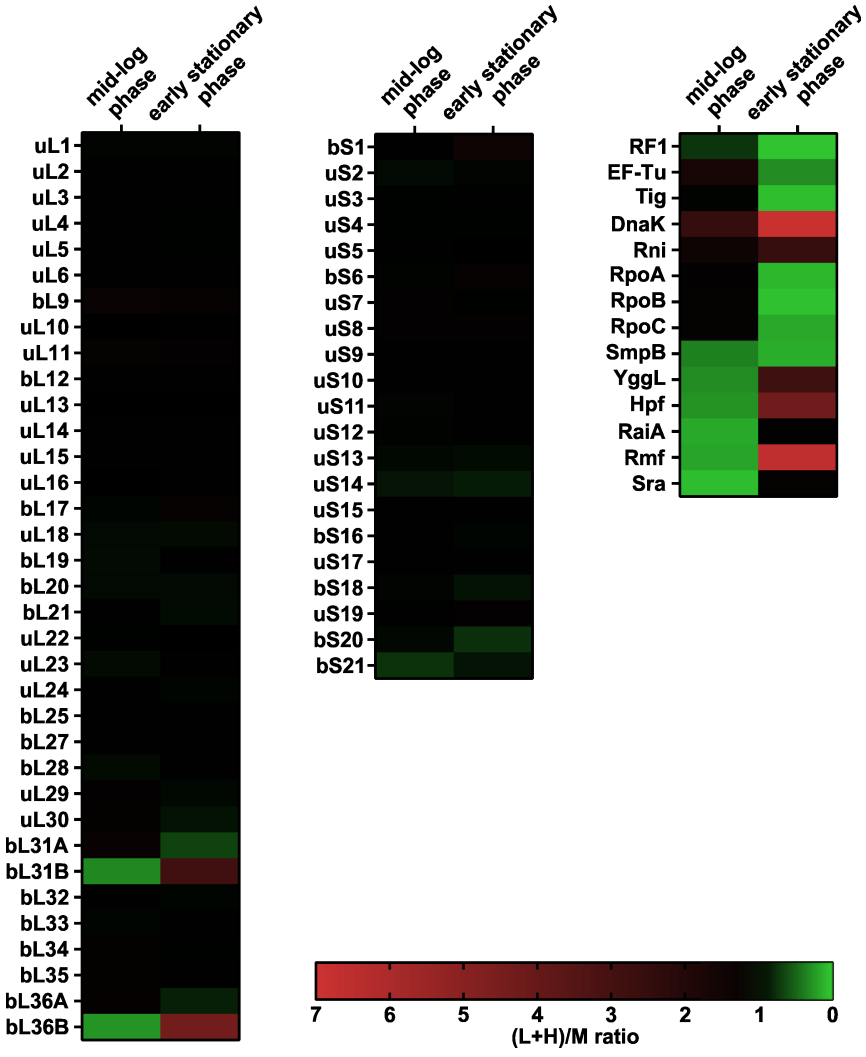

Figure 7 – Heatmap of ribosome protein composition in early stationary phase. Cells were grown in presence of heavy labeled arginine and lysine. At mid-log phase 20× excess of unlabeled arginine and lysine were added to the culture. Samples were collected at mid-log phase (OD~1) and early stationary phase (24h), 70S ribosomes were isolated, mixed in 1:1 molar ratio with reference 70S ribosomes containing medium-heavy labeled arginine ad lysine, and analyzed with quantitative mass spectrometry. (L+H)/M ratios were calculated from MS data for each protein. Mean of two experiments is shown.

4.4 The exchange of bL31B and bL36B against bL31A and bL36A in prolonged stationary phase

The stationary phase is not uniform, and while the period of cultures entering stationary phase has been observed in great detail, the prolonged stationary phase remains relatively uncharted. Therefore, our next aim was to determine the ribosome r-protein composition during prolonged stationary phase. The experimental approach remained the same, but cell culture growth was continued for up to 14 days. Ribosomes were isolated from them, mixed in 1:1 molar ratio with reference ribosomes containing medium-heavy arginine and lysine, and analyzed using quantitative mass spectrometry.

