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Bacterial ribosome heterogeneity on the example of bL31 paralogs in *Escherichia coli*

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

This dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Molecular Biology on July 4th, 2022 by the Council of the Institute of Molecular Cell Biology, University of Tartu.

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LIST OF ORIGINAL PUBLICATIONS

Current thesis is based on the following original Publications which will be referred to in the text by their Roman numerals:

I **Lilleorg, S;** Reier, K; Remme, J; Liiv, A (2017). The Intersubunit Bridge B1b of the Bacterial Ribosome Facilitates Initiation of Protein Synthesis and Maintenance of Translational Fidelity. Journal of molecular biology, 429 (7), 1067–1080. https://doi.org/10.1016/j.jmb.2017.02.015

II **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. https://doi.org/10.1016/j.biochi.2018.10.013. * These authors contributed equally.

III **Lilleorg, S.,** Reier, K., Volõnkin, P., Remme, J., Liiv, A. (2020). Phenotypic effects of paralogous ribosomal proteins bL31A and bL31B in *E. coli*. Sci Rep 10, 11682. https://doi.org/10.1038/s41598-020-68582-2

My contribution to the articles is as follows:

Ref I performed experiments (except for dual luciferase assay and quench-flow), analyzed data, wrote, and edited the manuscript

Ref II performed *in vitro* experiments, wrote, and edited the manuscript

Ref III performed growth competition assay, dual luciferase assay and FACS assay, analyzed data, wrote, and edited the manuscript

ABBREVIATIONS

aa	–	amino acids
aSD	–	anti-Shine-Dalgarno sequence
B. subtilis	–	*Bacillus subtilis*
CP	–	central protuberance
CRIM	–	conditional replication, integration and modular plasmids
DC	–	decoding center
E. coli	–	*Escherichia coli*
fMet-tRNA^{fMet}	–	initiator methionine transfer RNA
FS	–	frameshift
IC	–	initiation complex
lmRNA	–	leaderless messenger RNA
PIC	–	pre-initiation complex
PTC	–	peptidyl transferase center
PTM	–	post-translational modification
ROS	–	reactive oxygen species
r-proteins, rp	–	ribosomal proteins
SD sequence	–	Shine-Dalgarno sequence
T. thermophilus	–	*Thermus thermophilus*
UTR	–	untranslated region

INTRODUCTION

Ribosomes are universal to life meaning that they synthesize proteins in every cell and that ribosomes from all three domains of life (Bacteria, Archaea, Eukarya) share a common structural core. In addition, bacterial ribosomes, the main subject of this thesis, have bacterial and species-specific structural features of ribosomal RNA (rRNA) and ribosomal proteins (r-proteins). Accumulating evidence has demonstrated that various prokaryotic and eukaryotic organisms produce structurally different ribosomes under various physiological conditions and in response to stress. This ribosome heterogeneity can originate from rRNA and r-protein content variations, including differences in rRNA sequence and modifications, r-protein stoichiometry, post-translational modifications, and paralogous r-proteins. Importantly, ribosomes are more diverse and dynamic than commonly thought. Therefore, the intriguing question – of whether ribosome heterogeneity could contribute to translation regulation – rises. This possibility would mark a change in paradigm showing ribosomes as another determinant of translation outcome rather than passive protein synthesis factories. To date, the evidence for ribosome heterogeneity has been significantly more substantial than evidence for its functionality at the level of translation and physiology.

The first part of this thesis gives an overview of the structure of bacterial ribosomes, protein synthesis in bacteria, and the concept of ribosome heterogeneity in general as well as its state of research in bacteria. In the second experimental part of this thesis ribosome heterogeneity with respect to r-protein paralogs in Escherichia coli is characterized on the example of r-protein bL31 paralogs. Their functional importance for bacterial growth and various translation initiation and elongation aspects are evaluated.

1. REVIEW OF LITERATURE

1.1. Structure of the bacterial ribosome

From compositional point of view ribosome is a ribonucleoprotein particle meaning that it contains ribosomal RNA (rRNA) and ribosomal proteins (r-proteins). In addition to this, ribosome binds metal ions (Mg^{2+}, K^{+}, Zn^{2+}) required for its structural stability and translational activity (Akanuma, 2021; Nierhaus, 2014; Rozov et al., 2019). Ribosome mass is dominated by rRNA that in bacteria makes up about 2/3 of its molecular mass (about 2.4 MDa) whereas r-proteins constitute 1/3 (Reuveni et al., 2017). This proportion is achieved by 3 rRNA molecules (16S, 23S and 5S rRNA) and more than 50 r-proteins (their exact number slightly varies between bacterial species). Both rRNA and r-proteins are divided into two unequally sized subunits (small subunit 30S, large subunit 50S) that during translation initiation form a ribosome (70S) with a diameter of about 200 Å (Chen et al., 2016). *E. coli* has been the main bacterial model organism in the study of bacterial ribosome structure and function. Therefore, the numbering of rRNA and nomenclature of r-proteins is based on *E. coli* (Ban et al., 2014; Petrov et al., 2013).

The common core (about 2 MDa) shared by ribosomes from all three domains of life is composed of more than 4000 rRNA nucleotides and 34 r-proteins (15 in the small and 19 in the large subunit) meaning that approximately 97% of bacterial rRNA and 62% of bacterial r-proteins are included in it (Melnikov et al., 2012). *E. coli* ribosome contains 34 universally conserved and 20 bacterial-specific r-proteins (Melnikov et al., 2012). Even expansion segments – rRNA sequences between the universally conserved elements of rRNAs (Stepanov and Fox, 2021) – have been recently identified in 5S, 16S and 23S rRNA of several bacterial species (Kushwaha and Bhushan, 2020; Stepanov and Fox, 2021). rRNA expansion segments have been long considered to be present only in eukaryotic organisms (Melnikov et al., 2012; Parker et al., 2014).

In addition to the universal common core and bacterial-specific features bacterial ribosomes exhibit species-specific variations. At the level of rRNA these may include altered length and modification pattern of rRNA molecules with deletions, insertions and unique helices (Kirpekar et al., 2018; Kushwaha and Bhushan, 2020; Wimberly et al., 2000). R-proteins can have extensions or deletions, altered post-translational modification patter as shown in *Bacillus subtilis* compared to *E. coli* (Lauber et al., 2009). In addition, some r-proteins may be lost or gained during evolution, for example bS21 and bS22, bL37 in mycobacteria, respectively (Kushwaha and Bhushan, 2020).

Ribosome structure and function are dominated by rRNA. Ribosomal three-dimensional structure is mostly determined by rRNA's tertiary structure with r-proteins bound to it (Wimberly et al., 2000; Yusupov et al., 2001) as specified in the subsequent overview of the small and large subunit (chapter 1.1.1.). Ribosomal functional centers – decoding center where mRNA is decoded in the small subunit and peptidyl transferase center where peptide bond is catalyzed

between amino acids in the large subunit – are composed of 16S and 23S rRNA, respectively (Ban et al., 2000). Hence, ribosome is a ribozyme (Nissen et al., 2000). rRNA molecules considerably vary in size: in *E. coli* there are 1542 nucleotides in 16S rRNA, 120 nucleotides in 5S rRNA and 2904 nucleotides in 23S rRNA. In addition, the length of rRNA molecules depends on the specific bacterial species, for example in *Mycobacteria* the 23S rRNA is by more than 200 nucleotides longer than in *E. coli* whereas 16S and 5S rRNAs are slightly shorter (Kushwaha and Bhushan, 2020). Several rRNA positions are chemically modified. In *E. coli* the 70S ribosome has 36 modified nucleotides: 11 in the small subunit 16S rRNA and 25 in the large subunit 23S rRNA (Sergeeva et al., 2015). Three major rRNA modification types are pseudouridines, methylation of the 2' OH group of riboses and methylation of the base catalyzed mostly during ribosome biogenesis by site- or region-specific enzymes in bacteria (Decatur and Fournier, 2002). As a result of modifications properties of nucleotides can change, for example base stacking, hydrophobicity, structure flexibility (Antoine et al., 2021). This in turn can affect ribosome subunit assembly and activity considering that many modifications are located in the conserved functional centers of the ribosome (Sergeeva et al., 2015). Therefore, rRNA modifications are generally considered to fine tune ribosome structure and function (Antoine et al., 2021). In addition, they are thought to serve as quality control points during ribosome biogenesis and some rRNA modifications confer antibiotic resistance by for example preventing antibiotic binding to ribosome (Sergeeva et al., 2015).

Although ribosome structure and function are dominated by rRNA, r-proteins have important structural and functional roles. They participate in ribosome assembly, for example by stabilizing rRNA tertiary structure via binding rRNA helices (Ban et al., 2000; Wimberly et al., 2000), in intersubunit connection as bridge components (chapter 1.1.2.) (Liu and Fredrick, 2016) and in signal transmission within the ribosome (Poirot and Timsit, 2016). However, due to cooperativity within the ribosome it is often difficult to causally associate a single r-protein with a defined role in ribosome function (Wilson and Nierhaus, 2005).

R-proteins are relatively basic (average pI = 10.1) and small proteins in terms of molecular mass (4.4 – 61.2 kDa in *E. coli*) and length (38 – 557 amino acids in *E. coli*) (Stelzl et al., 2001) (Table 1 and 2). In *Bacteria* there are more than 50 r-proteins with the exact number slightly varying among species (Kushwaha and Bhushan, 2020). *E. coli* ribosome contains 54 r-proteins (21 in the small subunit, 33 in the large subunit) that are present in ribosomes in a single copy (Nikolay et al., 2015). The only exception is bL12 ranging from two to four copies of bL12 dimers in different bacterial species (two dimers in *E. coli*) while it is not known why ribosome uses several copies of bL12 (Chang et al., 2015). Importantly, a definition of r-proteins is based on their stoichiometry in ribosomes: r-proteins are those "present in stoichiometric amounts in the ribosome" (Wilson and Nierhaus, 2005). In general, every ribosomal protein is encoded by a single gene. Notable exceptions are some r-proteins that have paralogous genes (chapter 1.3.2.5). In *E. coli* two r-protein (bL31, bL36) encoding genes have paralogs (Makarova et al., 2001). Both paralogous proteins have been found in the X-ray

structure of *E. coli* 70S ribosomes with similar binding sites (Lilleorg et al., 2019; Watson et al., 2020) meaning that they bind to a given ribosome mutually exclusively. Therefore, the whole set of possible r-proteins in *E. coli* comprises 56 entities with 54 being simultaneously present in ribosomes. Interestingly, 25 of 54 r-proteins are not essential since corresponding *E. coli* single deletion strains are viable (Baba et al., 2006; Shoji et al., 2011) (Table 1 and 2).

Table 1. *E. coli* ribosomal proteins from the small subunit. R-protein names are from (Ban et al., 2014), gene names and length from (Stelzl et al., 2001), data about molecular mass and isoelectric points from (Nikolay et al., 2015). Data about essentiality for growth is based on (Baba et al., 2006; Shoji et al., 2011): yes – single deletion strain could not be constructed; no – single deletion strain viable.

name	gene	essentiality for growth	molecular mass (kDa)	length (number of amino acids)	isoelectric point (pI)
bS1	*rpsA*	yes	61.26	557	4.88
uS2	*rpsB*	yes	26.74	240	6.61
uS3	*rpsC*	yes	25.98	232	10.27
uS4	*rpsD*	yes	23.47	203	10.05
uS5	*rpsE*	yes	17.60	166	11.11
bS6	*rpsF*	no	15.70	135	4.90
uS7	*rpsG*	yes	20.02	177	10.36
uS8	*rpsH*	yes	14.13	129	9.44
uS9	*rpsI*	no	14.86	129	10.94
uS10	*rpsJ*	yes	11.74	103	9.68
uS11	*rpsK*	yes	13.84	128	11.33
uS12	*rpsL*	yes	13.74	123	10.88
uS13	*rpsN*	no	13.10	117	10.78
uS14	*rpsM*	yes	11.58	98	11.16
uS15	*rpsO*	no	10.23	88	10.40
bS16	*rpsP*	yes	9.19	82	10.54
uS17	*rpsQ*	no	9.70	83	9.64
bS18	*rpsR*	yes	8.99	74	10.60
uS19	*rpsS*	yes	10.43	91	10.52
bS20	*rpsT*	no	9.68	86	11.18
bS21	*rpsU*	no	8.50	7	11.15

Table 2. *E. coli* ribosomal proteins from the large subunit. R-protein names are from (Ban et al., 2014), gene names and length from (Stelzl et al., 2001), data about molecular mass and isoelectric points from (Nikolay et al., 2015). Data about essentiality for growth is based on (Baba et al., 2006; Shoji et al., 2011): yes – single deletion strain could not be constructed; no – single deletion strain viable.

name	gene	essentiality for growth	molecular mass (kDa)	length (number of amino acids)	isoelectric point (pI)
uL1	*rplA*	no	24.70	233	9.61
uL2	*rplB*	yes	29.86	272	10.93
uL3	*rplC*	yes	22.24	209	9.90
uL4	*rplD*	yes	22.09	201	9.72
uL5	*rplE*	yes	20.30	178	9.49
uL6	*rplF*	yes	18.90	176	9.71
bL7	*rplI*	yes	12.30	120	4.60
bL9	*rplJ*	no	15.77	148	6.15
uL10	*rplK*	yes	17.71	164	9.04
uL11	*rplL*	no	14.88	141	9.64
bL12	*rplI*	yes	16.02	120	9.91
uL13	*rplM*	yes	13.54	142	10.43
uL14	*rplN*	yes	14.98	123	11.18
uL15	*rplO*	no	15.28	144	11.22
ul16	*rplP*	yes	14.36	136	11.05
bL17	*rplQ*	yes	12.77	127	10.42
uL18	*rplR*	yes	13.13	117	10.62
bL19	*rplS*	yes	13.50	114	11.47
bL20	*rplT*	yes	11.56	117	9.85
bL21	*rplU*	no	12.23	103	10.23
uL22	*rplV*	yes	12.23	110	10.23
uL23	*rplW*	yes	11.20	100	9.94
uL24	*rplX*	no	11.32	103	10.21
bL25	*rplY*	no	10.69	94	9.60
bL27	*rpmA*	no	9.12	84	10.58
bL28	*rpmB*	yes	7.69	77	11.25
uL29	*rpmC*	no	7.27	63	9.98
uL30	*rpmD*	no	6.54	58	10.96
bL31A	*rpmE*	no	7.87	70	9.46
bL31B	*ykgM*	no	9.92	87	9.30
bL32	*rpmF*	no	6.54	56	11.03
bL33	*rpmG*	no	6.37	54	10.25
bL34	*rpmH*	no	5.38	46	13.00
bL35	*rpmI*	no	7.29	64	11.78
bL36A	*rpmE*	no	4.36	38	10.69
bL36B	*ykgO*	no	5.47	46	11.40

Several aspects of r-proteins are reflected in their nomenclature. R-protein names consist of three parts – a prefix, a capital letter and a number – referring to their evolutionary conservation, belonging to the respective subunit and size and acidity, respectively (Ban et al., 2014). First, based on homology r-protein names have been assigned prefixes: u for universal, b for bacterial, e for eukaryotic and a for archaeal (Ban et al., 2014). *E. coli* ribosome contains altogether 54 r-proteins, including 34 universally conserved and 20 bacterial-specific r-proteins (Melnikov et al., 2012). Second, whether a r-protein belongs to the small or large subunit is indicated by S or L in their names. Third, the size and acidity of r-proteins are associated with the numbering system as the original numbering was based on their location on the 2D polyacrylamide gel (Kaltschmidt and Wittmann, 1970). As a result of this large acidic r-proteins have smaller numbers (for example bS1, 61.2 kDa, isoelectric point 4.88) and small basic ones larger numbers (for example bL35, 7.2 kDa, isoelectric point 11.78) (Nikolay et al., 2015) (Table 1 and 2).

Several ribosomal proteins (11/54 in *E. coli*) are post-translationally chemically modified: six r-proteins are methylated (uL11, uL3, uS11, uL6, bL33 and bL7/bL12 whereas bL7 is the N-acylated form of bL12), three acetylated (uS5, bS18, bL7/L12), one methylthiolated (uS12), one glutamylated (bS6) (Nesterchuk et al., 2011). The majority of bacterial r-protein modifications is proposed to be constitutive and to contribute to ribosome biogenesis, however their biological function is not understood (Lammers, 2021; Nesterchuk et al., 2011).

R-proteins are distributed unevenly in the ribosome with the most r-protein rich regions located on the solvent side and the intersubunit side being dominated by rRNA (Ban et al., 2000; Wimberly et al., 2000). R-proteins have typically a globular domain that is usually located on the surface of the subunit and extension(s) that reach the rRNA rich interior of the respective subunit (Wilson and Nierhaus, 2005). Due to positive charge and high isoelectric point r-proteins interact with negatively charged rRNA. All r-proteins (except for bL12, bS1) interact with rRNA (Chang et al., 2015) with the majority of them having one or two r-protein interaction partners (Poirot and Timsit, 2016). In addition, there are 15 r-proteins that do not make any intrasubunit connections with another r-proteins (Poirot and Timsit, 2016). Analysis of 50S crystal structures has shown that the extensions of r-proteins form a network where certain r-proteins interact directly with ribosomal functional centers whereas other r-proteins connect them thereby enabling information flow during protein synthesis (Poirot and Timsit, 2016). Poirot and Timsit hypothesize that intrasubunit protein-protein interactions may be important for binding translation factors and coordinating complex molecular motions during translation (Poirot and Timsit, 2016).

1.1.1. Ribosomal subunits

The small subunit of the bacterial/ *E. coli* ribosome contains 16S rRNA (1542 nucleotides) and 21 r-proteins (bS1 – bS21) (Nikolay et al., 2015; Wilson and Nierhaus, 2005) (Table 1). The secondary structure of the 16S rRNA comprises 45 helices (denoted h1 – h45) and is divided into four domains – the 5', central, 3' major and 3' minor domain – that interact with r-proteins and form the structural domains of the small subunit: the head with the beak, the platform and the body with the shoulder and the spur (Schuwirth et al., 2005; Wimberly et al., 2000; Yusupov et al., 2001) (Figure 1). The head domain of the small subunit is composed of the 3' major domain of the 16S rRNA and r-proteins uS2, uS3, uS7, uS9, uS10, uS13, uS14, uS19 (Nikolay et al., 2015; Wilson and Nierhaus, 2005). The beak is formed by h33 of the 3' major domain of the 16S rRNA (Wilson and Nierhaus, 2003). The head of the 30S subunit is connected to the body via a flexible neck (h28) enabling the head to move during translocation (Korostelev et al., 2008; Yusupov et al., 2001). The platform comprises the central domain of the 16S rRNA and bS1, bS6, uS8, uS11, uS15, bS18 (Nikolay et al., 2015; Wilson and Nierhaus, 2005). The body domain contains 5' and 3' minor domains of the 16S rRNA with uS4, uS5, uS12, bS16, uS17 and bS20 (the spur is made of h6 from the 5' domain of the 16S rRNA (Yusupov et al., 2001)). 3' minor domain contains two helices (h44 and h45) at the subunit interface with h44 giving multiple intersubunit interactions (chapter 1.2.) (Wimberly et al., 2000). Additionally, the decoding center in the aminoacyl tRNA binding site (A-site) and the anti-Shine-Dalgarno sequence in h45 important for bacterial translation initiation (chapter 1.2.1.) are found in this rRNA domain (Wilson and Nierhaus, 2005).

During translation the small subunit binds mRNA, tRNAs, initiation factors (IF1, 2, 3) and is responsible for decoding, i.e. monitoring correct base-pairing between the mRNA codon and anticodon present in the corresponding tRNA (Yusupov et al., 2001). Hence, the functional centers in the small subunit are the mRNA path (with its entry and exit site), three tRNA binding sites (the A-, P- and E-site) and the decoding center (DC) (Figure 1). The path of mRNA on the small subunit binds about 30 nucleotides and is composed of an entry site, followed by a tunnel (diameter 15 Å) both consisting mainly of uS3, uS4, uS5 (Wilson and Nierhaus, 2005; Yusupova et al., 2001). Next comes a rRNA-dominated region where at least six mRNA nucleotides are exposed to the ribosomal subunit interface in the A- and P-site followed by a tunnel region surrounding about 15 nucleotides of the mRNA (Laursen et al., 2005; Wilson and Nierhaus, 2005). The mRNA path exit is surrounded by r-proteins uS7, uS11 and bS18 (Kurkcuoglu et al., 2008).

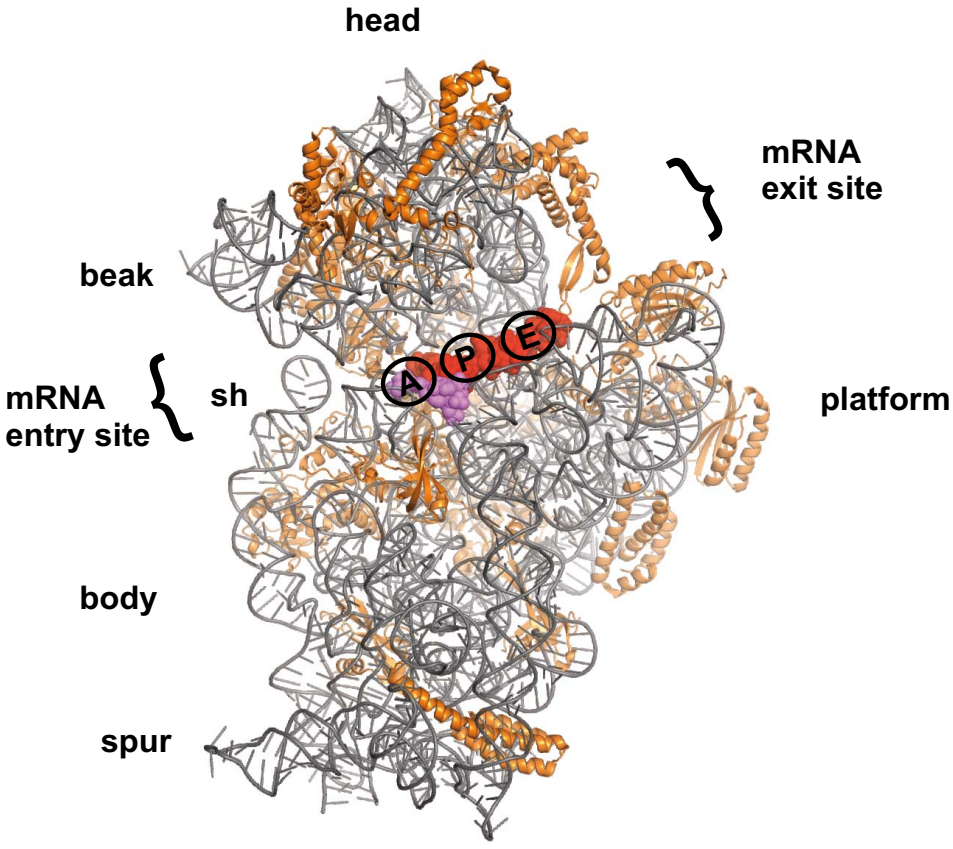

Figure 1. Intersubunit view of the bacterial ribosomal small subunit. 16S rRNA and r-proteins are grey and orange, respectively. Decoding center (pink) and mRNA (red) are indicated in spheres. Domains of the small subunit, approximate positions of the tRNA binding sites (A-, P- and E-site), mRNA entry and exit sites are indicated. sh – shoulder. This figure is based on the PBD coordinates 7K00 (Watson et al., 2020) rendered in PyMOL.

There is a groove between the head, body, and platform of the 30S subunit where three tRNAs bind (Yusupov et al., 2001). Small and large subunit have each three binding sites for tRNAs: the A-site binds the incoming aminoacyl tRNA (aa-tRNA), the P-site binds the peptidyl tRNA and the deacylated tRNA is in the E-site (exit site) (Yusupov et al., 2001) (Figures 1 and 2). At all three binding sites a given tRNA is in contact with both ribosomal subunits with its anticodon arm interacting with the 30S subunit and its acceptor arm with the 50S subunit (Khade and Joseph, 2010). Interactions between tRNAs and the ribosome are important for correct aminoacyl-tRNA selection to the A-site, maintaining the correct reading frame, translocation of tRNAs through the ribosome and peptide bond formation (Jenner et al., 2010a; Selmer et al., 2006; Yusupov et al., 2001).

In the A-site, interactions between mRNA codon and tRNA anticodon are monitored by three residues of 16S rRNA (A1492, A1493 of helix 44 and G530 from helix 18) (Khade and Joseph, 2010). After the correct base-pairing has been detected, the incoming aminoacyl tRNA will be accommodated to the large subunit presenting its amino acid to the peptidyl transferase center (PTC) (Jenner et al., 2010a; Selmer et al., 2006). From the small subunit side, the A-site is formed by uS12, uS13 and nucleotides from the 5' domain of the 16S rRNA whereas uL16, bL27 and nucleotides from domain II and V of the 23S rRNA form the A-site from the 50S subunit side (Jenner et al., 2010a; Khade and Joseph, 2010). The role of the P-site is to hold the tRNA to maintain the reading frame in the 30S subunit and to present it for peptidyl transferase reaction in the PTC of the 50S subunit (Selmer et al., 2006). The P-site tRNA contacts uS9 and uS13 and 3' major domain of the 16S rRNA, in addition to uL5, uL16, bL27 and elements from domains II and V of the 23S rRNA (Bowman et al., 2020; Khade and Joseph, 2010; Selmer et al., 2006). The E-site tRNA interacts with uS7 and uS11 and central domain of the 16S rRNA and uL1, bL28, bL33 and domain II and IV of the 23S rRNA (Bowman et al., 2020; Khade and Joseph, 2010).

The large subunit of the bacterial/ *E. coli* ribosome is composed of 23S and 5S rRNA (2904 and 120 nucleotides, respectively) and 33 r-proteins (uL1 – bL36) (Table 2). Three r-proteins are missing since pentamer of $(bL12)_4$ and uL10 was initially named L8, bL12 is a non-acetylated form of bL7 and L26 is identical with bS20 (Wilson and Nierhaus, 2005). 5S rRNA is a highly conserved molecule whose autonomous nature has been recently demonstrated by engineered ribosome with hybrid 5S-23S rRNA molecule to not to be required either for protein synthesis or any vital extra-ribosomal functions (Huang et al., 2020). Instead, the role of 5S rRNA as a separate molecule has been proposed to be in ribosome assembly (Huang et al., 2020). Secondary structure of the 23S rRNA is divided into seven domains (domain 0 – VI) consisting of 101 helices (denoted H1 – H101) (Petrov et al., 2013). In contrast to the small subunit, there is no direct relationship between secondary structure domains of 23S rRNA and morphological features of the 50S subunit. In other words, functional centers and structural landmarks of the large subunit are formed of different 23S rRNA domains that are connected with each other forming a hemispherical body of the 50S subunit.

The overall shape/ tertiary structure of the 50S subunit is characterized by three protuberances: the bL12 stalk, central protuberance and uL1 stalk (Figure 2). In the intersubunit view of the large subunit the bL12 stalk is located on the right and it is composed of uL10 (in complex with two bL12 dimers in *E. coli*) and uL11 that both bind to 23S rRNA domain II nucleotides 1030–1124 (Chang et al., 2015). C-terminal domains of the bL12 dimers interact with translation factors and they are proposed to deliver elongation factors EF-Tu, EF-G to the factor binding site on the large subunit (Chang et al., 2015).

The central protuberance (CP) comprises multiple r-proteins (uL5, uL16, uL18, bL25, bL27, bL31, bL33, bL35), 5S rRNA and nucleotides from 23S rRNA domains II and V (Korepanov et al., 2012; Yusupov et al., 2001). Several

r-proteins located in the central protuberance (uL5, uL16, bL25, bL27) are in contact with the A- and P-site tRNAs contributing to tRNA selection to the A-site and positioning it correctly for the peptide bond formation (Jenner et al., 2010a; Selmer et al., 2006). Ribosomal structures indicate that these r-proteins with elements from 23S rRNA form a dynamic network suggested to transmit the signal of correct decoding to the PTC via ribosome (Jenner et al., 2010a) thus contributing to translation accuracy. In this network intersubunit contacts of uL5 and bL31 with uS13, uS19 are of central importance (bridge B1b, chapter 1.1.2.).

The third structural landmark of the large subunit – the uL1 stalk – is located near the E-site and is composed of uL1 and 23S rRNA domain V H76-78 (Chang et al., 2015; Yusupov et al., 2001). The uL1 stalk is very mobile during translation adopting an open conformation when the E-site is vacant and a closed conformation interacting with the tRNA in the hybrid P/E-state (chapter 1.2.2.) (Chang et al., 2015). uL1 stalk is proposed to be important for the movement of deacylated tRNA from the P-site to the E-site and its removal from the ribosome (Schuwirth et al., 2005; Wilson and Nierhaus, 2005).

During translation, the large subunit is responsible for catalyzing peptide bond formation, directing the growing peptide out of the ribosome in addition to binding translation elongation factors and tRNAs. These tasks are accomplished in several functional centers: peptidyl transferase center, growing peptide tunnel, GTPase-associated center, three tRNA binding sites (described above). Peptidyl transferase center (PTC) is composed of five universally conserved nucleotides of 23S rRNA (C2063, A2451, U2506, U2585, and A2602) forming the inner core of PTC (Trappl and Polacek, 2011; Yusupov et al., 2001). The second layer of PTC is formed by 23S rRNA nucleotides that hold tRNAs in correct position for the peptidyltransferase reaction. R-protein bL27 has been shown to be close to the inner core of the PTC but it does not participate in the catalytic reactions (Jenner et al., 2010a; Trappl and Polacek, 2011).

Growing peptide starts to move through the large subunit outside the ribosome as more amino acids are incorporated. This path termed the growing peptide tunnel (synonymous with nascent chain tunnel, exit tunnel) stretches about 100Å from the PTC to the solvent side of the large subunit (Trappl and Polacek, 2011; Wilson and Nierhaus, 2005). The exit tunnel has a diameter of 10 – 20 Å and it is mainly composed of 23S rRNA from different domains and uL4, uL22, uL23, uL24 (Koubek et al., 2021; Liutkute et al., 2020). It has been estimated that about 1/3 of the *E. coli* proteins starts to fold in the tunnel (Ciryam et al., 2013). In addition, ribosome is able to sense interactions of the growing peptide with the tunnel resulting in regulation of translation elongation and termination (Liutkute et al., 2020; Wilson and Nierhaus, 2005). The exit of the growing peptide tunnel is surrounded by uL22, uL23, uL24 and uL29 (Wilson and Nierhaus, 2005) and it binds chaperones, for example trigger factor that assist the growing peptide in achieving its native fold.

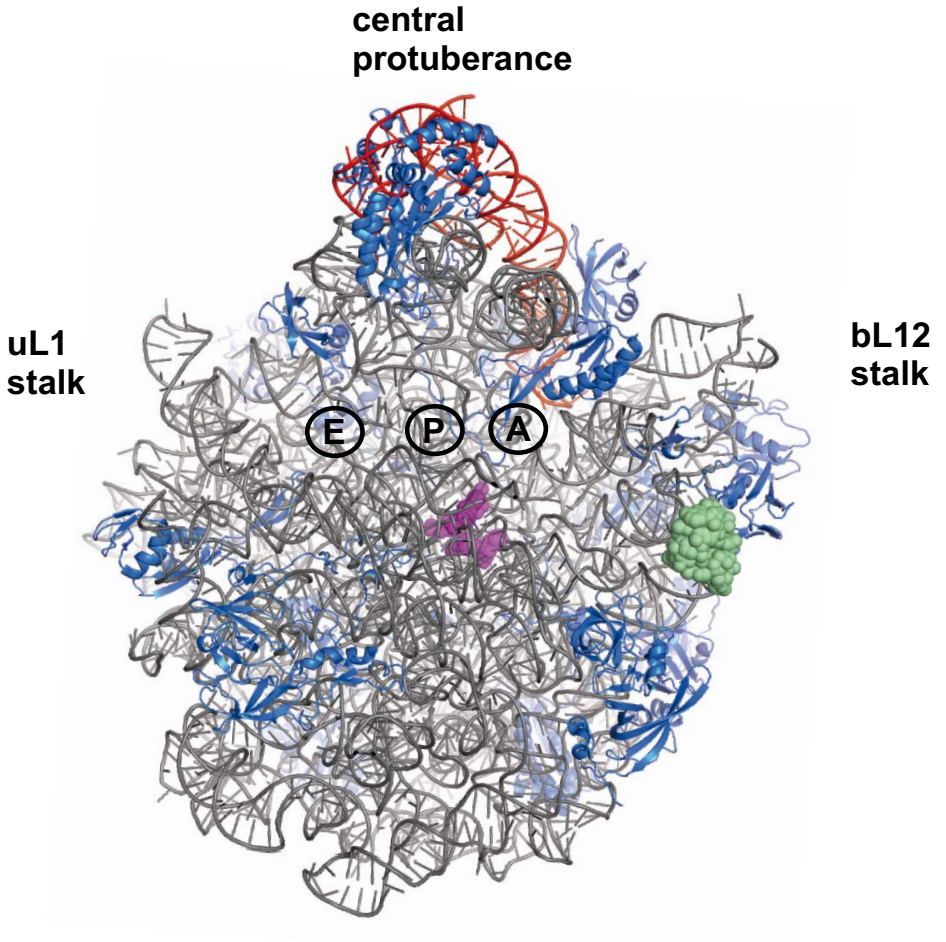

Figure 2. Intersubunit view of the bacterial ribosomal large subunit. 5S rRNA, 23S rRNA and r-proteins are red, grey, and blue, respectively. Structural landmarks of the large subunit (uL1 stalk, bL12 stalk, central protuberance), approximate positions of the tRNA binding sites (A-, P- and E-site) are indicated. Peptidyl transferase center and sarcin-ricin loop are pink and green, respectively. Peptide exit tunnel is not shown because it starts from the P-site and reaches the solvent side of the large subunit. This figure is based on the PBD coordinates 7K00 (Watson et al., 2020) rendered in PyMOL.

GTPase-associated center (GAC) is composed of H42-44 of the domain II of 23S rRNA and uL11, uL10 and bL12 (two dimers in *E. coli*) (Clementi and Polacek, 2010). It has been proposed that the role of this center composed of the bL12 stalk is to recruit translation elongation factors (Clementi and Polacek, 2010). The second binding site for elongation factors – the sarcin-ricin loop (SRL) in the IV domain of 23S rRNA – is likely to activate GTPases with its adenine in position 2660 (Clementi et al., 2010; Yusupov et al., 2001).

1.1.2. Intersubunit bridges

During translation small and large ribosomal subunits are bound to each other via molecular contacts (termed bridges) as demonstrated by high resolution crystal structure of 70S from *Thermus thermophilus* (Yusupov et al., 2001). Each of the 12 bridges is composed of several single interactions and based on the type of interacting partners bridges are classified into three sections: five rRNA-rRNA, six rRNA-r-protein or one r-protein-r-protein bridges (Figure 3) (Yusupov et al., 2001). Chrystal structure of the 70S ribosome of *T. thermophilus* demonstrates that rRNA residues forming intersubunit bridges are not distributed evenly on the secondary structure of rRNA but clustered to the IV domain of 23S rRNA (except for H38 and H34 from the domain II forming B1a and B4, respectively) in the large subunit. In the small subunit most bridge forming rRNA residues are in the central and 3' minor domain of the 16S rRNA (in addition to h14 from the 5' domain forming B8) (Yusupov et al., 2001) with the hot spot in h44 participating in four bridges (B2a, B3, B5, B6, Table 3) (Yusupov et al., 2001). Altogether, nine r-proteins are involved in bridge interactions: uS13, uS19 and uS14 from the 30S head domain and uS15 from the 30S platform in addition to uL2, uL14 and bL19 from the 50S body and uL5 and bL31 from the central protuberance (Table 3). In general, r-protein positions involved in bridge interactions exhibit higher bacterial species specific sequence variation than rRNA positions (for example B3, B1b, B4, B7a) (Liu and Fredrick, 2016). However, comparisons of the 70S structures of *T. thermophilus* and *E. coli* have shown that the general architecture of some bridges (B4, B6) is largely the same despite species-specific sequence variations in bridge interactions (Liu and Fredrick, 2016).

In addition to holding subunits together ribosomal bridges facilitate inter-subunit movements essential for translation. These movements include a counter-clockwise rotation of the 30S relative to the 50S (i.e. the 30S spur towards the bL12 stalk) and rotation of the 30S head relative to the rest of the 30S subunit domains and the 50S towards the E-site (head swiveling) (Liu and Fredrick, 2016). These ribosomal movements are essential for translocation, i.e. stepwise transport of the tRNA-mRNA complex through the ribosomal A-, P- and E-sites (chapter 1.2.2.). The pivot point of intersubunit rotation (up to 10 degrees) is suggested to be near the bridge B3 (Figure 3) and other bridges are broken or rearrange in different extent during this movement (Korostelev et al., 2008; Liu and Fredrick, 2016). rRNA-rRNA type bridges in the central area of the ribosome are the least dynamic during translocation whereas the only protein-protein bridge B1b located in the 30S labile head domain is the most dynamic according to x-ray crystal structures of the *E. coli* 70S (Zhang et al., 2009).

rRNA-rRNA bridges (B2a, B2b, B2c, B3 and B7a) are located centrally in the ribosomal core being the least dynamic during translation (Yusupov et al., 2001; Zhang et al., 2009). Bridges B2a, B2b and B2c form a large area of intersubunit contacts between the 30S platform and the 50S body near the tRNA binding sites (Liu and Fredrick, 2016) (Figure 3).

Table 3. Components of the intersubunit bridges of the bacterial ribosome. Bridge nomenclature and components are from (Liu and Fredrick, 2016).

bridge type	name	30S components	50S components	location
rRNA-rRNA	B2a	h44, h24, h45	H69	
	B2b	h24, h45	H68, H71	
	B2c	h24, h27	H67	near the tRNA binding sites
	B3	h44	H71, uL14	
	B7a	h23	H68	
rRNA-rp	B5	h44	H62	
	B6	h44	bL19	near the binding site for translational GTPases
	B8	h14	uL14, bL19	
	B4	uS15	H34	body, platform + uL1 stalk
	B7b	h23, h24	uL2	body, platform + uL1 stalk
	B1a	uS13	H38	head + CP
rp-rp	B1b	uS13	uL5, bL31	head + CP
	B1c	uS14, uS19, h42	bL31	head + CP

Located near the decoding center B2a is formed by h44, h24 and h45 of the 16S rRNA and H69 of the 23S rRNA (Liu and Fredrick, 2016) (Table 3). Mutations in H69 have shown that B2a is important for ribosomal processivity, translation initiation and subunit association *in vitro* (Kipper et al., 2009). B2b is formed by h24, h45 of the 16S rRNA and H68 and H71 from the 23S rRNA (Table 3). B2c is formed by h24 and h27 and H67, in addition Mg^{2+} ions coordinate bridge interactions between these helices in *T. thermophilus'* ribosomes and most likely in *E. coli* as well (Liu and Fredrick, 2016). B3 bridge connects the 30S body with the body of the 50S and is formed by h44 and H71 (Liu and Fredrick, 2016) (Figure 3, Table 3). Comparison of ribosomes in different rotation states indicates that the pivot point of ratcheting may be near this bridge (Liu and Fredrick, 2016). During intersubunit rotation B3 maintains its contacts whereas bridge B2a rearranges and B2b, B2c are broken (Liu and Fredrick, 2016). Bridges B7a and B7b together with B4 bridge connect the body and platform of the 30S subunit with the base of the uL1 stalk of the 50S body (Figure 3). B7a is an rRNA-rRNA bridge (h23 and H68) whereas B7b and B4 are formed by rRNA and r-proteins (B4: uS15 and H34 and B7b: h23, h24 and uL2) (Liu and Fredrick, 2016). On the other side of the subunit interface three rRNA – r-protein bridges B5, B6 and B8 connect the 30S body with the 50S body near the GTPase associated center (Figure 3).