While most r-proteins stayed in stoichiometry in ribosomes during stationary phase, there were exceptions. R-proteins bS1 and bS21 were found in non-stoichiometric amounts during the later stages of stationary phase (See Figure 4 in ref. II). At the end of the experiment, r-protein bS1 was missing from approximately 50% and bS21 respectively from 25% of ribosomes (See Figure 4 In ref. II). To our surprise, we observed changes in the quantities of bL31 and bL36 paralogs in ribosomes when cell cultures were grown for 14 days. After approximately 6 to 8 days into stationary phase, bL31B and bL36B were exchanged for bL31A and bL36A in ribosomes (See Figure 3 in ref. III). RAPs were also quantified, revealing a decrease in the quantity of hibernation factors bound to ribosomes and an increase in translation factors as the culture progressed into prolonged stationary phase (See Figures 2 and S3 in ref. III).

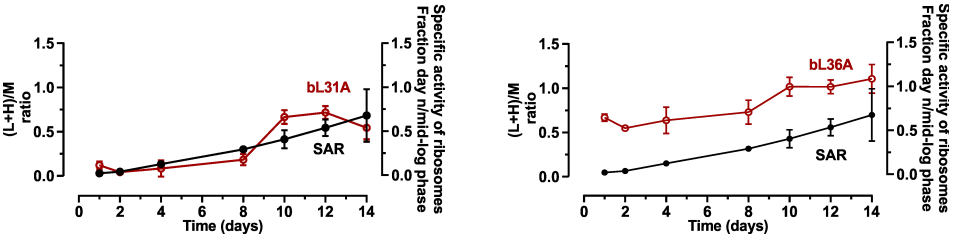

Figure 8 – bL31A and bL36A quantity in ribosomes compared to specific activity of ribosomes during stationary phase.
E. coli batch culture was grown and samples were collected over the course of 14 days. 70S ribosomes were isolated and mixed in a 1:1 ratio with medium-heavy labeled reference 70S ribosomes for r-protein quantification using LC-MS/MS. (A) The relative quantity of bL31A and bL36A is presented as the (L+H)/M ratio (L+H = sample; M = reference) and normalized against the average of (L+H)/M ratio of all 50S r-proteins and respective r-protein fraction in the reference ribosomes (see materials and methods). The specific activity of ribosomes during stationary phase: ratio between Dpm/A_{600} and ribosomes per cell was calculated and normalized against mid-log phase values. (A) Quantity of bL31A in 70S ribosomes (red, empty) and specific activity of ribosomes (SAR) (black, filled) during 14 days of stationary phase. (B) Quantity of bL36A in 70S ribosomes (red, empty) and specific activity of ribosomes (SAR) (black, filled) during 14 days of stationary phase. Values shown are the mean of three independent biological experiments with standard deviation (n=3; mean ± SD).

It has been shown that bS1 can be absent from non-translating ribosomes (Van Knippenberg, Hooykaas et al., 1974). Also, bS1 and bS21 are important for efficient translation initiation in *E. coli* (Held, Nomura et al., 1974; Van Duin and Wijnands, 1981; Watson, Ward et al., 2020). The non-stoichiometry of bS1 and bS21 in ribosomes is most likely another translation regulation mechanism during stationary phase to reduce translation initiation.

The gradual exchange of bL31B and bL36B against their A paralogs during prolonged stationary phase is an intriguing phenomenon. Also, a gradual increase of translation factors in ribosome fraction was observed during prolonged stationary phase (See Figure S3 in ref. III). The translation activity is strongly inhibited in the early stationary phase but shows a small increase from day 4 (Figure 6, see Figure 1 in ref. III). These results show that while the translation is strongly inhibited in the early stationary phase, then in the prolonged stationary phase translation activity starts to increase again. It is important to note that the translation activity per ribosome in prolonged stationary phase is partially restored (Figure 8).

Based on these observations, we postulate that the bL31A and bL36A alongside with their genetic paralogs regulate some aspect of translation activity: bL31A and bL36A are present in more translationally active ribosomes, while bL31B and bL36B are switched into composition of ribosomes during suppression of translation activity. In that regard, *in vitro* analysis of ribosome translation efficiency in presence of bL31A or bL31B shows increased frameshifting in presence of bL31B (Lilleorg, Reier et al. 2020). R-protein bL31A may confer to more precise ribosomes that are required in regards to increased translation activity of the cells, while bL31B allows limited translation, improves the formation of hibernation complexes, or helps with ribosome rescue mechanisms under growth-restricting conditions.

CONCLUSIONS

The ribosome, a macromolecular structure comprising nucleic acids and proteins, operates to enable protein synthesis across all organisms. The catalytic center for peptide bond formation is mostly composed of RNA, however, the cooperative nature of ribosome components accentuates the importance of r-proteins in ribosome functionality. The following thesis was composed to widen our knowledge on how r-protein quantities change in *E. coli* ribosomes and proteome during stationary phase. Stationary phase can be divided into early stationary phase when cells adapt to non-optimal growth conditions and prolonged stationary phase when cells are accustomed to those conditions. The main results and conclusions are following:

During the transition from mid-log to early stationary phase:

- R-proteins bL31A and bL36A that are abundant in mid-log phase ribosomes are exchanged against r-proteins bL31B and bL36B, that are prominent in early stationary phase ribosomes. R-protein exchange is accompanied by changes in ribosome associated proteins – translation factors disappear from ribosomes, while hibernation factors become abundant.
- Alongside the appearance of hibernating 100S ribosomes and r-protein bL31 and bL36 B paralogs in ribosomes correlates with 40-fold drop in the translation activity during the early stationary phase. This indicates that the A paralogs are attuned to translationally active ribosomes, while the B paralogs are prominent in ribosomes during diminished translation activity.

In the prolonged stationary phase:

- The number of ribosomes per cell starts to decrease gradually as cells reprogram themselves for non-optimal growth. During ribosome degradation, 70S ribosomes are dissociated into 50S and 30S subunits, and 50S subunits are more stable than 30S subunits. It is likely that this disparity in subunit stability occurs because inherent problems that free 30S subunits can create in translation system.
- 21 r-proteins are degraded with varying rates concurrently with rRNA, while 30 r-proteins remain stable in whole proteome and start to accumulate as free pools. However, 70S ribosomes still contain most r-proteins in stoichiometric amounts throughout stationary phase. Notable exceptions are bS1 and bS21 that are present in non-stochiometric amounts. Thus, most r-proteins are present in stochiometric quantities in ribosomes, but non-stoichiometric amounts in the proteome of *E. coli* during prolonged stationary phase.

- Translation activity in prolonged stationary phase increases about two times compared to early stationary phase, concurrently the fraction of 100S ribosomes in the cells decreases. Also, during prolonged stationary phase, translation factors (EF-G, EF-Tu, EF-Ts) and RNA polymerase subunits (RpoA, RpoB and RpoC) start to reappear in ribosome fraction again.
- After the early stationary phase bL31B ja bL36B are partially exchanged against bL31A and bL36A. Both exchanges happen gradually over long period of time (days). However, bL31A and bL31B are present in almost equal quantities after 14 days of growth, while with bL36A and bL36B the situation is in reverse – paralogs are unequally represented in ribosomes after 14 days of growth. This again correlates well with previous statement that A paralogs are present in more translationally active ribosomes, while B paralogs are prominent in ribosomes during lower translation activity.

In conclusion, the data presented in this work clearly shows that ribosome r-protein composition undergoes specific changes while entering into stationary phase conditions. Also, r-proteins show surprising differences in stability, not observed in previous studies, during stationary phase. This information improves our comprehension of translational machinery under non-optimal growth conditions.

SUMMARY IN ESTONIAN

Muutused ribosoomi ja ribosoomiga seotud valkude kogustes soolekepikese statsionaarses kasvufaasis

Ribosoomid on makromolekulaarsed kompleksid, mis viivad läbi valkude sünteesi kõigis elusloodu domeenides. *E. coli*-s koosnevad ribosoomid kolmest ribosoomi RNA-st (rRNA) ning 54 ribosoomivalgust (r-valgud). Need komponendid omakorda jaotuvad kahe ebavõrdse alamühiku vahel – 50S (23S rRNA, 5S rRNA ja 33 r-valku) ning 30S (16S rRNA ja 21 r-valku). Valgusünteesi käigus sünteesitakse ribosoomis peptiidsidemed aminohapete vahel. Aminohappelise järjetus põhineb geneetilise informatsioonil DNAst, mida vahendab ribosoomidele mRNA.