One expected consequence of mutations in rRNA residues involved in bridges could be that they most likely alter subunit association. Indeed, sucrose gradient

analyses of 16S and 23S rRNA bridge mutants have shown that some bridge mutants have a substantial effect on subunit association *in vivo* as shown in increased fractions of 30S and 50S as compared to 70S (16S rRNA B3, B7b mutants) (Sun et al., 2011). At the same time, other bridge mutants (B5, B6, B8) seem to have no effect on subunit association (Liiv and O'Connor, 2006; Sun et al., 2011). This picture becomes more heterogeneous with one bridge having a substantial, slight or no effect on association depending on the specific rRNA position mutated (for example B7a, B7b) (Liiv and O'Connor, 2006; Sun et al., 2011). These results illustrate the cooperativity of bridging interactions inside ribosome bearing in mind that a single bridge is composed of several interactions and bacterial ribosomal subunits are held together by twelve bridges. However, the effects of 23S rRNA mutations on subunit association become evident *in vitro* when due to the absence of any ligands (like tRNAs) subunit joining depends more on bridges (Liiv and O'Connor, 2006).

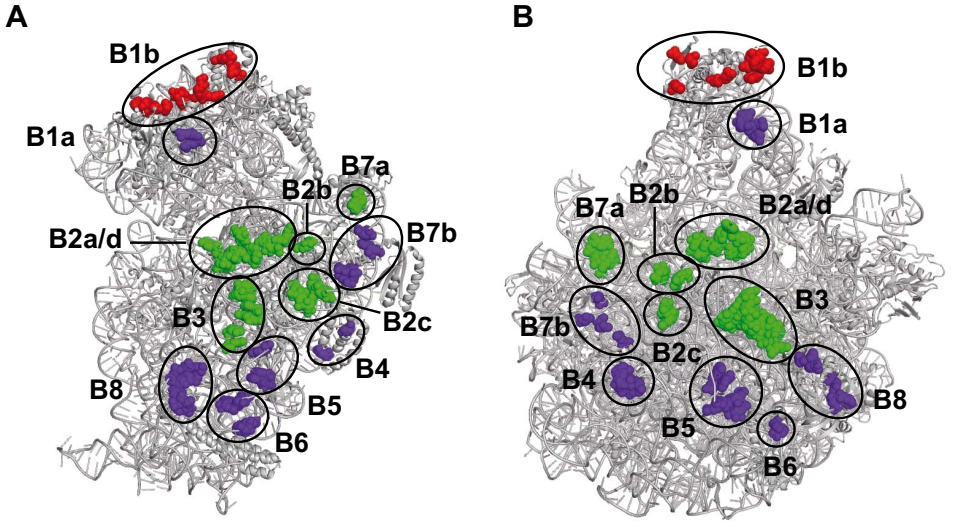

Figure 3. Intersubunit bridges of the bacterial ribosome. rRNA and r-proteins are grey. rRNA – rRNA bridges are green spheres, rRNA – r-protein bridges purple and r-protein – r-protein bridges red. Coordinates of the small subunit are from PDB entry 7K00 (Watson et al., 2020) and coordinates of the bridges are from PDB entries 3R8T (Dunkle et al., 2011) and 5AFI in the case of B1b and B7b (Fischer et al., 2015). A Intersubunit bridges of the bacterial ribosomal small subunit. B Intersubunit bridges of the bacterial ribosomal large subunit.

Interestingly, mutations in several bridges located distant from the decoding center have been shown to affect the fidelity of decoding. Substitution mutations in 16S rRNA residues involved in bridges result in increased stop codon read-through (bridges B5, B6, B8) or recognition of AUG translation start codon (B3, B5, B7b) as evidenced by β-galactosidase assay in the *E. coli* strain expressing only plasmid-encoded mutated 16S rRNA (Sun et al., 2011). The mechanism how these bridges could affect decoding fidelity is unknown. However, no effect on

reading frame maintenance was detected. Moreover, the effect of several bridges on translational fidelity is confirmed by mutations in 23S rRNA residues forming bridges (B1a, B2b, B2c, B3, B4, B5, B7a) that also result in many cases in increased stop codon readthrough and in no effect on frameshifting as evidenced by β-galactosidase assay (Liiv and O'Connor, 2006).

Bridges B1a and B1b connect the 30S head with the 50S central protuberance lying above the A- and P-site (Figure 3). Bridge B1a is formed by uS13 from the small subunit and H38 from the large subunit (Yusupov et al., 2001) (Table 3). Mobile helix 38 (the A-site finger) protrudes from the CP about 100 Å to the subunit interface directly contacting the A-site tRNA (Yusupov et al., 2001). According to crystal structure of 70S from *Thermus thermophilus* B1a bridge is intact in the post-translocational ribosomes but broken in the ratcheted ribosome (Chen et al., 2013). The only protein-protein bridge B1b is composed of uS13 and uL5 and bL31 (Chen et al., 2013; Fischer et al., 2015; Jenner et al., 2010a; Selmer et al., 2006) (Table 3). The N-terminus of bL31 interacts with uL5 and uS13 while its C-terminus interacts with uS19 and uS14 and h42 (Fischer et al., 2015). These C-terminal interactions of bL31 have been proposed to form the B1c bridge (Jenner et al., 2010a; Liu and Fredrick, 2016). High resolution *E. coli* cryo-EM structures have shown that during translocation bL31 maintains its interactions with both subunits (Fischer et al., 2015). This is facilitated by its flexible linker region between the N- and C-termini that adopts an extended and compressed conformation in non-rotated and rotated ribosome, respectively (Fischer et al., 2015). B1b bridge has been demonstrated by analysis of x-ray crystal structures of *E. coli* ribosomes to be the most dynamic bridge during translocation as it is able to accommodate movements of approximately 20 Å (Zhang et al., 2009).

Functional studies of B1a and B1b have suggested that these bridges can play a role in translation fidelity, translocation and signal transmission within the ribosome (Cukras and Green, 2005; Jenner et al., 2010a; Komoda et al., 2006). B1a and B1b bridges have been studied from the 30S side by deleting uS13 or mutating its various regions giving intersubunit interactions (B1a, B1b) or P-site tRNA contacts (Cukras and Green, 2005). Although uS13 is not essential for growth in *E. coli* its deletion results in substantial subunit association defects *in vivo* and *in vitro* in addition to translation initiation complex formation *in vitro* (Cukras and Green, 2005). uS13 mutants lacking B1b bridge or P-site tRNA contacts exhibited growth defect and increased levels of frameshifting and stop codon read through as evidenced by β-galactosidase assay (Cukras and Green, 2005). The effect of bL31 as a bridge B1b component on ribosome functioning is within the scope of this dissertation.

B1a and B1b connect the labile 30S head with the central protuberance (Figure 3). During translocation the 30S head moves with respect to the CP (1) as part of subunit rotation (up to 10 Å) and (2) head swiveling (up to 20 Å) (Zhang et al., 2009). These movements are important for controlling the position of tRNAs in the ribosome and moving them towards the P- or E-site. Both 30S head bridges have been proposed to act as translocation attenuators controlling the extent of the head swiveling during translocation (Liu and Fredrick, 2016; Shasmal

et al., 2010). Biochemical study of ribosomes with shortened H38 proposes that B1a bridge may act as regulator of translocation to maintain translational reading frame whereas its contribution to subunit association is moderate (Komoda et al., 2006). Next, many components of these bridges interact directly with the A-site tRNA (H38, uS13) or P-site tRNA (uL5, uS13) (Yusupov et al., 2001). Comparison of ribosomal structures at the cognate tRNA selection step suggests that B1a and B1b bridges may contribute to intraribosomal signal transmission between the DC in the 30S subunit and PTC of the 50S subunit (Jenner et al., 2010a). This idea has been extensively studied in *Saccharomyces cerevisiae* (Bowen et al., 2015; Rhodin and Dinman, 2011).

1.2. Bacterial translation

Ribosomes are – from functional point of view – conceptualized as translators since they mediate the transfer of the genetic information carried in mRNA nucleotides to protein sequence. Translation is carried out in ribosomes with the help of several proteins – translation factors. In the previous chapter ribosome has been depicted as a rather static macromolecular complex composing of rRNA and r-proteins. This chapter sheds light on a translating ribosome, i.e., ribosome in action with its interacting partners (substrates and factors) at a given time point during translation cycle. Translation in bacteria is described according to its four stages (initiation, elongation, termination, and ribosome recycling) by describing boundaries between them (start and end points), participating molecules and events. X-ray chrystallography and cryo-electromicroscopy have been used to obtain information about the structure of translating ribosomes in complex with various partners during different steps of protein synthesis (Jenner et al., 2010a; Milon and Rodnina, 2012; Zhou et al., 2012). With the help of single molecule fluorescence resonance energy transfer and rapid kinetics timing of translation events has been elucidated (Milon et al., 2012; Wasserman et al., 2016).

1.2.1. Translation initiation

In bacteria, translation can be initiated during mRNA synthesis or post-transcriptionally with the latter way being the prevalent one. This is indicated by the comparison of mRNA median lifetimes (about 5 minutes in *E. coli* (Bernstein et al., 2002)) and duration of transcription elongation (depending on the gene sequence approximately 28–40 seconds for 1 kb gene (Proshkin et al., 2010)). One mRNA is usually translated by several ribosomes, collectively termed polysomes. Most experimental data has been acquired using free mRNAs, i.e. those not bound to the RNA polymerase or another ribosome (Rodnina, 2018). Therefore, very little is known about translation initiation in ribosome-RNA polymerase complex (Kohler et al., 2017) and in polysomes (Borujeni and Salis, 2016; Rodnina, 2018).

During initiation the message to be translated (a mRNA and a reading frame) is selected and the enzyme catalyzing peptide bond synthesis is formed from both of its subunits. Bacterial mRNAs differ in the presence of the Shine-Dalgarno (SD) sequence in its ribosomal binding site and in the presence of the 5' untranslated region (5' UTR). Based on this, mRNAs are classified as SD-led, non-SD-led and leaderless mRNAs (without 5' UTR) (Chang et al., 2006; Shine and Dalgarno, 1974). The proportion of these mRNA types varies between bacterial species ranging from about 10% to 90% for SD-led mRNAs as shown by analysis of 141 bacterial genomes (Chang et al., 2006). In *E. coli* about 54% of genes contains SD sequence (consensus sequence GGAGG) (Nakagawa et al., 2010). Therefore, there are different translation initiation pathways to the elongation competent ribosome that binds an initiator tRNA and start codon of mRNA in its P-site.

The efficiency of translation initiation depends on several mRNA features. Translation initiation region of an mRNA involves nucleotides –20 to +15 around start codon and it typically (but not absolutely) involves the Shine-Dalgarno sequence that pairs with the corresponding anti-Shine-Dalgarno (aSD) sequence near the 3' end of 16S rRNA (Milon and Rodnina, 2012; Shine and Dalgarno, 1974; Steitz and Jakes, 1975). As a result of SD-aSD interaction the start codon (AUG, GUG, UUG) is placed into the P-site where it is recognized by initiator tRNA (Milon and Rodnina, 2012). In addition to the SD sequence the efficiency of translation initiation of a certain mRNA is affected by the start codon, stability of its secondary structure near the start codon and A/U-rich elements of mRNA that are recognized by r-protein bS1 (Milon and Rodnina, 2012). For effectiveness of translation initiation features of mRNA may be complemented by ribosome composition that has been shown to be heterogeneous in various organisms from all three domains of life (chapter 1.3).

The majority of knowledge about translation initiation comes from experiments with SD-led mRNAs followed by leaderless mRNAs that bind directly to 70S (Milon and Rodnina, 2012). In contrast, it is unknown how translation is initiated on non-SD-led mRNAs. Therefore, the following overview of translation initiation is based/focused on SD sequence containing mRNAs followed by leaderless mRNAs. Lastly, as bacterial mRNAs are polycictronic a ribosome may initiate translation of the next cistron on the same mRNA after translation termination without ribosome recycling step (70S scanning mechanism). Although there are many pathways to the elongation competent 70S ribosome, bacterial translation initiation involves following participants: 30S subunit, three initiation factors (IF1, 2, 3), mRNA, initiator- tRNA (fMet-tRNA^{fMet}), 50S subunit.

30S binding (or canonical pathway) proceeds via three assembly intermediates: 30S preinitiation complex (30S PIC), 30S initiation complex (30S IC) and 70S initiation complex (70S IC) (Milon and Rodnina, 2012) (Figure 4A). First, 30S PIC is formed by three initiation factors (IF1, 2, 3), initiator tRNA (fMet-tRNA^{fMet}) and mRNA associating with the small subunit. Interestingly, there is no strict order of factor binding to 30S subunit, i.e. initiation factors and mRNA can bind to the small subunit independently of each other (Rodnina, 2018).

Nevertheless, a kinetically preferred order has been suggested based on arrival times calculated using association rates and *in vivo* concentrations of initiation factors in *E. coli* (Rodnina, 2018). According to this IF3 and IF2 bind first (Figure 4A, step 1) followed by IF1 (step 2), initiator tRNA (step 3) and mRNA (step 4) (Milon and Rodnina, 2012). The 30S PIC formation typically starts with IF3 binding either to a free 30S subunit (Figure 4A, step 1) or to a 30S subunit in complex with a translated mRNA and a deacylated tRNA during ribosome recycling (Figure 6, step 7a). The latter pathway results in release of the translated mRNA and tRNA from the previous round of translation (Laursen et al., 2005; Milon and Rodnina, 2012). In this way IF3 binding connects ribosome recycling and translation initiation thereby closing the cycle of translation (initiation-elongation-termination-recycling) (Milon and Rodnina, 2012). IF3 (180 aa, 20.56 kDa in *E. coli*) binds to the platform of 30S subunit and inhibits ribosomal subunit association (Milon and Rodnina, 2012).

IF2 (890 aa, 97.35 kDa), a translational GTPase, binds to the subunit interface interacting with 30S platform and body and GTPase-associated center in the 50S subunit (Laursen et al., 2005). IF2 binds the initiator tRNA either on the 30S subunit or before binding to the 30S bringing it to the 30S PIC (Milon and Rodnina, 2012; Rodnina, 2018). IF2 distinguishes initiator tRNA from elongator tRNAs based on their structural differences (Laursen et al., 2005) like the N-formulated methionine in the 3' CCA-end of the initiator tRNA. This leads to the exclusion of this type of tRNA from translation elongation where methionine is transported by the elongator $tRNA^{Met}$ in complex with EF-Tu (Laursen et al., 2005). Third factor IF1 (72 aa, 8.25 kDa) binds to the A-site of the 30S subunit close to uS12, h44 of the 16S rRNA (Milon and Rodnina, 2012) thereby occupying the A-site and preventing tRNAs from binding there until the 70S IC has been formed (Laursen et al., 2005). IF1 strengthens the binding of IF2 and IF3 to the small subunit and controls the conformational dynamics thus affecting mRNA and initiator-tRNA selection (Milon and Rodnina, 2012).

After initiation factors and initiator-tRNA have been attached to the 30S subunit mRNA is thought to bind to 30S (Figure 4A, step 4). Important aspects of initial mRNA binding to the 30S are its intracellular concentration, the secondary structure of its translation initiation region, the presence of SD sequence and accessibility of A/U sequences that bind ribosomal protein bS1 (Milon and Rodnina, 2012). mRNA binds to the platform of 30S subunit close to uS2, uS7, uS11, bS18, bS21 and bS1 (Jenner et al., 2005; Marzi et al., 2007). After binding mRNA secondary structures have to unfold to make stretches of SD sequence and start codon single-stranded (Marzi et al., 2007). After establishment of SD-aSD interactions mRNA start codon is placed into the P-site of 30S subunit so that is can interact with the initiator tRNA (Milon and Rodnina, 2012). Importantly, it is the start codon recognition in the P-site of 30S subunit by initiator tRNA that converts the labile 30S PIC to the more stable 30S IC (Figure 4A, step 5) (Milon and Rodnina, 2012; Rodnina, 2018).

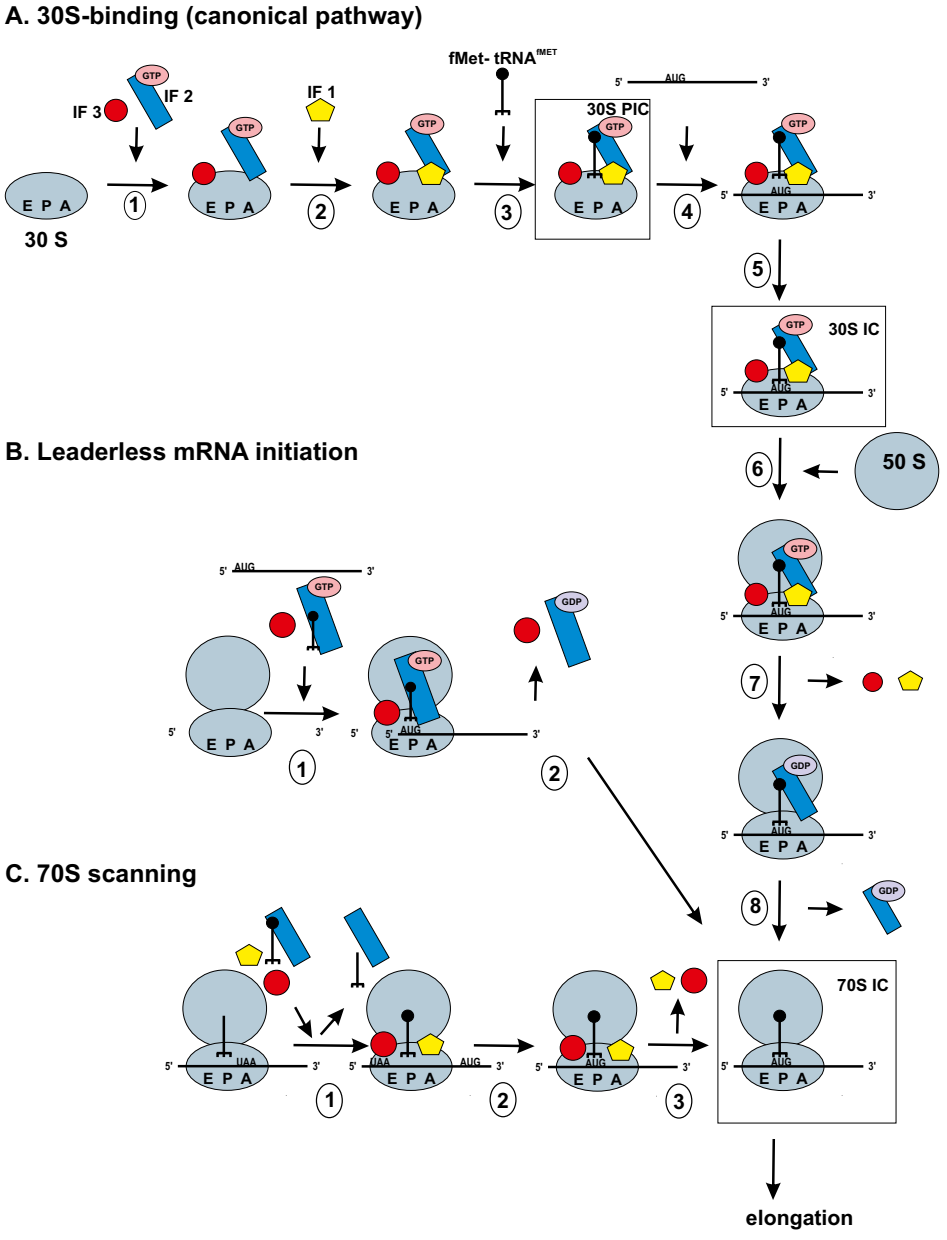

Figure 4. Model of the three pathways of bacterial translation initiation (with steps described in the main text). **I. Canonical or 30S binding pathway.** Formation of the 30S pre-initiation complex (30S PIC). 1 – binding of IF3, GTP-bound IF2. 2 – binding of IF1. 3 – binding of initiator tRNA. Formation of the 30S initiation complex (30S IC). 4 – binding of mRNA. 5 – start codon recognition. Formation of the 70S initiation complex (70S IC). 6 – 50S binding. 7 – GTP hydrolysis, dissociation of IF3, IF1. 8 – dissociation of GDP-bound IF2. Based on (Milon and Rodnina, 2012). **II Leaderless mRNA initiation.** 1 – binding of IF3, GTP-bound IF2, initiator tRNA, mRNA. 2 – GTP hydrolysis, dissociation of GDP-bound IF2, IF3. Based on (Yamamoto et al., 2016). **III 70S scanning.** 1 – binding of IF1, IF3, IF2, initiator tRNA, dissociation of IF2, deacylated tRNA. 2 – 70S scanning. 3 – dissociation of IF1, IF3. (Yamamoto et al., 2016)

During start codon recognition translational reading frame is established by interactions between the mRNA start codon (AUG, GUG, UUG exist in approximately 90%, 8% and 1% of mRNAs in *E. coli* (Laursen et al., 2005)) and anticodon of the initiator tRNA that recognizes different start codons.

The last step of SD-led initiation is the formation of 70S IC. First, a 50S subunit binds to the 30S IC where IF2 provides a large docking site for it (Figure 4A, step 6). Large subunit binding promotes GTP hydrolysis by IF2 and release of IF3 and IF1 (Figure 4A, step 7). In the GDP-bound conformation IF2 has lost several interactions with initiator tRNA and the ribosome and is hence ready to leave the initiation complex (Figure 4A, step 8) (Myasnikov et al., 2005). The resulting 70S IC is composed of associated small and large ribosomal subunits with a mRNA bound to the small subunit so that its start codon and initiator tRNA are placed in the P-site leaving the A-site empty for the incoming aminoacyl elongator tRNA. In addition to mRNAs with SD sequence whose translation is initiated via canonical pathway, there are bacterial mRNAs without SD sequence in their 5' UTR but very little is known about their translation initiation (Rodnina, 2018).

In addition to SD-led mRNAs there is a considerable number of mRNAs without 5' UTR (leaderless mRNAs, lmRNAs). *In silico* analysis of 953 bacterial genomes indicates that about 20% of them contains leaderless genes with the average proportion of leaderless genes ranging from 2.9 to 39.4% in different taxonomic groups (Zheng et al., 2011). In gamma-proteobacteria, including *E. coli* about 4.5% of genes are leaderless (Zheng et al., 2011). Characteristically these mRNAs do not contain any other ribosome binding sites except for the AUG start codon (Moll et al., 2004) that has been shown to be required for ribosome binding according to toeprint data (Brock et al., 2008). The absence of canonical ribosome binding sites found in SD-led mRNAs raises the question of how a ribosome can initiate translation of lmRNAs. *In vivo* and *in vitro* evidence indicates that 70S monosomes are capable of translation initiation of naturally occurring lmRNAs in *E. coli* (Moll et al., 2004; Udagawa et al., 2004). However, r-protein bS1 is not required (Moll et al., 2004) as opposed to the canonical translation initiation pathway where bS1 binds to mRNA enhancer sequences upstream of the SD and helps to unfold mRNA secondary structures (Eugenio Leiva and Katz, 2022; Milon and Rodnina, 2012). Translation is initiated by 70S ribosomes that bind a leaderless mRNA, IF3 and IF2*GTP in complex with initiator tRNA (Figure 4B, step 1). Interestingly, IF3 can bind 70S ribosome (with slightly different binding site than in 30S) being essential for translation initiation of leaderless mRNAs and 70S scanning mechanism discussed later in this chapter (Figure 4C) (Yamamoto et al., 2016). After dissociation of IF3 and GTP hydrolysis by IF2 followed by its release (Figure 4B, step 2), the 70S IC is formed.

The third mechanism of translation initiation in bacteria is 70S scanning. In this case a 70S ribosome does not undergo recycling after it has ended the translation of a cistron. Instead, it continues to move on mRNA until it encounters the next start codon and initiates its translation (Yamamoto et al., 2016). The discovery of this mechanism raises the question whether ribosomal subunits must be dissociated after translation termination in order to initiate translation of the next

cistron on the same mRNA. According to 70S scanning model the post-termination complex (70S ribosome, mRNA and deacylated tRNA in the P-site) binds IF3, IF1 and IF2 in complex with the initiator tRNA (Figure 4C, step 1) resulting in placing it into the P-site instead of the deacylated tRNA and release of IF2. This ribosomal complex scans mRNA sequence around translation stop codon searching for the next SD sequence and start codon (Yamamoto et al., 2016) (Figure 4C, step 2). At the same time IF1 in the A-site prevents aminoacylated elongator tRNA in complex with EF-Tu from binding to ribosome. After the SD-aSD interactions have been established and start codon recognized by initiator tRNA IF1 and IF3 are released and the 70S initiation complex is formed (Figure 4C, step 3) (Yamamoto et al., 2016). This 70S-scanning mechanism has been estimated to be utilized in the case of approximately half of translation initiation events (Yamamoto et al., 2016). It has been proposed that monocistronic mRNAs with 5' UTRs shorter than its median length (37 nucleotides in *E. coli*) may use 70S scanning mechanism. This is based on the result that 70S ribosomes are capable of resolving secondary structures with $\Delta G = -6$ kcal/mol (5' UTRs less than 37 nucleotides contain mRNA secondary structures of –5 kcal/mol on average) (Yamamoto et al., 2016). In conclusion, biological processes are stochastic *an sich* meaning that translation initiation may likely proceed *via* different routes even within the same pathway yielding in elongation competent 70S IC (Chen et al., 2016).

1.2.2. Translation elongation

During translation elongation ribosome decodes genetic information presented in the mRNA codon by selecting an aminoacyl tRNA complementary to the respective mRNA codon in a process termed decoding. Next it catalyzes peptidyl transferase reaction resulting in addition of an amino acid to the growing peptide followed by translocation, i.e. the movement of tRNAs and mRNA by one codon to place the next codon to be translated to the A-site. Translation elongation is a cyclic process composing of three steps: tRNA selection/decoding, peptidyl transferase reaction/ peptide bond formation and translocation. Translation elongation starts as soon as an initiator tRNA is in the P-site of 70S and the A-site is vacant and accessible for the elongator tRNA (Rodnina, 2018) and it ends when a stop codon reaches the A-site.

The first step in elongation cycle is tRNA selection that is divided into initial selection and proofreading. During initial selection, aminoacyl-tRNA in complex with elongation factor Tu (EF-Tu, 394 aa, 43,28 kDa in *E. coli*) and GTP is brought to ribosome. This complex binds the bL12 stalk so that the tRNA anticodon is placed in the A-site whereas its acceptor stem carrying an amino acid is still bound to EF-Tu and lies about 70Å away from the PTC (Figure 5, step 1) (Rodnina, 2018; Steitz, 2008). The A-site of the 30S subunit contains the mRNA codon to be translated that has become accessible to elongator tRNAs after initiation or the last cycle of elongation (Milon and Rodnina, 2012). Depending on whether tRNA anticodon matches mRNA codon in all three positions or in

two or one positions tRNA are termed cognate, near-cognate or no-cognate, respectively (Jenner et al., 2010a). The ability to distinguish the cognate tRNA from the near- and non-cognate tRNAs is crucial for accurate decoding.

In the decoding center, the geometry of the codon-anticodon basepairs is monitored by 16S rRNA residues A1492, A1493 and G530 (Rodnina, 2018). In the case of cognate tRNA, all three nucleotides from the anticodon base pair with the mRNA codon followed by three 16S rRNA bases of the A-site (A1492, A1493, G530) changing their conformation from open to close so that they will interact with the anticodon (Steitz, 2008). These local 16S rRNA conformational changes do not take place in the case of non-cognate (and some near-cognate) base pairs indicating how 16S rRNA detects and stabilizes correct codon-anticodon interactions and eliminates non-cognate tRNAs (Steitz, 2008). Simultaneously the conformational change of the bL12 stalk of the large subunit brings tRNA in complex with EF-Tu closer to the sarcin-ricin loop of 23S rRNA that activates the GTPase activity of EF-Tu (Rodnina, 2018) (Figure 5, step 2). GTP hydrolysis marks the end of the initial tRNA selection and starts proofreading step of decoding.

In the proofreading phase those near-cognate tRNAs that have managed to bypass initial selection based on codon-anticodon interactions are rejected by the ribosome (Jenner et al., 2010a). After GTP hydrolysis EF-Tu changes its conformation to GDP bound state, releases tRNA and dissociates from the ribosome (Figure 5, step 2). The elbow region and acceptor end of the cognate aminoacyl-tRNA can now move into the A-site of the large subunit in a process termed accommodation (Figure 5, step 3). Importantly, rRNA and several r-proteins of the large subunit (uS13, uS19, uL16, bL25, bL27 and bL31) have been proposed to scan the elbow region of the tRNA to determine whether to accommodate the acceptor end to the PTC (Jenner et al., 2010a). To ensure cognate tRNA selection the signal of correct codon-anticodon interactions needs to be transferred from the decoding center in the 30S to the GTPase-associated center (to trigger GTP hydrolysis on EF-Tu) and to the PTC. Signals can be transferred via intersubunit connections and via tRNA that is in contact with mRNA as well as with EF-Tu (Rodnina and Wintermeyer, 2001). In the proofreading step conformational changes in H89, r-proteins uL16, bL27 and intersubunit bridges B1a and B1b are suggested to transmit the signal of correct decoding (Jenner et al., 2010a). As a result of conformational changes and multiple interactions between mRNA, tRNA, EF-Tu and the ribosome the average error frequency of tRNA selection has been estimated to be 1/1000 to 1/10 000 misincorporations (Rodnina and Wintermeyer, 2001).

Accommodation of the CCA-end of the aminoacyl-tRNA into the PTC induces conformational change in PTC-forming rRNA (U2506, G2583, U2584, U2585) (Korostelev et al., 2008; Steitz, 2008). As a result of this the α-amino group of the aminoacyl-tRNA in the A-site is placed optimally to attack the carbonyl group of the ester bond in peptidyl-tRNA (Rodnina, 2018; Steitz, 2008). Peptide bond is formed, and the growing peptide is transferred from the P-site tRNA to the A-site tRNA leaving the P-site tRNA deacylated and A-site tRNA with the peptide (Figure 5, step 4).

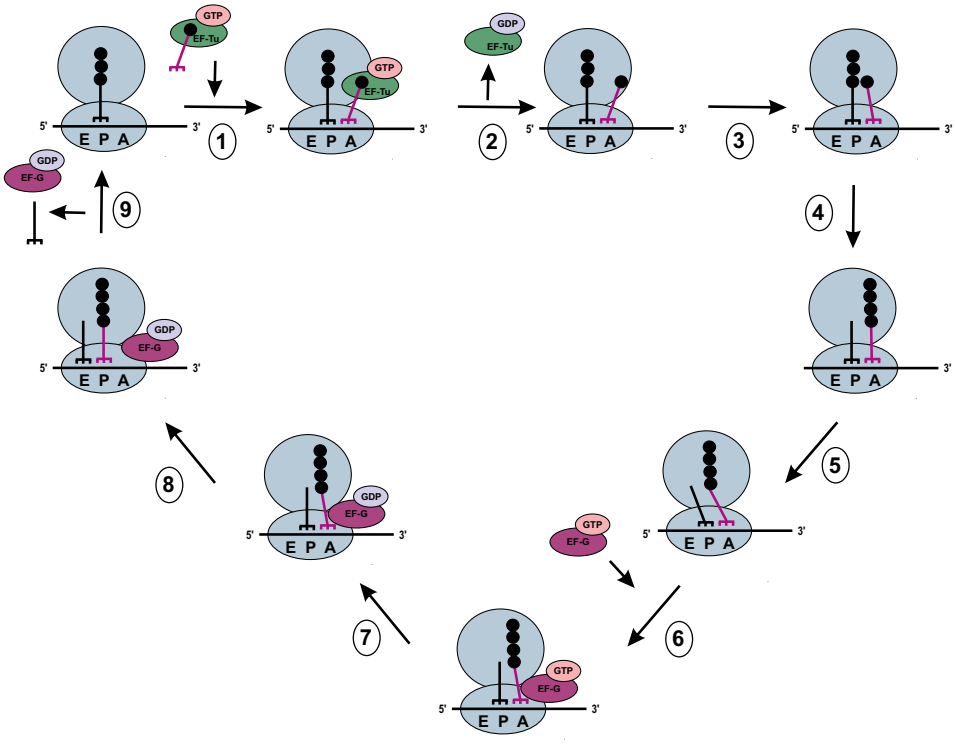

Figure 5. Model of the bacterial translation elongation cycle (with steps described in the main text). 1 – delivery of an aminoacyl-tRNA in complex with GTP-bound EF-Tu. 2 – GTP hydrolysis, dissociation of GDP-bound EF-Tu. 3 – accommodation of aa-tRNA in the A-site of the large subunit. 4 – peptide bond formation. 5 – hybrid state formation. 6 – binding of GTP-bound EF-G. 7 – GTP hydrolysis. 8 – translocation. 9 – dissociation of GDP-bound EF-G and E-site tRNA. (Steitz, 2008)

After peptide bond formation ribosomal subunits are in the non-rotated state (Rodnina, 2018). For the next round of elongation, tRNAs need to be moved from the A- and P-site to the P- and E-site, respectively, and mRNA by one codon. As a result of spontaneous intersubunit rotation tRNAs achieve hybrid states with their acceptor stems in the next binding site within the 50S subunit whereas their anticodon stems are still in the same binding site on 30S subunit (A/P and P/E state, Figure 5, step 5) (Dunkle et al., 2011). Concomitantly with this the uL1 stalk of the 50S subunit moves toward the P-site (Chen et al., 2016). Before translocation ribosomes fluctuate between non-rotated and rotated states and after translocation they are back in the non-rotated state (Rodnina, 2018).

Next, tRNAs and mRNA are moved with respect to the small subunit. This is catalyzed by elongation factor G (EF-G, 704 aa, 77.58 kDa in *E. coli*), another translational GTPase that binds first to the bL12 stalk in the ribosome (Schmeing and Ramakrishnan, 2009; Yamamoto et al., 2014) (Figure 5, step 6). A domain of EF-G is placed into the A-site where it prevents back-translocation (Yamamoto et al., 2014). EF-G binding to the ribosome stabilizes tRNAs in hybrid state, the

head of 30S subunit in swiveled position (about 18 Å toward the E-site) and the uL1 stalk in closed conformation (Chen et al., 2016). Similarly to EF-Tu the sarcin-ricin loop of the 23S rRNA is important for stimulating the GTPase activity of EF-G (Schmeing and Ramakrishnan, 2009) (Figure 5, step 7). After GTP hydrolysis tRNAs and mRNA are translocated (Figure 5, step 8). The head of the small subunit is moved towards the E-site (swiveled position) opening the "gate" formed by 16S nucleotides between the P- and E-site thereby creating space for tRNA movement. The body and head of 30S subunit move leading to opening of the decoding region in 30S to allow mRNA-tRNA complex to move by one codon (Chen et al., 2016). After translocation the tRNAs are in P- and E-sites and the E-site tRNA dissociates from the ribosome as well as EF-G (Figure 5, step 9) leaving uL1 stalk in open conformation (Chen et al., 2016). Ribosome is ready for the next elongation cycle.

mRNA movement by more or less than one codon changes the initial reading frame resulting in +1 or –1 frameshifting (Jenner et al., 2010b). Detailed molecular mechanisms of frameshifting are not known but it is assumed that they are different for different directions (Caliskan et al., 2015; Chen et al., 2014; Dunkle and Dunham, 2015).

1.2.3. Translation termination and ribosome recycling

After a stop codon has reached the A-site of the ribosome, translation is terminated. This means that first, stop codon has to be recognized, then the ester bond of the peptidyl-tRNA hydrolyzed followed by dissociation of translation termination factors from the ribosome. In the first step, a stop codon in the A-site of the ribosome is recognized by release factors RF1 (UAG, UUA) or RF2 (UGA, UAA) (Figure 6, step 1) (Youngman et al., 2008). Both RF1 (360 aa, 40.52 kDa *in E. coli*) and RF2 (365 aa, 41.25 kDa) bind to the A-site. More specifically, a conserved tripeptide recognition motif of RF1 or RF2 interacts with the nucleotides of the mRNA stop codon (Dunkle and Cate, 2010; Rodnina, 2018). Stop codon recognition in the 30S A-site is communicated to the PTC where the peptide hydrolysis reaction is catalyzed (Dunkle and Cate, 2010). This communication has been proposed to be facilitated by a switch region in the RF1 or RF2 that interacts with the A-site elements of 50S and 30S subunits and rearranges upon stop codon recognition (Dunkle and Cate, 2010). These conformational changes potentially result in placing the conserved GGQ motif into the PTC where peptidyl-tRNA hydrolysis is catalyzed resulting in release of the newly synthesized peptide whereas the deacylated tRNA remains in the P-site (Figure 6, step 2). Binding of another termination factor RF3*GTP that does not depend on peptide release (Figure 6, step 2) is required to dissociate RF1 or RF2. After the peptide has been released RF3*GTP binding to ribosome is stabilized, favoring RF1 or RF2 dissociation (Figure 6, step 3) followed by GTP hydrolysis and subsequent dissociation of RF3*GDP (Figure 6, step 4) (Rodnina, 2018).

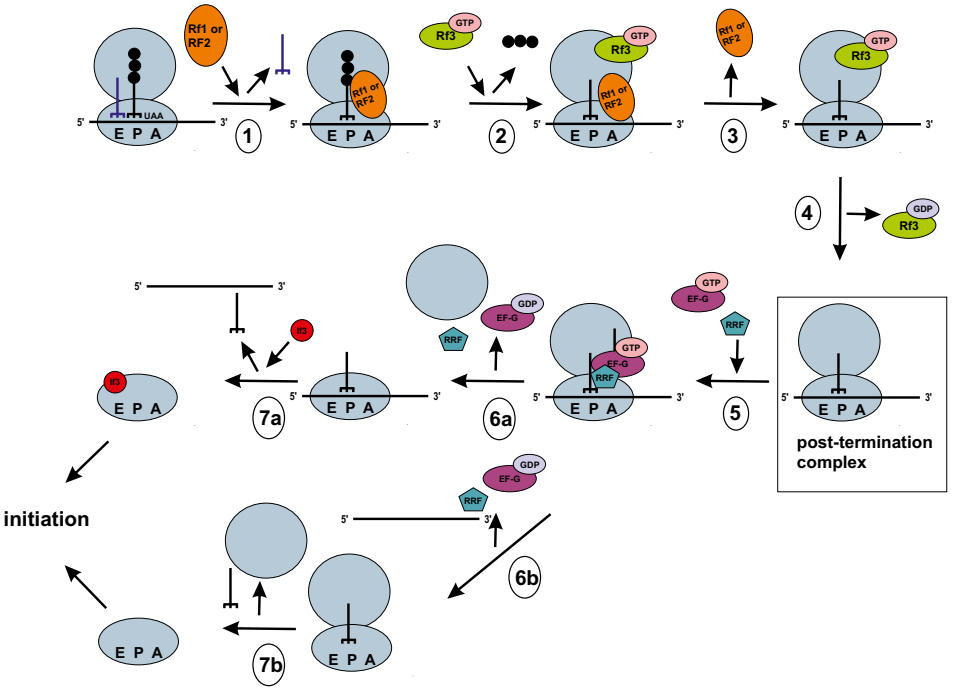

Figure 6. Model of bacterial translation termination and ribosome recycling (with steps described in the main text). Translation termination: 1 – binding of RF1 or RF2. 2 – peptide release, binding of GTP-bound RF3. 3 – dissociation of RF1 or RF2. 4 – GTP hydrolysis, dissociation of GDP-bound RF3. Ribosome recycling: 5 – binding of GTP-bound EF-G, RRF. In the presence of a SD containing mRNA: 6a – dissociation of GDP-bound EF-G, RRF, 50S subunit. 7a – binding of IF3, dissociation of tRNA. (Steitz, 2008). In the absence of an upstream SD sequence: 6b – dissociation of GDP-bound EF-G, RRF, mRNA. 7b – dissociation of tRNA and 50S subunit. (Chen et al., 2017).