Valkudel endil on oluline roll elusloodustes: mõned neist viivad läbi katalüütilisi reaktsioone, samas kui teised täidavad rakkudes struktuurseid või mehhaanilisi funktsioone. Tähelepanuväärne on fakt, et r-valgud moodustavad ligi 40% ribosoomi massist. R-valgud osalevad mitmesugustes ribosoomi tööülesannetes, nagu näiteks: mRNA sidumine ribosoomile (bS1), valgusünteesiks vajalike faktorite sidumine ribosoomile (bL7/bL12), seondumine kasvava peptiidahelaga (uL4, uL22) ning tšaperonide liitumine ribosoomile valgu struktuuri moodustumiseks (uL23, uL29).

Varem on näidatud, et ribosoomide r-valguline koostis võib muutuda nii *in vitro* kui ka *in vivo*. Nende muutuste põhjused võivad olla seotud ribosoomide parandamise mehhanismi või valgusünteesi reguleerimisega spetsialiseeritud ribosoomide kaudu. Samas võib ribosoomide valgulise koostise muutumine aidata rakkudel kohaneda keskkonnamõjudega, näiteks statsionaarsesse kasvufaasi ülemineku. Käesoleva töö eesmärk oli välja selgitada, kuidas ja millisel määral muutuvad r-valkude tasemed *E. coli* varajase ja hilise statsionaarse kasvufaasi ribosoomides ning proteoomis.

Üks suuremaid muutusi ribosoomide r-valgu koostises toimub rakukultuuri üleminekul eksponentsiaalsest→statsionaarsesse kasvufaasi. R-valgud bL31A ja bL36A asenduvad ribosoomides nende paraloogidega, bL31B ja bL36B-ga. Samal ajal toimuvad muutused ka teistes ribosoomide ja rakkude aspektides: ribosoomidega seotud translatsioonifaktoreid jääb vähemaks, ribosoomidele seonduvad hibernatsioonifaktorid, translatsiooni aktiivsus rakkudes langeb ligikaudu 40 korda ning 100S ribosoomid hulk rakus tõuseb. Nendest vaatlustest tulenevalt näib, et bL31A ja bL36A esinevad ribosoomides eksponentsiaalse kasvufaasi ajal, mil translatsioon on aktiivne ning bL31B ja bL36B on seotud ribosoomidega statsionaarse kasvufaasis vähemaktiivse translatsiooni perioodil.

Hilises statsionaarses kasvufaasis toimuvad muutused nii ribosoomides kui ka rakkude proteoomis. Enamike ribosoomivalkude tase ribosoomides ei muutu hilise statsionaarse kasvufaasi käigus, eranditeks on bS1 ja bS21 ning jällegi bL31B ja bL36B. Statsionaarse kasvufaasi süvenedes asenduvad bL31B ja bL36B järkjärgult bL31A ja bL36A-ga. Huvitav on asjaolu, et samal ajal suureneb translatsiooni aktiivsus ligi kaks korda võrreldes varajase statsionaarse kasvufaasiga.

Sammuti kinnituvad ribosoomidele taas translatsioonifaktorid (EF-G, EF-Tu, EF-Ts). Käesoleva töö andmete põhjal joonistub välja selge korrelatsioon: bL31A ja bL36A esinevad translatsiooniliselt aktiivsemates ribosoomides, samas kui bL31B ja bL36B esinevad ribosoomides, mille translatsiooniline aktiivsus on madalam. Lisaks väheneb statsionaarse kasvufaasi käigus järk-järgult ribosoomide bS1 ja bS21 sisaldus. bS1 ja bS21 kohta on teada, et nad osalevad translatsiooni initsiatsioonil. Hilises statsionaarses kasvufaasis on bS1 ja bS21 olemas vastavalt 50% ja 75% ribosoomides. Nende valkude mitte- stöhhiomeetriline esindatus hilise statsionaarse kasvufaasi ribosoomides võiks olla üks võimalikest mehhanismidest, mis mõjutab translatsiooni aktiivsust läbi initsiatsiooni pärssimise.