After termination a ribosome contains a mRNA and a deacylated tRNA in the P-site. For the next round of translation these RNA molecules are thought to be required to be removed and ribosomal subunits to be dissociated. The latter is catalyzed by the ribosome recycling factor RRF (185 aa, 20.64 kDa) and EF-G*GTP. For efficient ribosome recycling RRF binding that stabilizes ribosome in its rotated state precedes EF-G*GTP binding (Rodnina, 2018) (Figure 6, step 5). Interestingly, the sequence of next events in ribosome recycling is not clear yet. Depending on the nature of the model mRNA two scenarios have been proposed explaining how the post-termination complex would be recycled. According to one model it is the GTP hydrolysis by EF-G that leads to subunit dissociation (Figure 6, step 6a) resulting in 30S subunit still in complex with tRNA and mRNA. Release of the tRNA is promoted by IF3 binding (Figure 6, step 7a) and mRNA is proposed to leave spontaneously (Peske et al., 2005). Another more recent model suggests that GTP hydrolysis promotes mRNA dissociation (Figure 6, step 6b), followed by tRNA release and subunit splitting (Figure 6, step 7b) (Chen et al., 2017). This model is based on the kinetic study

of post-termination complex with mRNA devoid of SD sequence. It is possible that depending on the nature of mRNA (the SD sequence possibly stabilizing mRNA binding to the small subunit) both pathways may function *in vivo*.

1.3. Ribosome heterogeneity

1.3.1. General aspects of ribosome heterogeneity

Ribosome heterogeneity is defined as the intra- or intercellular existence of ribosomal subpopulations with different rRNA or r-protein composition (Emmott et al., 2019; Ferretti and Karbstein, 2019). This phenomenon is universal to life as it exists in Bacteria (Byrgazov et al., 2013), Archaea (Lopez-Lopez et al., 2007) and Eukarya (Genuth and Barna, 2018a). Ribosomal heterogeneity can arise from differences in rRNA as well as in r-protein composition. rRNA molecules expressed from different loci can differ in sequence and chemical modification pattern (Georgeson and Schwartz, 2021). R-proteins can differ in their stoichiometry in ribosomes, posttranslational modifications, and exist as paralogs (Xue and Barna, 2012).

In general, ribosome heterogeneity can be created by subtraction or substitution (Sulima and Dinman, 2019). The former means that a component (rRNA and r-protein modifications, r-proteins) is removed from ribosome or not added to it during biogenesis. Ribosome biogenesis is an extremely complex process involving synthesis and modification of rRNA and r-proteins that need to be assembled into ribosomal subunits (Shajani et al., 2011). Since ribosome assembly has multiple parallel pathways (Shajani et al., 2011) it is conceivable that some ribosomes may not have all r-proteins (Ferretti and Karbstein, 2019). In case of substitution a component is incorporated into maturing subunit instead of another component (for example rRNA sequence variants, r-protein paralogs) during ribosome biogenesis or replaced on mature ribosomal subunit (r-protein paralogs) (Sulima and Dinman, 2019). In addition to this, addition of modifications to rRNA and r-protein in mature ribosomal subunits may also be conceivable (Ero et al., 2010; Pletnev et al., 2019). Different types of rRNA and r-protein heterogeneity can appear in ribosomes at the same time meaning that the number of unique ribosomes is extremely large (Sulima and Dinman, 2019) and that alterations in ribosome structure may be combined to have biological consequences. Related concepts to ribosome heterogeneity that remain out of the scope of this thesis are heterogeneity arising from ribosome-associated proteins, i.e. ribointeractome (Simsek et al., 2017) and from other parts of translational machinery (Sauert et al., 2015), e.g. some translation initiation factors in eukaryotes (Genuth and Barna, 2018b).

According to the widespread view there is “the ribosome” – a unique macromolecular complex (with domain- and species-specific features) that is able to translate a vast number of mRNAs in response to a vast number of extra- and intracellular stimuli (Dinman, 2016). However, to achieve high fitness and

maintain homeostasis in changing environment natural variation – ribosome populations with different composition – is crucial (Dinman, 2016). Selective pressure would act against nonfunctional ribosomes but not with slightly differently functional ribosomes (Ferretti and Karbstein, 2019). Additionally, ribosome heterogeneity between individuals of the same species is important for speciation. Dinman defines "the ribosome" as all possible ribosomes that are competent for supporting vital cellular functions. Depending on the environmental conditions optimal ribosome population could be different and the expression of defective ribosomes may lead to disease (Dinman, 2016).

Heterogeneous ribosomes can be conceptualized as intermediates of ribosome biogenesis (Shajani et al., 2011) implying that their production is not specifically regulated in response to changed environmental conditions. Considering the extreme complexity of ribosome biogenesis (Shajani et al., 2011), it seems conceivable that some ribosome variants deviating from the standard ribosome composition may be created. They may exist if they are translation competent and are therefore not degraded. Such ribosomes may not have been initially specialized but as material for natural selection to operate on they may acquire specialization over time.

Another possible explanation to the existence of heterogeneous ribosomes holds that some of them may be stable ribosome degradation intermediates (Ferretti and Karbstein, 2019) and therefore non-functional in terms of translation. In *E. coli*, specialized ribosomes and their leaderless mRNA substrates were proposed to be created by MazF endoribonuclease under stress (Vesper et al., 2011). In contrast, a genome-wide study of the targets of MazF in *E. coli* demonstrates that MazF creates neither leaderless mRNAs nor specialized ribosomes for their preferential translation (Culviner and Laub, 2018) but it cleaves most mRNAs, r-protein transcripts and rRNA precursors leading to inhibition of ribosome biogenesis.

Next, ribosome heterogeneity has been proposed to be important for stress adaptation, especially in bacteria. Indeed, one of the fastest and most energy-efficient mechanisms for stress adaption would be to reversibly tune composition of mature ribosomes (Gilbert, 2011). This idea has found experimental support in bacteria where replacement of r-protein paralogs differing in zinc-binding motifs has been proposed to be important for maintaining zinc homeostasis (zinc reservoir hypothesis) (Akanuma, 2021; Nanamiya et al., 2004; Panina et al., 2003). In addition, r-protein paralogs having redox sensitive cysteine residues have been hypothesized to act as sensors of reactive oxygen species in *Saccharomyces cerevisiae* (Topf et al., 2018). Both hypothesizes are discussed in detail in the chapter 1.3.2.5.

If the structure of a molecular complex is altered, it is reasonable to assume that this may potentially change its function. In the context of ribosome heterogeneity this would result in specialized ribosomes defined as "ribosomes with preferential regulation of any aspect of translational control" (Genuth and Barna, 2018a) e.g. translation initiation, mRNA selectivity, speed and fidelity of translation elongation. Preferential means that ribosomes, although possibly specialized,

can nevertheless translate several types of mRNAs. It is important to distinguish ribosome heterogeneity from ribosome specialization as heterogeneous ribosomes without special functions in terms of translation regulation may exist (Emmott et al., 2019; Ferretti and Karbstein, 2019). To be considered specialized ribosomes need to satisfy two conditions (Gilbert, 2011). First, they are biochemically distinct and produced under different growth conditions (i.e. heterogeneous *in vivo*), and second, they affect cell physiology *via* translation. Ribosome specialization is defined as "variations in ribosome composition that influence its activity, thereby changing the output of translation" (Ferretti and Karbstein, 2019). Different types of ribosome heterogeneity may be expressed simultaneously to regulate translation of specific mRNAs as proposed in ribosome code hypothesis (Komili et al., 2007). To date this hypothesis has remained speculative since studies have been focused on the effect of a single type of heterogeneous ribosomes on translation nevertheless being aware of the possible simultaneous existence of several types of heterogeneous ribosomes (Chen et al., 2020).

The central hypothesis of the functional importance of heterogeneous ribosomes can be summarized as follows:

ribosome heterogeneity → ribosome specialization →
altered proteome → phenotype

Major research focus is on the first step, i.e. to elucidate whether ribosomal structural diversity leads to its altered functional properties (Dinman, 2016; Genuth and Barna, 2018a). Next step would be to causally associate the activity of specialized ribosomes to changes in proteome resulting in specific phenotypes. Since ribosomes and translation are connected to multiple cellular processes (Bowman et al., 2020) it is challenging to achieve.

Why would cells use ribosome composition to regulate translation? First, it enables bidirectional regulation. Translation of some mRNA-s is upregulated while translation of other mRNAs is simultaneously downregulated (Emmott 2019, Ferretti, Karbstein 2019) if changes in ribosome composition result in ribosomes with altered preference for binding different mRNAs. This flexibility, i.e. bidirectional regulation of mRNA translation contrasts with the unidirectional regulation in the ribosome concentration model (see below). Second, tuning ribosome r-protein composition takes less time than transcriptional regulation (including recruiting transcriptional machinery). For example, in rapidly changing environment is it important for bacteria to be able to react fast and adequately. Since incorporating rRNA sequence variants and creating rRNA modification pattern takes place during ribosome biogenesis this type of ribosome heterogeneity would be too slow to utilize for achieving fast results (Ferretti, Karbstein 2019). Third, translation regulation by ribosome composition needs less energy since changes in ribosome composition can be made during its assembly and by tuning the r-protein composition of existing ribosomes. Therefore, ribosomes need not to be degraded (that also needs energy) but tuned and reused to respond to

changed environmental conditions, for example, ribosome repair by replacement of certain damaged r-proteins has been shown in *E. coli* (Pulk et al., 2010).

The central question is whether heterogeneous ribosomes are functionally equivalent. The notion that most likely not all heterogeneous ribosomes are specialized raises the question of how to study ribosome specialization. Initially, ribosome's ability to bind mRNAs with different efficiency (the ribosome filter hypothesis (Mauro and Edelman, 2002) was the main starting point and it still prevails in ribosome heterogeneity research (Segev and Gerst, 2018). However, based on the definition of specialized ribosomes (see above) any aspect of translation can be affected. The need to look beyond mRNA selectivity when studying ribosome specialization has been emphasized (Emmott et al., 2019; Ferretti and Karbstein, 2019; Gilbert, 2011) since "the efficiency, selectivity, fidelity or rate of any ribosome-dependent reaction could be affected by ribosome specialization." (Gilbert, 2011).

Ribosome heterogeneity, if functional, may contribute to translational heterogeneity (Sonneveld et al., 2020), i.e. the differential translation of the same mRNA or different mRNAs. Similar to ribosomes, translation seems to be more heterogeneous that previously thought challenging the textbook view of each mRNA encoding a single protein and mRNAs transcribed from the same gene to be decoded in the same way (Sonneveld et al., 2020). Translational heterogeneity can be divided into cell-to-cell, intergenic and intragenic types. First, cell-to-cell translational heterogeneity states that the same mRNA is translated differently in different cell types. As noted above, there is an open question of whether ribosome heterogeneity is an inter- or intracellular phenomenon. Second, intergenic translational heterogeneity means that mRNAs from different genes are translated differently in a single cell. This has been the main perspective in the ribosome heterogeneity research, although results have been based on the average of the cell population (Chen et al., 2020) and not (yet) at the single cell resolution. The ribosome filter hypothesis stating that ribosomes may be able to preferentially select certain mRNAs for translation (Mauro and Edelman, 2002) is conceptually related to intergenic translational heterogeneity although with a narrower focus on the translation initiation. Third, intragenic translational heterogeneity refers to mRNA molecules from the same gene in a single cell that are differently translated. In this context, ribosome heterogeneity has been mentioned as a potential origin in addition to mRNA features (structure, sequence, modifications, binding proteins) (Sonneveld et al., 2020). The question is to what extent ribosome heterogeneity contributes to the differential translation of the same substrate mRNA. As with the topic of ribosome heterogeneity, functions of translational heterogeneity are under debate (Sonneveld et al., 2020). Is it important for proteome diversification? Or is it a rather stochastic phenomenon? Translation has to be flexible to some extent to be able to enable proteome diversification. At the same time a proper balance between robustness and flexibility is important to ensure that vital cellular functions are supported while unsuitable heterogeneity (like aberrant proteins) is avoided (Sonneveld et al., 2020).

Very recently ribosomes have been started to be recognized as potential translation regulators (Gay et al., 2022; Goscinska and Topf, 2020; Sonneveld et al., 2020). Ribosome-mediated translation regulation is proposed to be facilitated by changes in ribosome core composition (ribosome specialization hypothesis) and ribosome-associated proteins or abundancy (ribosome concentration hypothesis) (Gay et al., 2022). Notably, according to ribosome concentration hypothesis ribosome abundancy may regulate translation (in addition to ribosome composition) because the availability of free ribosomal subunits for initiation can differently affect translation of different mRNAs depending on mRNA features (like the length of 5' untranslated regions and consensus sequence of translation initiation site) (Ferretti and Karbstein, 2019; Mills and Green, 2017). Poorly translated eukaryotic mRNAs tend to have long 5' untranslated regions and weak consensus sequence and their translation is predicted to depend on the concentration of both ribosomal subunits (Ferretti and Karbstein, 2019). It has been predicted that translation of well-translated mRNAs may not significantly depend on the free subunit availability. As ribosome abundancy has been shown to be decreased in several experimental systems (Ferretti and Karbstein, 2019) it is an important confounding factor when interpreting results. Preferential translation of certain mRNAs may result due to reduced ribosome numbers rather than altered ribosome composition. Translatability of an mRNA depends on its intrinsic features and on the availability of free ribosomal subunits. Due to reduced ribosome numbers translation of certain mRNAs can be decreased more than others (Mills and Green, 2017). However, the effect is unidirectional (translation of all mRNAs decreases but to a different degree) as opposed to bidirectional translation regulation according to ribosome specialization hypothesis (some mRNAs are more translated, others less translated) (Emmott et al., 2019). It is important to note that both hypotheses may be relevant for ribosome-mediated translation regulation.

There is consensus that heterogeneous ribosomes exist in bacteria as well as in eucaryotic organisms (Byrgazov et al., 2013; Norris et al., 2021). However, several questions concerning origin and functioning of ribosome heterogeneity have remained elusive (Emmott et al., 2019; Gay et al., 2022; Genuth and Barna, 2018b, 2018a; Norris et al., 2021; Shi and Barna, 2015; Xue and Barna, 2012). How are heterogeneous ribosomes created and when (during ribosome biogenesis or *via* remodeling of mature ribosomal subunits)? What other processes regulate generation of heterogeneous ribosomes? What is the number of combinations of alternative ribosome components present in ribosomes *in vivo*? Is there evidence for ribosome code, i.e. ribosome modifications acting in concert to regulate translation? Is ribosome heterogeneity an intra- or intercellular phenomenon?

In contrast to the concept of ribosome heterogeneity that is relatively well established in the field ribosome specialization hypothesis needs further conclusive evidence to be widely accepted (Ferretti and Karbstein, 2019; Gay et al., 2022; Genuth and Barna, 2018b; Norris et al., 2021; Xue and Barna, 2012). Do specialized ribosomes exist *in vivo*? How do heterogeneous ribosomes affect translation? Are paralogous r-proteins functionally equivalent? What criteria

should be met to confidently claim the existence of specialized ribosomes (Emmott et al., 2019; Ferretti and Karbstein, 2019)?

In conclusion, ribosome heterogeneity is a naturally occurring widespread phenomenon in *Bacteria, Archaea* and *Eukarya*. To date it has been most extensively studied in diverse eukaryotic model organisms and systems (Genuth and Barna, 2018b) explaining why major concepts and hypothesis are of eukaryotic origin. Since ribosome are central to life (Bowman et al., 2020) the study of their specialization is inherently complex. Therefore, the evidence for ribosome heterogeneity is considerably stronger than that of ribosome specialization and its physiological roles as will be outlined in the next chapter about ribosome heterogeneity in bacteria.

1.3.2. Ribosome heterogeneity in bacteria

In this chapter all five sources of ribosome heterogeneity (originating from rRNA variants, rRNA modifications, r-protein stoichiometry, post-translational modifications, and paralogs) are reviewed based on experimental evidence in *Bacteria*. State of research will be presented with gaps in our knowledge. Each type of ribosome heterogeneity is dissected as follows. First, genetic base for each type of ribosome heterogeneity is outlined followed by experimental evidence for its presence in ribosomes and potential effect on translation outcome and bacterial physiology. The question is whether the genetic potential for ribosome heterogeneity realizes as ribosomes with slightly different composition. Next, does altered composition alter translation outcome that affects bacterial physiology? Although evidence from different bacterial species is discussed the emphasis is on *E. coli* as the model organism of this doctoral thesis.

1.3.2.1. rRNA sequence heterogeneity

In *Bacteria* there are up to 15 rDNA operons (encoding rRNA) per genome (Pei et al., 2010). The genome of *E. coli* encodes 7 rDNA operons (*rrn* operons) with similar gene organization: two tandem promoters, 16S rRNA gene, tRNA genes, 23S rRNA gene, 5S rRNA gene (Blattner et al., 1997). Sequence variation known as microheterogeneity is greater in the promoter region as compared to the rRNA and tRNA coding regions (Maeda et al., 2015). Importantly, rRNA gene sequence of *E. coli* is absolutely conserved (there are no differences) in regions involved in peptide bond formation and at positions where rRNA nucleotides are chemically modified (Maeda et al., 2015). Variable rRNA sequences tend to be located at the surface of the ribosomal subunits involved in contacts with r-proteins and ribosome-associated proteins (Maeda et al., 2015). Altogether there are several variable positions in 16S rRNA (23/1542, number of variable positions/ length of the rRNA), in 23S rRNA (35/2904) and in 5S rRNA (4/120) (Kurylo et al., 2018).

All 7 *rrn* operons are constitutively expressed in *E. coli* (Condon et al., 1992; Kurylo et al., 2018) suggesting that ribosomes are heterogeneous *in vivo* with

respect to their rRNA sequence. The relative expression of *rrn* operons is suggested to depend on bacterial growth phase and growth rate (Condon et al., 1995; Maeda et al., 2015). In addition, *E. coli* mutant strains with less than 7 operons have difficulties with rapid growth and recovery from stress (Condon et al., 1995). Therefore it was assumed that multiple *rrn* operons are primary necessary to ensure that there is sufficient rRNA for ribosome biogenesis during rapid growth (Condon et al., 1995; Pei et al., 2010).

Different rRNA operons may additionally be needed to adapt to altered environmental conditions – an idea that is supported by temperature-dependent changes in rRNA operon expression in the halophilic archaeon *Haloarcula marismortui* (Lopez-Lopez et al., 2007). At higher temperatures, the expression of the *rnnB* operon is induced with the resulting rRNA variant predicted to be more stable than the other two variants (Lopez-Lopez et al., 2007). In line with this, the *rrnB* inactivated mutant strain displays a growth defect at higher temperatures. The authors propose that different rRNA variants may complement each other and confer higher fitness to the organism at various temperatures (Lopez-Lopez et al., 2007).

In recent years first studies focusing on the role of rRNA sequence variation in ribosome heterogeneity and its relation to ribosome specialization hypothesis (both reviewed in the previous chapter) have been published. Kurylo *et al* showed by RNA-seq of total RNA and polysomal RNA (representing actively translating ribosomes) that all seven *rrn* operons are expressed and their rRNA is incorporated into ribosomes during nutrient limitation in *E. coli* (Kurylo et al., 2018). Six of the seven *rnn* operons were differently expressed in minimal and complex media with the *rnnH* operon expression increased in minimal media by more than 20% (Kurylo et al., 2018). Clearly, ribosomes are heterogeneous *in vivo* with respect to their 16S rRNA sequence, and the proportion of different ribosome populations can be changed in response to nutrient limitation-induced stress. In addition, cells expressing ribosomes containing *rrnH* 16S rRNA variant in their 30S subunit (H-ribosomes) exhibited increased biofilm formation and decreased cell motility – both characteristics of the general stress response. In addition, increased tolerance to tetracycline-class antibiotics was found and functionally linked to altered drug binding and translation elongation mechanism of the H-ribosomes (Kurylo et al., 2018). Altogether *rrnH* operon contributes to bacterial fitness during nutrient limitation. However, the question of how does this rRNA variant alter translation on H-ribosomes remains for further studies.

Another example of bacterial "stress" ribosomes translating preferentially a subset of genes necessary for stress response comes from a pathogenic marine bacterium *Vibrio vulnificus* (Leppek and Barna, 2019). Song *et al* have shown that ribosomes containing rRNA from the *rrnI* operon (I-ribosomes) translate preferentially a subset of mRNAs (no data on the mechanism) leading to fast adaptation of *Vibrio vulnificus* to temperature and nutrient shifts (Song et al., 2019). Therefore, this type of ribosome heterogeneity can be considered as gene expression regulator similar to the results of Kurylo *et al*. In contrast to the increased proportion of H-ribosomes during nutrient limitation induced stress

(Kurylo et al., 2018) the proportion of the I-ribosomes is constant during heat shock. The authors propose that the level of target mRNAs is increased during stress (Song et al., 2019). Moreover, they propose that due to rRNA heterogeneity ribosomes are able to modulate proteome in response to stress resulting in adequate adaptation to changed environmental conditions (Song et al., 2019). Interestingly, I-ribosomes and their target mRNAs were found to be functionally important for virulence of *Vibrio vulnificus* in mice.

In conclusion, rRNA sequence variants can direct ribosome to preferentially translate a subset of mRNAs via unknown mechanisms leading to changes in bacterial proteome. These changes help bacteria to cope with stress originating from temperature shifts or nutrient limitation. Hence, ribosome heterogeneity with respect to rRNA sequence belongs to mechanisms that bacteria seem to use for stress management and potentially during infection.

1.3.2.2. rRNA modifications

In addition to rRNA sequence ribosomes can potentially differ in their rRNA modifications, and this difference may lead to functional consequences (alterations in translational properties, proteome, fitness). This hypothesis is supported by several lines of evidence. First, the majority of rRNA modifications are located near the ribosomal functional centers (DC, PTC, peptide exit tunnel, tRNA binding sites) (Antoine et al., 2021). Second, modification changes chemical properties of the respective rRNA residue as compared to its unmodified counterpart and these changes may affect ribosome function. For example, pseudouridine has an extra hydrogen bond donor leading to stabilization of rRNA structure (Spenkuch et al., 2014).

Third, the expression pattern of several rRNA modifying enzymes varies in response to environmental conditions (Baldridge and Contreras, 2014; Gupta et al., 2013) suggesting that corresponding rRNA modifications may be catalyzed in response to changes in environmental conditions (as opposed to being constitutively present in ribosomes). The expression of multiple rRNA methyltransferases varies under heat, cold and oxidative stress (Baldridge and Contreras, 2014). Based merely on transcriptome data this conclusion gives a hint to search for ribosome heterogeneity. Hence, the extent of possible ribosome heterogeneity with respect to rRNA methylations and its dynamics under diverse stress conditions remains to be elucidated along with its effect on translation and cell physiology.

Another example of an rRNA modification potentially variable and dynamic in ribosomes is the dimethylation of A2058 in 23S rRNA (Gupta et al., 2013). It is catalyzed by an erythromycin resistance methylatransferase ErmC induced in the presence of erythromycin (Gupta et al., 2013). In addition to conferring antibiotic resistance, this rRNA modification has been demonstrated to be substoichiometrically (60%) present in the clinical strain of *S. aureus* (where ErmC was constitutively overexpressed from a plasmid). Importantly, it leads to reduction of translation elongation speed *in vitro* resulting in differential translation of

a subset of mRNAs (as shown by increased ribosome pausing on the example of a downregulated protein) (Gupta et al., 2013). The effect of dimethylated A2058 on translation elongation was proposed to be caused by the changed interactions between ErmC-modified ribosome peptide exit tunnel and the growing peptide. Next it would be necessary to quantify the extent of this rRNA modification in wild type *S. aureus* strain during various stages of infection. Does the abundancy of modified ribosomes correlate with specific changes in proteome that lead to biological consequences? Interestingly, *ermC* expression reduces cellular fitness in the absence of the antibiotic as shown in growth competition experiments (Gupta et al., 2013). Consequently, it would not be beneficial for bacteria to maintain continuous erythromycin resistance since the same rRNA modification leads to skewed proteome via decreasing translation elongation rate.

Bacterial rRNA modifications as a source of ribosome heterogeneity is a relatively new topic in the field as the first study addressing it on the example of the dynamics of 5-hydroxycytidine (ho^5C) in *E. coli* been published very recently. Interestingly, C2501 in 23S rRNA is dynamically modified in a growth phase dependent manner: its level increases from about 30% of ribosomes in exponential phase to about 80% in the stationary phase wild type *E. coli* cells (Fasnacht et al., 2022). Moreover, the level of this rRNA modification does not change rapidly in response to cold, heat shock or oxidative stress – a result that can be explained by the downregulation of ribosome biogenesis during stress. These results are also compatible with the idea of ribosome heterogeneity in rRNA composition to be a rather slower way to respond to stress conditions due to its introduction in ribosome biogenesis (Ferretti and Karbstein, 2019). The effect of ho^5C2501 modification on the cellular physiology seems to depend on the stress condition. This rRNA modification may be harmful at high temperatures based on its reduced levels under heat shock and reduced *in vitro* translational activity in case of stoichiometric levels (Fasnacht et al., 2022). During oxidative stress, however, this rRNA modification provides a strong growth advantage and promotes *in vivo* translation.

Altogether, ribosome heterogeneity with respect to rRNA is hypothesized to be less dynamic that modifying its r-protein content (Ferretti and Karbstein, 2019). Less dynamic means more time and energy consuming since ribosome biogenesis and degradation are needed for introduction of rRNA heterogeneity and changing its relative proportions, respectively. Therefore, rRNA heterogeneity would be useful to stress adaptation that is not particularly time critical. First conclusive examples of bacterial ribosome heterogeneity with respect to rRNA sequence and modification exist, however there is much left to discover about the extent, regulation, evolution and functional consequences of ribosome heterogeneity (Georgeson and Schwartz, 2021).

1.3.2.3. R-protein stoichiometry

In addition to rRNA ribosomes can differ in their r-protein composition. Comparison of ribosome heterogeneity with respect to rRNA or r-proteins shows several principal differences. First, every r-protein is generally encoded by a single gene (except for r-protein paralogs discussed in chapter 1.3.2.5) (Aseev and Boni, 2011) whereas every rRNA (16S, 23S and 5S) is encoded by several genes varying in sequence (Pei et al., 2010). Genes encoding ribosomal proteins are organized into operons (21 operons in *E.coli* (Aseev et al., 2020)). In most cases (15 from 21 operons) r-protein biosynthesis is regulated autogenously at the level of translation (Aseev et al., 2020). This means that one r-protein encoded in the same operon binds to the mRNA transcribed from this operon and therefore inhibits its translation.

Second, not all r-proteins are essential for ribosome function and cell viability as shown by the viability of 25 r-protein single deletion strains (Table 1 and 2) (Baba et al., 2006; Shoji et al., 2011). However, it is not known which r-proteins constitute the minimal set required for translation. This is in strong contrast with the fact that 16S, 23S and 5S rRNA are all indispensable for translation. This means that ribosomes may translate without the full set of r-proteins *in vivo*, but they cannot function without rRNAs. It therefore raises the question of r-protein stoichiometry in ribosomes (that has been considered to be one for the whole set). Indeed, a recent preprint demonstrates that in *E. coli* the stoichiometry of most r-proteins is about 1 and it does not change during extended stationary phase (14 days) as shown by mass spectrometry based stable isotope labeled amino acids in cell culture (Reier et al., 2022). Notable exceptions are bS1, bS21 and bL31 and bL36 paralogs that are substoichiometrically present in stationary phase ribosomes. These results challenge the definition of r-proteins as those “present in stoichiometric amounts in the ribosome, whereas translation factors are present on the ribosome with a copy number less than one per ribosome.” (Wilson and Nierhaus, 2005). For example, is bS1 that is weakly and reversibly associated with the ribosome (Delvillani et al., 2011) a r-protein or a translation factor? bS1 is proposed to bring mRNA to the 30S subunit and assists its interactions with anti-Shine-Dalgarno sequence in 16S rRNA during translation initiation (Wilson and Nierhaus, 2005). In line with this, bS1-deficient ribosomes are still able to translate leaderless mRNAs (i.e. mRNAs beginning with 5’ AUG start codon) (Moll et al., 2002).

This evaluation of the effect of stationary phase conditions on ribosome r-protein composition (Reier et al., 2022) is the first systematic study of ribosome heterogeneity in r-protein stoichiometry in bacteria although first observations that ribosomes can differ in their r-protein stoichiometry depending on growth rate date back to the 1970ies (Bickle et al., 1973; Deusser, 1972; Milne et al., 1975). In addition to this there are some examples of unexpected findings from studies of other biological phenomena like translation of leaderless mRNAs in the presence of the antibiotic kasugamycin (Kaberdina et al., 2009) or translation suppression in persister cells (Cho et al., 2015). In the presence of kasugamycin

r-protein deficient ribosomes form. These ribosomes (61S ribosomes) lack bS1, uS2, bS6, uS12, bS18 and bS21 and the amount of r-proteins uS3, uS5, uS11, bS16 and uS17 was reduced by more than 50% (Kaberdina et al., 2009), but the composition of the 50S subunit was not altered. These 61S ribosomes lack several r-proteins close to the docking site for mRNAs at the 30S platform (uS2, uS11, bS18, bS21) (Milon and Rodnina, 2012). Indeed, such ribosomes translate selectively leaderless mRNAs in the presence of kasugamycin *in vivo* and *in vitro* (Kaberdina et al., 2009).

R-protein-deficient ribosomes have also been identified in *E. coli* persister cells as a small fraction of intact ribosomes (less than 25%) characterized by reduced amount of 7 ribosomal proteins: uS3, uS5, uS10, uS11, uS14, bS21, bL25 (Cho et al., 2015). The majority of ribosomes are inactive and likely degradation intermediates. This explains translation suppression in persisters that is important for the formation and maintenance of persisters (Cho et al., 2015). However, whether these r-protein-deficient ribosomes are translation-competent and how would the simultaneous loss of several r-proteins affect ribosome function and proteome composition is left for future studies. Additionally, it would be important to determine the effect of different stress conditions on r-protein stoichiometry.

Third aspect differing in ribosome rRNA and r-protein heterogeneity is the timing of its introducing. R-protein heterogeneity can be introduced during ribosome biogenesis and in contrast to rRNA heterogeneity by modifying existing ribosomes (ribosome remodeling). The latter way enables to change ribosome composition with less time and energy as compared to ribosome biogenesis making it a useful mechanism for stress response (Gilbert, 2011). It has been shown that in *E. coli* a subset of r-proteins (18) is exchangeable on mature ribosomes *in vitro* (Pulk et al., 2010). Moreover, the translational activity of chemically damaged ribosomes can be restored by replacement of damaged r-proteins *in vitro* (Pulk et al., 2010). This ribosome repair mechanism implies that the r-protein composition of ribosomes can be dynamic, at least with respect to certain r-proteins. Some genes encoding exchangeable r-proteins are organized into the same operons allowing their expression without translation of non-exchangeable r-proteins (Pulk et al., 2010).

1.3.2.4. Posttranslational modifications of r-proteins

In *Bacteria*, r-proteins are methylated (bL7/L12, uL11, uL3, uS11, uL6, bL33), acetylated (uS5, bS18, bL7/L12), methylthiolated (uS12) and glutamylated (bS6) but their biological function is not understood (Lammers, 2021; Nesterchuk et al., 2011). bS6 protein has an unique PTM: several glutaminic acids are added to the C-terminus of the protein by the ligase RimK (Nesterchuk et al., 2011). Why are these amino acid residues not encoded in the respective gene? Could this exceptional r-protein PTM hint at its potential regulatory capacity? This hypothesis is supported by experimental data indicating that oligoglutamination of bS6 is induced during transition from exponential to stationary growth phase although

mRNA of *rimK* is expressed in both phases (Pletnev et al., 2019). Interestingly, RimK modifies assembled small subunits (i.e. bS6 in 30S, 70S or 100S particles) and not free bS6 *in vitro*. Considering this the existence of ribosome heterogeneity is very likely. However, the effects of this PTM on ribosome structure and function, translation outcome and bacterial physiology wait for clarification.

In *Pseudomonas fluorescence*, this PTM has been linked to altered translation of a subset of genes including factors needed for surface attachment (Grenga et al., 2020). Based on Ribo-Seq data glutamylation of r-protein bS6 is proposed to facilitate fast proteome adaptation to changed environmental conditions in rhizosphere contributing to more effective plant root colonization (Grenga et al., 2020). The question of how this PTM may affect ribosome function, for example how the modified ribosome distinguishes mRNAs to be preferentially translated remains to be answered (Grenga et al., 2020; Little et al., 2016). Next studies will hopefully shed light on the potential ribosome heterogeneity with respect to oligoglutamination of S6. Altogether, bacterial ribosome heterogeneity with respect to r-protein stoichiometry and post-translational modifications is at present an underexplored field of study with some examples illustrating its potential for ribosome heterogeneity research.

1.3.2.5. R-protein paralogs

In most cases r-proteins are encoded by a single gene (Makarova et al., 2001). A notable exception present r-proteins having paralogs. Paralogs are defined as proteins encoded by genes that derive from the same ancestral gene, are located now at different positions in the same genome and whose protein products differ in at least one amino acid (Gay et al., 2022). In general, about half of the analyzed 995 completely sequenced bacterial genomes encodes r-protein paralogs whereas several phyla encode none (Yutin et al., 2012). R-protein genes exhibit a low level of paralogy (Yutin et al., 2012) and some r-proteins seem to have no paralogs (bS6, bS20, S22, S31, bL9, bL20, bL27, bL35) (Yutin et al., 2012). In contrast, several r-proteins have paralogs in many bacterial genomes: top 5 are bL33, bL31, bL36, uS14, bL28 (310, 179, 144, 138, 57 genomes, respectively) (Yutin et al., 2012). Their paralogs usually differ significantly in their amino acid sequence (< 50% identical amino acids) (Yutin et al., 2012).

Typically, one paralog has a zinc-binding motif usually consisting of two pairs of conserved cysteine residues (referred to as C+ paralog) whereas the other has not (C– paralog) (Makarova et al., 2001). In many cases C+/– r-protein paralogs are located in different operons (Makarova et al., 2001). Terminology for r-protein paralogs has been largely inconsistent depending on the research group. The C+ and C– paralogs have been referred to in different ways: capitalized gene names, 1 and 2 paralog, primary and alternative paralogs, C+ and C– paralogs (Akanuma, 2021; Dow and Prisic, 2018; Li et al., 2018; Nanamiya et al., 2004; Prisic et al., 2015). In this thesis termini C+ and C– paralogs are used, however, to keep the same style as in my papers the C+ paralogs of bL31 and bL36 are referred to as bL31A and bL36A and the C– paralogs as bL31B and bL36B.

The genome of *E. coli* encodes paralogs of two r-proteins: bL31 (*rpmE* and *ykgM* genes) and bL36 (*rpmJ, ykgO*) (Makarova et al., 2001). The genes of C+ paralogs are located in different operons (*rpmE* in the *rpmE* operon, *rpmJ* in the *spc* operon) (Aseev et al., 2020; Makarova et al., 2001) and those of the C– paralogs in the same operon (the *ykgMO* operon) (Makarova et al., 2001). The expression of the *rpmE* operon is regulated at the level of translation by bL31A binding to the mRNA and thereby inhibiting its translation (Aseev et al., 2020). Similarly, the expression of the *spc* operon is regulated by uS8 encoded in the same operon (Aseev and Boni, 2011). The *ykgMO* operon is transcriptionally regulated by Zur repressor (zinc uptake regulator) that under sufficient zinc conditions contains zinc and thereby is able to bind DNA inhibiting transcription (Shin and Helmann, 2016). Under zinc deficiency Zur becomes zinc-deficient, leaves the *ykgMO* operon thereby allowing its expression (Shin and Helmann, 2016).

Functions of r-protein paralogs

The zinc reservoir hypothesis

The difference in the existence of the Zn-binding motif in r-protein paralogs and the zinc-dependent regulation of C– paralog expression by Zur repressor has led to the zinc-reservoir hypothesis (Panina et al., 2003). According to this C+ paralogs may act as zinc storage proteins that under zinc deficient conditions may be replaced by their C– paralogs in ribosomes. After dissociation from ribosomes C+ paralogs may become degraded and thereby provide free zinc ions inside cells. In this context, the function of C– paralogs is thought to be in primary maintaining ribosome function under zinc-deficient conditions and perhaps in conferring additional features to ribosome.

The zinc reservoir hypothesis has been the prevailing theoretical framework for the study of the functions of r-protein paralogs in bacteria. Ribosome heterogeneity introduced via zinc-dependent C+ paralog replacement by C– paralog has been shown on the example of bL31 and uS14 in *Bacillus subtilis* (Nanamiya et al., 2004; Natori et al., 2007), bS18 in *Mycobacterium tuberculosis* (Chen et al., 2020). In *E. coli* it was not known in the beginning of the present study whether ribosomes are heterogeneous with respect to bL31 and bL36 paralogs. Recently this has been independently confirmed (Ueta et al., 2020).

The extent of replacing the C+ paralog with its C– paralog seems to be different depending on the concrete location of r-protein in the ribosome. For example in *B. subtilis*, bL31 paralogs positioned on the surface of the 50S subunit (Fischer et al., 2015) and loosely bound to the ribosome (Eistetter et al., 1999) are more extensively replaced as compared to those of the essential uS14 located in the head of 30S subunit (Akanuma et al., 2006; Natori et al., 2007). uS14C– paralog is proposed to be involved in *de novo* synthesis of 30S subunit (since depletion of the uS14 C+ paralog results in deficient 30S subunits) rather than actively replacing uS14 C+ paralog possibly leading to increased intracellular

zinc concentration (Natori et al., 2007). Interestingly, as zinc concentration decreases operons regulated by Zur are expressed stepwise starting from the C– paralogs of bL31-bL33 followed by the ZnuACB uptake system and YciC metallochaperone and then uS14 C– paralog and FolE2 (Shin and Helmann, 2016). Therefore, uS14C– paralog appears in a smaller amount of ribosomes and later than the bL31C– paralog (Natori et al., 2007).

The zinc reservoir hypothesis has found sound experimental evidence as detailed above. However, there are some points to consider. Importantly, the number of C+ ribosomes is estimated to be thousands fold higher than that of other zinc requiring proteins in cells (Cheng-Guang and Gualerzi, 2021). Therefore, paralog replacement in all ribosomes would not be necessary for bacterial cells to maintain optimal intracellular zinc concentration. Indeed, cells need relatively low free zinc concentration (in picomolar range) (Wang et al., 2011). This suggests that contributing to zinc homeostasis may not be the only function of r-protein paralogs. At the time of starting this PhD research r-protein paralog exchange in ribosomes lacked adequate direct quantification (Nanamiya et al., 2004; Natori et al., 2007; Prisic et al., 2015) (as many studies relied on radical free and highly reducing 2-D electrophoresis (Nanamiya et al., 2004; Natori et al., 2007) that enable identification rather than precise r-protein quantification in ribosomes.)