Proteoomianalüüsi tulemused näitasid, et varajases statsionaarses kasvufaasis esinevad ribosoomivalgud proteoomis ja ribosoomides stöhhiomeetrilistes kogustes. Hilises statsionaarses kasvufaasis aga tuli esile väga oluline tendents: ribosoomides püsib enamike r-valkude sisaldus muutumatuna, kuid proteoomis jagunevad ribosoomivalgud kahte rühma. Identifitseeriti 30 stabiilset r-valku, mille tase ei muutu statsionaarse kasvufaasi käigus ning 21 ebastabiilset r-valku, mille tase proteoomis väheneb varieeruva kiirusega – ehk neid lagundatakse. Võrreldes r-valkude koguseid proteoomis ribosoomi hulgaga, ilmneb teine oluline seos: ebastabiilsete valkude kogused vähenevad samaväärselt ribosoomide kogusega. Sellest järeldub, et ebastabiilsed r-valgud lagundatakse samaaegselt ribosoomidega ning stabiilsed r-valgud moodustavad raku sees vabade valkude kogumi.

Kokkuvõtvalt näitavad selles töös esitatud tulemused, et ribosoomide valguline koostis muutub, kui rakud kohanevad statsionaarse kasvufaasi tingimustega. Lisaks tuvastasime, et vabadel ribosoomivalkudel on erinevad stabiilsused raku proteoomis, mida varem statsionaarses kasvufaasis ei ole kirjeldatud. Need tulemused täiendavad meie arusaama ribosoomide funktsioneerimisest ebasoodsatel kasvutingimustel.

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PUBLICATIONS

CURRICULUM VITAE

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Education

2017– University of Tartu, Institute of Molecular and Cell Biology, PhD studies, molecular biology
2016 University of Tartu, Institute of Molecular and Cell Biology, MSc degree, gene technology
2014 University of Tartu, Institute of Molecular and Cell Biology, BSc degree, gene technology

Professional Career

2023– Specialist, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu
2022–2023 Laboratory assistant, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu
2020–2022 Junior research fellow, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu
2013–2019 Laboratory assistant, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu

Scientific and research activity

My main research focus has been quantitative molecular biology in bacterial lifeforms with specialized usage and understanding of quantitative mass-spectrometric methods. Furthermore, using the data from quantitative analyses, modeling the dynamics and kinetics of protein and macromolecular structure quantities under in vivo conditions. Main fields of research CERCS: P320 Nucleic acids, protein synthesis; P370 Macromolecular chemistry.

List of publications

Reier, Kaspar; Liiv, Aivar; Remme, Jaanus (2023). Ribosome Protein Composition Mediates Translation during the Escherichia coli Stationary Phase. International Journal of Molecular Sciences, 24 (4). https//dio.org/10.3390/ijms24043128.

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Teaching and supervision in University of Tartu

Practical Course in Chemistry of Nucleic Acids
Practical Course in Laboratory projects, supervision of student project
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CERCS teaduserialad: P320 Nukleiinhappetesüntees, proteiinisüntees; P370 makromolekulaarkeemia.

Publikatsioonid

Reier, Kaspar; Liiv, Aivar; Remme, Jaanus (2023). Ribosome Protein Composition Mediates Translation during the Escherichia coli Stationary Phase. International Journal of Molecular Sciences, 24 (4). https//doi.org/10.3390/ijms24043128.

Reier, Kaspar; Lahtvee, Petri-Jaan; Liiv, Aivar; Remme, Jaanus (2022). A Conundrum of r-Protein Stability: Unbalanced Stoichiometry of r-Proteins during Stationary Phase in Escherichia coli. mBio, 13 (5). https//doi.org/10.1128/mbio.01873-22.

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Õppetöö ning juhendamised Tartu Ülikoolis

Nukleiinhapete keemia praktikum
Õpilasprojekti juhendamine aine laboratoorsed porjektid raames.
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