After almost two decades of research several open questions have remained (Cheng-Guang and Gualerzi, 2021). What signals promote r-paralog replacement in ribosomes? How does this replacement take place? What happens after the replacement so that intracellular free zinc availability increases? And most importantly in the context of ribosome heterogeneity – are r-protein paralogs functionally equivalent, i.e. in terms of affecting ribosome working cycle? bL31 paralogs have been proposed to be functionally redundant based on subunit association, *in vitro* translation and 100S formation (Ueta et al., 2020). Therefore, the role of r-protein paralogs is suggested to store and supply zinc (C+ paralogs) or maintain translation under zinc-deficient conditions (C– paralogs). However, in the study of Ueta et al bL31 paralogs were expressed in different genomic context (Ueta et al., 2020) that could result in different expression and paralog level (Ferretti and Karbstein, 2019) thereby not allowing for adequate comparison of their effects on translation. These possible confounding factors were minimized in our experimental strategy based on expression of bL31 paralogs in the same genomic location and context (Ref III).

Reactive oxygen species (ROS) sensor hypothesis

Zinc-binding CXXC motifs in r-proteins have been shown to be prone to oxidation in *Saccharomyces cerevisiae* (Topf et al., 2018) leading to zinc release (Cremers and Jakob, 2013). This can possibly alter the structure and function of the r-protein. Considering that oxidative stress results in cytosolic translation inhibition that can be reversed as physiological conditions are restored it has been proposed that r-proteins may function as sensors for oxidative stress in *S. cerevisiae* (Topf

et al., 2018). To date it is not known which r-proteins might affect translation inhibition and whether this is the only consequence of cysteine oxidation of r-proteins (Shcherbik and Pestov, 2019). Does oxidation pattern of r-proteins modulate ribosome function? If so, could ROS-induced ribosome heterogeneity contribute to adaptation to oxidative stress?

In *E. coli*, bL31A contains four cysteine residues forming a zinc-binding motif whereas bL31B does not have it (Hensley et al., 2012; Makarova et al., 2001). In addition to cysteines zinc-binding motifs may contain histidines, for example in bL36A (Hard et al., 2000). Therefore, bL31A and bL36A have the potential to belong to the redox switches of the translation apparatus. This implies that under reducing conditions both C+ paralogs would contain zinc and be present in ribosomes. In response to ROS cysteine residues would become oxidized thereby releasing zinc leading to altered protein structure and dissociation from the ribosome. Ribosomes could remain "empty" or alternatively bL31B without any cysteines could become incorporated in the same binding site as its paralog. The ROS sensor hypothesis has not (yet) been addressed in bacteria, but it might be relevant as, for example, zinc limited *M. tuberculosis* are more resistant to oxidative stress (Dow et al., 2021) and it is reasonable to assume that C– paralogs may facilitate this phenotype.

Ribosome hibernation hypothesis

In recent years, r-protein paralogs have been proposed to play a role in ribosome hibernation in mycobacteria. During infection mycobacterial cells encounter different microenvironments in the host. For example in macrophages the zinc concentration is sufficient for leading to the expression of C+ ribosomes (Li et al., 2021a). After lysis of infected macrophages bacterial cells have to potentially cope with low zinc concentration in the extracellular milieu as zinc is bound by calprotectin secreted from neutrophiles (Dow et al., 2021). There free zinc concentration is low as it is bound to the zinc-binding protein calprotectin secreted from neutrophiles (Dow et al., 2021). In response to this, bacterial cells begin to express C– ribosomes (Li et al., 2018, 2021a; Prisic et al., 2015). Decreasing zinc concentration induces first ribosome remodeling (i.e. replacement of C+ paralogs with C– paralogs in ribosomes) and as it continues to decrease ribosome hibernation in mycobacteria (Li et al., 2018, 2020). Remodeled, C– ribosomes are translationally active until the decreasing zinc concentration reaches threshold level. After that C– ribosomes start to bind mycobacterial protein Y (MPY) and MPY-recruitment factor (MRF) leading to translationally inactive, i.e. hibernating ribosomes that become drug resistant (Li et al., 2018). These ribosomes are significantly less sensitive to aminoglycoside antibiotics like kanamycin, streptomycin (Li et al., 2018, 2020). Therefore, hibernating C– ribosomes may be important for survival of growth-arrested cells under low zinc concentration and their antibiotic tolerance (Li et al., 2021a). However, it is not clear whether MPY associates exclusively with the C– ribosomes (Chen et al., 2020; Li et al., 2019; Tobiasson et al., 2019). In addition, could ribosomal r-protein paralog

content correlate with differences in antibiotic sensitivity. The extent of intra- and intercellular ribosome heterogeneity (i.e. the proportion of C– ribosomes in the whole ribosome populations) and its effect on translation outcome awaits for closer investigation.

Zinc starvation induced ribosome hibernation via ribosome remodeling is also hypothesized to be one important mechanism how *Mycobacterium tuberculosis* generates drug-tolerant non-replicating cells (persisters) (Li et al., 2021a). Persisters are defined as genetically uniform but phenotypically heterogeneous cells without mutations in DNA and generated at low frequency (less than 1% of population) (Song and Wood, 2021). Persister cells survive drug treatment and they resume growth after stress is over leading to the latent infection and/or recurrent disease episodes (Wood et al., 2019). Persister resuscitation has been shown to depend on ribosome abundancy (Kim et al., 2018). However, the role of ribosome composition has not been evaluated in this context. The medically relevant question is how to attack dormant *M. tuberculosis'* cells (in latent infection) representing a reservoir of tuberculosis. Conventional antibiotics are ineffective since they target translationally active ribosomes, but translation is shut down in dormant cells. Therefore, C– ribosomes may become a potential drug target for tuberculosis (Kumar et al., 2021; Li et al., 2021a). Although intriguing and with some supporting experimental evidence the ribosome hibernation hypothesis needs further investigation to gain a deeper insight into its importance for persistence of mycobacteria (Li et al., 2021a).

As mentioned above, ribosome remodeling can result in C– ribosomes significantly more drug resistant (Li et al., 2018, 2020). For example replacement of the uS14C+ paralog with its C– paralog in ribosomes under zinc starvation results in about 2-fold reduced binding of spectinamide (a preclinical drug candidate for tuberculosis) (Li et al., 2021b). This difference is most likely caused by altered interactions of the C– paralog with the ribosome due to two additional contact points with the 16S rRNA in the 30S head and body region. Based on this the authors hypothesize that its binding to the ribosome could reduce the movements of the 30S head during translation potentially reducing spectinamide binding and leading to slower translation rate (Li et al., 2021b).

C– paralogs in bacterial cell physiology beyond zinc storage

C– paralogs have been proposed to be involved in facilitating morphological changes in response to zinc depletion in mycobacteria (Dow and Prisic, 2018; Dow et al., 2021). This is based on the evidence that *Mycobacterium smegmatis* devoid of four C– paralogs (uS14, bS18, bL28, bL33) is not able to undergo morphogenesis (including cell elongation, changes in the cell wall structure, nucleoid condensation and so on) in response to decreasing zinc concentration (Dow and Prisic, 2018). In addition, this deletion strain is not able to grow at low zinc concentration indicating that C– paralogs can support bacterial growth.

During infection *Mycobacterium tuberculosis* encounters zinc-replete and zinc-deplete microenvironments in macrophages and extracellular milieu respectively (Li et al., 2021a). It has been hypothesized that zinc availability induces phenotypic heterogeneity of *Mycobacterium tuberculosis* in the host organism (Dow et al., 2021). This is based on the evidence that under zinc deficiency bacterial cells grown outside the host exhibit changes in cell surface morphology, lipid metabolism and expression of virulence factors (Dow et al., 2021). Importantly, these bacteria are more resistant to oxidative stress and show increased replication in the host (Dow et al., 2021). These results wait for validation in the host organism, however these distinct cell populations may affect disease progression and response to treatment (Dow et al., 2021). The mechanism how zinc deficiency may induce bacterial phenotypic heterogeneity is currently not known. It needs to be clarified whether heterogeneous ribosome subpopulations differing in their functional properties exist and whether they may contribute to altered proteome composition and thereby to altered phenotype of zinc-limited *M. tuberculosis*.

Translation regulation by r-protein paralogs

Under zinc deficient conditions C– paralogs have been shown in mycobacteria to maintain translation (Dow and Prisic, 2018; Li et al., 2020), to make ribosomes prone to hibernation thus becoming translationally inactive (Li et al., 2018) and to contribute to cell morphological changes as a stress response (Dow and Prisic, 2018; Dow et al., 2021). The question of ribosome heterogeneity leading to specialized ribosomes has been only recently addressed. In *Mycobacterium smegmatis* ribosome subpopulations differing in bS18 paralog content were isolated from the strain expressing both paralogs (Chen et al., 2020). According to polysome profiles C– ribosomes are translationally active, however ribosome profiling indicates that they translate several genes with different efficiency as compared to C+ ribosomes. Accumulation of sequencing reads in 5' end of mRNAs and less efficient translation initiation complex formation *in vitro* suggest defective translation initiation of C– ribosomes (Chen et al., 2020). However, as the authors note, several combinations of C– ribosomes may exist that cannot be distinguished by their tagging and purification strategy. This is the first direct comparison of C+ and C– ribosomes in bacteria challenging the widespread view of their functional redundancy.

2. AIMS OF THE THESIS

Structures of bacterial ribosomes demonstrate that r-protein bL31A is a part of the intersubunit bridge B1b connecting the central protuberance of the large subunit with the head domain of the small subunit (Figure 7) (Fischer et al., 2015; Jenner et al., 2010a). Bridge B1b is the most dynamic bridge during translocation facilitating intersubunit rotation and 30S head swiveling (Zhang et al., 2009). Additionally, bL31A interacts with r-proteins uS13 and uS19 that contact A- and P-site tRNAs (Jenner et al., 2010a). Based on its position in ribosome bL31A has been proposed to be involved in tRNA selection to the ribosomal A-site and in controlling intersubunit movements thereby contributing to translation fidelity (Jenner et al., 2010a; Shasmal et al., 2010).

Interestingly, r-protein bL31 is the second most frequent r-protein to have paralogs in bacteria according to genomic analysis of 995 sequenced bacterial genomes (Yutin et al., 2012). In *Bacillus subtilis*, bL31A and bL31B have been shown to be present in ribosomes with their functional importance interpreted in the context of zinc concentration hypothesis (Nanamiya et al., 2004). At the start of the current study it was unknown whether the genetic potential for ribosome heterogeneity with respect to r-protein paralogs realizes in *E. coli*.

Therefore, this study aims to find out

- whether there is ribosome heterogeneity with respect to r-protein paralogs in *E. coli*; (Ref II)
- how does this ribosome heterogeneity in bL31 paralog content affect bacterial growth (Ref I, III);
- what are the functions of r-protein bL31 as a major component of the inter-subunit bridge B1b in the *E. coli* ribosome during translation cycle (Ref I, III).

3. RESULTS AND DISCUSSION

3.1. Ribosome heterogeneity and effects of bL31 paralogs on bacterial growth

3.1.1. *E. coli* ribosomes are heterogeneous with respect to r-protein paralog content *in vivo*

Starting point of the present study constitute previous mass spectrometry results from our laboratory indicating that the composition of *E. coli* 70S ribosomes is qualitatively and quantitatively similar in exponential as well as in stationary growth phase (Ref II, Figure 1). The only exceptions are r-proteins bL31 and bL36 that have each two paralogous genes in *E. coli* (Makarova et al., 2001; Panina et al., 2003). Clearly, the A paralogs are overrepresented in the exponential phase ribosomes as compared to the stationary phase ribosomes and the B paralogs are prevalent in the stationary phase ribosomes (Ref II, Figure 1).

Can some ribosomes bind the A paralog whereas others contain the B paralog of bL31 or bL36? Ribosomes extracted from the exponential, late exponential and stationary phase *E. coli* wild type strain cultivated at 37 °C were analyzed by mass spectrometry for their r-protein levels. From this experiment on, two reference strains with known expression level of A or B paralogs were used for quantification. Consistent with previous results (Ref II, Figure 1) r-protein composition (except for bL31 and bL36) is highly similar in exponentially growing bacteria as well as in bacteria from the stationary phase (Ref II, Figure S2). In contrast, the level of bL31 and bL36 paralogs strongly varies between growth phases: in exponential phase the majority of ribosomes (more than 90%) contains the A paralog (bL31A or bL36A), their fraction decreases in late exponential phase with simultaneous increase in the fraction of B paralogs leading to most ribosomes binding B paralogs (bL31B, bL36B) in the stationary phase (Ref II, Figure 2, S3). The change in r-protein paralog proportions during bacterial growth is greater in the case of bL31 as shown by almost reversible abundancies of bL31A and bL31B in stationary phase as compared to the exponential phase. The proportion of bL36A containing ribosomes decreases by about 3x on average during growth phase progression (25% or 43% of ribosomes contain the bL36A paralog depending on the biological replicate). Importantly, at all three time points bacterial cells contain ribosome subpopulations differing in their bL31 and bL36 paralog content demonstrating conclusively that *E. coli* ribosomes are heterogeneous with respect to r-protein paralogs *in vivo* under conditions used.

Similar results were obtained in a qualitative r-protein composition analysis of *B. subtilis*' ribosomes from exponential and stationary growth phase (Nanamiya et al., 2004) demonstrating that bL31A is the dominant paralog in exponential phase and bL31B in the stationary phase ribosomes. However, two-dimensional polyacrylamide gel electrophoresis could not identify some r-proteins (uS14B, bL9, bL33B, bL36) and the extent of change in ribosomal bL31 paralogs

composition was not quantified. We speculate that the growth phase specific molecular switch in ribosomal bL31 paralogs may be part of the more general "switch" (adaptation mechanism) ensuring that cells are able to adapt to and survive under conditions of limited nutrients in the stationary phase (Wolfe, 2005).

Our results regarding ribosome heterogeneity with respect to r-protein paralogs in *E. coli* have been supported by a study published shortly after our paper (Ueta et al., 2020). First, *E. coli* ribosome heterogeneity was confirmed in *zur* mutant cells containing ribosomes with different bL31 and bL36 paralog content in exponential as well as in stationary phase (24h, 72h post-inoculation) (Ueta et al., 2020). Unfortunately, the authors did not present any data about the ribosomal r-protein composition in the wild type strain. Expectedly, the proportion of ribosomes with different r-protein paralogs is approximately the same regardless of growth phase, suggesting that incorporation of r-protein paralogs is non-phase-dependent in the strain tested. The dominant presence of bL31B and the relatively high proportion of bL36B containing ribosomes in *zur* mutant cells in exponential phase cells quantified by radical-free and highly reducing 2D-PAAG gel (Ueta et al., 2020) can be partially explained by the loss of Zur repressor. In wild type strain Zur would inhibit transcription of the *ykgMO* operon encoding bL31B and bL36B (Shin and Helmann, 2016). Based on this and on the minor presence of bL31A in ribosomes it can be speculated that bL31B may bind or be incorporated into ribosomes *in vivo* with greater affinity than bL31A (Ueta et al., 2020). It remains unclear why the proportion of bL36B in ribosomes remains constantly and considerably smaller than that of bL36A. Data about mRNA and cellular protein levels of all four r-protein paralogs in exponential and stationary phase would be needed to explain the issues discussed above.

Our quantification of r-protein paralogs in ribosomes from different growth phases demonstrates that the proportion of ribosomes with different bL31 paralogs changes in considerably larger extent than that of ribosomes with bL36 paralogs (Ref II, Figure 2, S3). Why does the proportion of bL31A containing ribosomes decrease significantly more than that of bL36A? This result can be rationalized considering their different location in ribosome. bL31A is situated on the solvent side of the 50S subunit contacting uL5, 5S rRNA from the large subunit and additionally in 70S ribosome uS13, uS19 from the small subunit (Jenner et al., 2010a) (Figure 7). Moreover, bL31A has been shown to be exchangeable between ribosome-bound and unbound state *in vitro* and *in vivo* by quantitative mass-spectrometry (Pulk et al., 2010). In contrast, bL36A is located inside the 50S subunit near the bL12 stalk contacting mainly 23S rRNA (Fischer et al., 2015) (Figure 7A). Results of chemical protection experiments with 50S subunits suggest that bL36A may be important for organizing 23S rRNA structure (Maeder and Draper, 2005). Comparison of ribosomal locations of bL31A and bL36A suggests why dissociation from the ribosomal subunit would be more conceivable for bL31A than for bL36A. Dissociation of the A paralogs would be even more complicated in the context of 70S, because bL31A has several additional contacts with the small subunit and the 30S subunit may possibly physically block dissociation of bL36A from the large subunit.

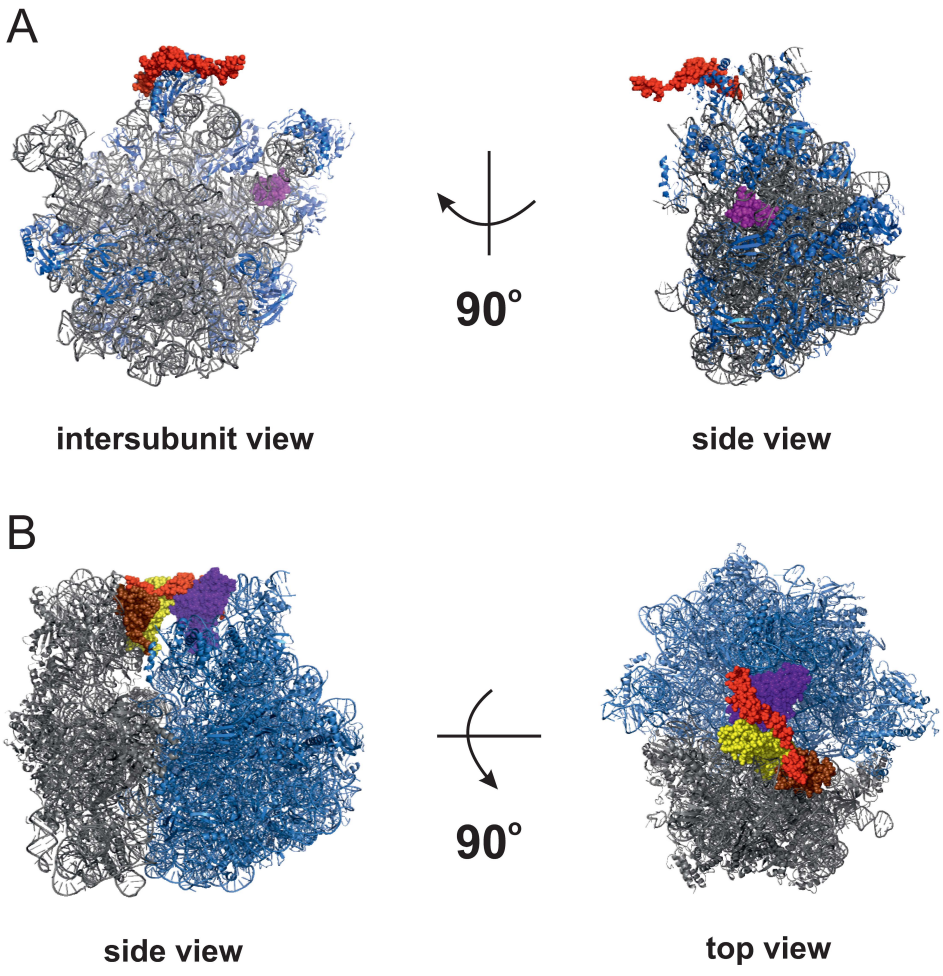

Figure 7. R-protein paralogs and the ribosomal intersubunit bridge B1b in the ribosome of *E. coli*. (A) R-protein paralogs in the large ribosomal subunit of *E. coli*. bL31 (red) is located in the central protuberance, bL36 (pink) lies near the base of the bL12 stalk. rRNA is in grey and r-proteins in blue. Intersubunit and side views of the large subunit are presented left and right, respectively. (B) In 70S ribosome small subunit (grey) and large subunit (blue) are held together by 12 intersubunit bridges. Components of the B1b bridge are shown as spheres: uS13 (yellow), uS19 (brown) from the small subunit and bL31 (red), uL5 (purple) from the large subunit. (A) side view, (B) top view. Side view and top view of the 70S ribosome are presented left and right, respectively. Ribosomal structures were generated with PyMOL using coordinates from PDB entry 5AFI (Fischer et al., 2015).

How to explain so profound change in the proportion of ribosomes binding the A or B paralogs during growth phase progression? First, r-protein paralogs can be incorporated into ribosomal subunits during ribosome biogenesis like all other r-proteins. However, this pathway seems to be less relevant here since ribosome biogenesis is highly downregulated (Bremer and Dennis, 2008) whereas ribosome degradation is increased before bacterial culture enters into stationary phase (Piir et al., 2011) both processes possibly resulting in decreasing proportion of bL31A containing ribosomes. At the same time, the proportion of the B-paralog containing ribosomes continues to increase. Therefore, it seems reasonable to assume that B-paralogs may be incorporated mostly into existing ribosomal subunits (ribosome remodeling). Modifying a fraction of assembled ribosomes has been proposed to be a time and energy saving way to fine-tune translation (Ferretti and Karbstein, 2019).

3.1.2. Ribosome heterogeneity with respect to bL31 paralogs can arise via ribosome remodeling *in vitro*

Next, we aimed to clarify mechanisms of ribosome remodeling in the context of r-protein paralogs. Why and by which mechanism(s) does the level of the A paralogs in ribosomes decrease and the level of the B paralogs increase during bacterial growth phase progression? The change in ribosomal protein paralog composition takes place as bacterial cells transition from exponential to stationary growth phase (Ref II, Figure 2) in agreement with other studies using *B. subtilis* (Nanamiya et al., 2004). During fast growth under aerobic conditions *E. coli* produces acetate (Wolfe, 2005) resulting in transiently decreased intracellular pH (Diez-Gonzalez and Russell, 1997). Therefore, the effect of acidic pH on r-protein composition was tested *in vitro* by incubating wild type 70S or 50S particles at pH 5.5. and pH 7.6 followed by quantification of their r-protein composition with mass spectrometry. At pH 7.6 about 60% of wild type ribosomes contain the bL31A paralog and about 40% have the bL31B paralog. The amount of bL31A containing ribosomes was decreased by about 30% after incubation at pH 5.5 whereas the level of bL31B, bL36A and bL36B did not change upon treatment under low pH (Ref II, Fig 3, S4). These results confirm ribosome heterogeneity identified in the previous growth progression experiment (Ref II, Figure 2, S3) and indicate partial pH dependent dissociation of bL31A from ribosomes. Acidic pH induced dissociation of bL31A was additionally demonstrated by the analysis of r-protein protein dissociation under pH 5.5 in SDS-PAAG (Ref II, Figure S5). Next, as bL31A selectively dissociates from ribosomes under acidic pH vacant ribosomes (i.e. ribosomes without any bL31 paralogs) arise. We then aimed to find out whether bL31B is able to associate with ribosomes without any bL31 paralogs. Indeed, incubation of vacant ribosomes from bL31 paralogs' deletion strain with purified bL31B resulted in about 80% of bL31B containing ribosomes (Ref II, Figure 4).

pH dependent dissociation of bL31A was estimated to account for about 30% of its decrease in mid-exponential phase ribosomes *in vitro* (Ref II, Figure 3). However, under *in vivo* conditions stationary phase ribosomes contain more than 5x less bL31A than exponential phase ribosomes (Ref II, Figure 2, S3). The difference between *in vivo* and *in vitro* results indicates that aspects affecting bL31A dissociation other than acidic pH need to be considered. Possibly, additional factors missing under *in vitro* conditions but acting *in vivo* may be involved in dissociation of bL31A and bL36A, the latter being unaffected by acidic pH (Ref II, Figure S4). Additionally, low level of ribosome biogenesis with increased rRNA degradation during transition from exponential to stationary phase (Piir et al., 2011) may help to explain the decrease in ribosomes having A paralogs *in vivo*.

Ribosomes are heterogeneous with respect to r-protein paralogs in exponentially growing *E. coli* cells. The presence of the B paralogs in exponential phase ribosomes (Ref II, Figure 3, 4) implies that their genes need to be expressed during fast growth already. This suggests that their expression should be regulated by other means in addition to zinc dependent Zur repressor (chapter 1.3.2.5). This contrasts the zinc concentration hypothesis proposing that its intracellular concentration is the critical factor controlling alternation of bL31 paralogs in *B. subtilis* ribosome (Nanamiya et al., 2004). More specifically, bL31A does not bind a zinc ion under zinc deficient conditions resulting in its instability and dissociation from ribosomes (Akanuma et al., 2006; Nanamiya et al., 2004). We have not tested the effect of zinc concentration on ribosomal r-protein paralog content. However, it seems rather hard to imagine that exponential phase cells would experience zinc deficiency leading to expression of the B paralogs in ribosomes. Intracellular zinc concentration measurements along with r-protein paralog expression data at mRNA and protein level would be useful to clarify this discrepancy. Nevertheless, it can be assumed that our results cannot explained by potential zinc-deficiency only.

Altogether, based on the r-protein quantification experiment during bacterial growth phase progression in combination with *in vitro* experiments with acidic pH and purified bL31B the following picture emerges about ribosome heterogeneity of r-protein paralogs. In exponential growth phase, most ribosomes contain bL31A. As bacterial cells transition from exponential to stationary phase, they experience transient intracellular acidic pH and a proportion of bL31A dissociates from ribosomes leaving its ribosomal binding site vacant for bL31B that in turn associates with vacant ribosomes. Notably, in addition to lower pH other environmental conditions (for example decreased zinc concentration) and possibly additional presently unknown factors may contribute to bL31 paralog exchange on ribosomes. Indeed, it is not known whether such extrinsic factors are involved in protein exchange on ribosomes or what could be the physiological meaning of this process (Pulk et al., 2010).

The situation is different with bL36 paralogs although in exponential growth phase, most ribosomes contain bL36A. During transition to stationary phase its fraction in ribosomes decreases by about 3x, i.e. to a smaller extent if compared to bL31A. In contrast to bL31A, acidic pH does not induce dissociation of bL36A

from ribosomes (Ref II, Figure S4). One aspect probably making it more difficult to accomplish without the help of additional factors is its position inside the ribosome as opposed to the location of bL31A on the solvent side (Figure 7A). Therefore, it seems more feasible that instead of ribosome remodeling bL36B may be incorporated into newly synthesized ribosomes.

Preliminary results from our laboratory had shown that bL36 paralogs seem to have similar to wild type effect on (1) the ribosomes' ability of catalyzing PTC reaction as assessed by puromycin reaction, (2) general translation capability and processivity as evidenced by poly(U) poly(Phe) dependent translation system (Volõnkin, 2015). In contrast, bL31 seemed to have a greater potential for translation regulation owing to its intriguing position in ribosome (chapter 1.1.2., Figure 7). Therefore, this dissertation about bacterial ribosome heterogeneity concentrates on the role of bL31 paralogs for bacterial growth and translation.

3.1.3. bL31A and bL31B are important but not equivalent for optimal growth at lower temperatures

To investigate the functional importance of bL31 paralogs following *E. coli* mutants were constructed. First, a double deletion *E. coli* strain lacking *rpmE* and *ykgM* genes encoding bL31A and bL31B, respectively, was constructed in the MG1655 background (referred to as MGΔAB) by stepwise P1 phage transduction (Ref I, Materials and Methods, Table 3). To compare the effects of bL31A or bL31B on bacterial growth and translation parameters, two strains expressing only *rpmE* or *ykgM* in the same chromosomal context were constructed (referred to as the A-strain and the B-strain). For that *rpmE* or *ykgM* gene was introduced into the MGΔAB strain (in the Δ*lacI*Δ*lacZ* background) under *tac* promoter using the conditional-replication, integration and modular (CRIM) plasmids (Ref III, Materials and Methods, Table 1) (Haldimann and Wanner, 2001). This approach enables to express *rpmE* or *ykgM* in the identical chromosomal context yielding in their stoichiometric presence in ribosomes as shown by r-protein quantification by MS (Ref III, Figure S2). Expression of each paralog in the same untranslated region has been proposed to be included into best practices of investigating functionality of ribosome heterogeneity because it aims to clarify whether the open reading frame or non-coding regions cause differential effects (Ferretti and Karbstein, 2019).

The loss of both bL31 paralogs (the MGΔAB strain) leads to considerable cold-sensitive growth defect as compared to the wild type strain. This is demonstrated by the slower growth rate in liquid medium: at 37 °C the double deletion strain grows about 1.7x slower than the wild type strain (generation times 37.3 min vs 21.4 min, respectively) whereas at 30 °C, the difference in generation times is about 2.5x (101.2 min vs 39.5 min, respectively, Ref I, Table 1). These results are supported by growth analysis by serial dilutions test at different temperatures on solid media. At 30 °C *E. coli* cells without any bL31 paralogs grow

slower than the wild type strain whereas the MGΔAB strain is barely able to form colonies at 25 °C, 20 °C (Ref III, Figure 1a).

Strains expressing a single copy of only one bL31 paralog (*rpmE* or *ykgM*) in the same chromosomal context (the A-strain, B-strain, respectively) exhibit different growth characteristics. The A-strain demonstrates generation times similar to the wild type strain at all tested temperatures (37 °C, 30 °C 25 °C) whereas the B-strain grows slightly slower (up to 1,3x) exhibiting cold-sensitive growth phenotype as compared to the wild type (Ref III, Figure 1b–g). These results can be explained by the fact that most ribosomes contain bL31A in the exponential phase (Ref II, Figure 2). Growth analysis on solid medium indicates that the A-strain grows like the wild type strain at all tested temperatures whereas the B-strain forms visually less colonies than the wild type strain at 25 °C, 20 °C (Ref III, Figure 1a). In conclusion, these results demonstrate that the loss of both bL31 paralogs leads to cold sensitive growth phenotype as demonstrated by generation time measurements and serial dilution spot tests. Interestingly, bL31A and bL31B are important but not equivalent for optimal growth at suboptimal temperatures.

In addition, in *E. coli* the effect of bL31A on bacterial growth has been studied on the basis of its deletion strain at 37 °C two other research groups (Aseev et al., 2020; Ueta et al., 2017, 2020). In contrast to our results, the loss of bL31A results in slower growth in liquid and on solid media as compared to the wild type strain (Aseev et al., 2020; Ueta et al., 2017, 2020). Aseev et al propose that the *ykgMO* opeon may have been partly open under conditions used in Ref II resulting in bL31B compensating for the loss of bL31A. This possibility is in agreement with our results demonstrating that depending on the time point during exponential phase up to about 40% of wild type ribosomes contain bL31B (Ref II, Figure 2–4). Importantly, all three studies have their shortcomings: both studies of Ueta *et al* lacked complementation with *rpmE* gene/ bL31A and no growth data about the wild type strain was presented by Aseev *et al*. In addition, bL31B deletion strain or double deletion strain lacking both bL31 paralogs have been excluded from the growth experiments although both strains were used in other experiments (Ueta et al., 2020). Therefore, the effect of bL31 paralogs on bacterial growth has not been addressed systematically (in terms of growth conditions and strains) and results from other studies have remained largely inconclusive.

Next, the effect of bL31A and bL31B on bacterial fitness was compared in growth competition assay (at 37 °C and 25 °C). For that, liquid cultures of the A-strain and the B-strain were mixed at equal ratio, divided into two batches, and grown in parallel. One batch (stationary phase culture) was allowed to grow in the same media during the whole experiment (30 days) (Ref III, Figure 2b). The other batch (cyclic growth culture) was rediluted into the fresh medium after sampling at every six days (altogether five cycles, Ref III, Figure 2a). From both cultures samples were taken after every six days to assess the ratio of both strains in the mixed cultures by quantifying agarose gel bands corresponding to chromosomal PCR products of the *rpmE* and *ykgM* genes (Ref III, Figure 2c). In the stationary phase culture, the ratio of the A and B strains remains approximately

1:1 for the whole experiment at 25 °C as well as at 37 °C (Ref III, Figure 2e, g). In contrast, in the cyclic growth culture the fraction of the A-strain increases during five cycles concomitant with the decrease of the B strain (Ref III, Figure 2d, f). The fraction of the A strain increases from 0.5 to 0.8 at 25 °C and from 0.5 to 0.6 at 37 °C with approximately 3.7x and 1.4x increase, respectively (Ref III, Figure 2d, f). The growth advantage of the A strain over the B strain in mixed culture becomes more evident at lower temperature similar to the growth comparisons on solid and in liquid media (Ref III, Figure 1). Interestingly, bL31A confers higher fitness to *E. coli* as compared to bL31B during cyclic growth but not during stationary phase. During cyclic growth bacterial cells need to adapt to fast growth conditions and maximize biomass/ growth rate. The A-strain seems to be more successful as shown by growth competition with the B-strain and suggested by its shorter lag phase and generation time in comparison to the B-strain at 25 °C (Ref III, Figure 1d, g) when grown separately.

3.2. Effects of bL31 paralogs on translation

The central question is whether ribosome heterogeneity with respect to bL31 paralogs is functional. In other words: does the specific r-protein paralog composition affect translation outcome? And may altered translation have a role in cell's physiology leading to changed growth? Growth monitoring experiments in liquid and on solid media at various temperatures have shown that bL31A and bL31B are important but not equivalent for optimal growth at lower temperatures whereas the loss of both bL31 paralogs results in serious cold sensitive growth defect (Ref I, Table 1, Ref III, Figure 1).

This leads to hypothesis that bL31A and bL31B may have slightly different effect on ribosome functioning during translation cycle. The functional importance of bL31 was not known in the beginning of this study but computational analyses have proposed and ribosomal structural analyses shown that bL31A is a part of the intersubunit bridge B1b connecting the dynamic head of the 30S subunit with the central protuberance of the 50S subunit (Jenner et al., 2010a; Selmer et al., 2006; Shasmal et al., 2010) (Figure 7). Based on its location in the ribosome this bridge has been hypothesized to be involved in several steps of translation initiation and elongation (formation of 70S initiation complex and elongator tRNA selection, translocation, respectively) (chapter bridges 1.2.). Therefore, it seems very likely that bL31A may play a role in translation regulation.

It is worth to note that, generally bL31A has been (and is still largely) considered to be *the* bL31 in scientific literature since bL31B had not been found in *E. coli* ribosome structure before our study (Ref II). This reasoning was also used in the Ref I, since bL31B was demonstrated to be a true ribosomal protein by MS and X-ray crystallography of 70S later in the Ref II (70S structure solved by Arto Pulk). Although bL31A (encoded by *rpmE* gene) and bL31B (encoded by *ykgM* gene) have a rather low sequence identity (36.8%, Ref II, Figure 6) they share a

similar ribosomal binding site (Ref II, Figure 5b) indicating that they mutually exclusively bind to a ribosome. Their ribosome-bound structures exhibit local structural differences: bL31B has a loop structure of unknown function in its N-terminal part protruding to the solvent (Ref II, Figure 5d) and bL31A has a zinc-binding motif (Fischer et al., 2015). Therefore, it is reasonable to ask whether mutually exclusively ribosome bound r-proteins bL31A and bL31B are functionally equivalent.

3.2.1. bL31A and bL31B make comparable contributions to translation initiation rate and ribosomal subunit association *in vivo* and *in vitro*

The effect of bL31 paralogs on translation initiation was analyzed *in vivo* and *in vitro*. Initially experiments published in the Ref I were performed with the MG wild type strain, the bL31 paralogs' double deletion strain (MGΔAB) and its complementation with expression of bL31A *in trans*. After confirmation of the presence of bL31B in wild type ribosomes *in vivo* (Ref II, Figure 2, S3, 5), complementation with bL31B was included on modified versions of the respective figures from Ref I.

First, the effect of bL31 paralogs on the rate of initiation was assessed in exponentially growing cells at 37 °C by β-galactosidase time course assay (Samhita et al., 2012). Initiation rate is represented by the slope of the β-galactosidase activity curve and elongation rate by the lag corresponding to translation of the first full-length protein (Samhita et al., 2012) (Ref I, Figure 4a). In the absence of bL31 the rate of translation initiation is reduced by about 38% as compared to the wild type strain (5.02 and 8.11, respectively) (Ref I, Table 2). The expression of bL31A or bL31B *in trans* results in translation rate similar to that in the wild type strain indicating that the presence of either one paralog (bL31A or bL31B) is important for translation initiation (Figure 8, Table 4; Ref I, Table 2).

Table 4. The effect of bL31 on translation parameters measured by β-galactosidase time course assay. This table is based on Table 2, Ref I with additional data about MGΔbL31AB+pHBT-bL31B.

strain	plasmid	rate of initiation	time of elongation (min)	rate of elongation (aa/s)
MG1655	pHBT	8.11 ± 0.28	3.18 ± 0.16	5.39 ± 0.28
MGΔAB	pHBT	5.02 ± 0.22	2.97 ± 0.23	5.79 ± 0.44
MGΔAB	pHBT-bL31A	8.70 ± 0.44	2.99 ± 0.13	5.73 ± 0.25
MGΔAB	pHBT-bL31B	8.97 ± 1.00	2.97 ± 0.25	5.81 ± 0.50

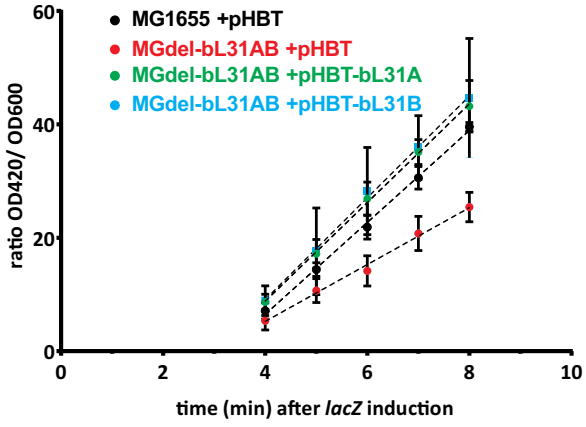

Figure 8. Efficiency of translation initiation depends on the presence of bL31. Wild-type (MG1655; black), bL31 deletion strain (MGdel-bL31AB; red) and bL31 deletion strain harboring bL31A expressing plasmid (MGdel-bL31AB+pHBT-bL31A; green) or bL31B expressing plasmid (MGdel-bL31AB+pHBT-bL31B; blue). The results are presented as the ratio of the β-galactosidase reaction product (spectrophotometrically detected at A420) to the amount of cells (A600). Means of at least three independent experiments are shown with standard errors. This figure is based on Figure 4b, Ref I with additional data about MGΔbL31AB+pHBT-bL31B.

Next, the contribution of bL31 paralogs to translation initiation was evaluated more specifically by assessing the kinetics of functional 70S initiation complex formation *in vitro* using quench-flow techniques. In the absence of both bL31 paralogs the rate of 70S initiation complex formation (expressed by observed rate constant k_{obs}) is about 50% reduced as compared to the reaction with wild type 50S subunits (Ref I, Figure 5) that have been demonstrated to contain bL31A by quantitative MS (Ref I, Figure S1). In conclusion, pre-steady-state kinetics of 70S initiation complex formation and β-galactosidase time course assay indicate that the presence of bL31 in large subunit is important for optimal rate of translation initiation *in vitro* and *in vivo*.

The importance of bL31 paralogs in ribosomal subunit association was additionally evaluated *in vivo* by qualitative analysis of subunit association states from exponentially growing and stationary phase (24h) *E. coli*. The loss of both bL31 paralogs results in substantial decrease in 70S fraction as compared to the wild type strain and most ribosomes sediment at approximately 60S during exponential as well as stationary phase (Ref I, Figure 6). This shows that without bL31 and hence B1b bridge ribosomal subunits are able to associate. However, their reduced sedimentation rate pointing to "loose-couple" ribosomes (Hapke and Noll, 1976) suggests their association to be weaker. Given that bacterial ribosome has 12 intersubunit bridges each comprising several contacts it is not surprising that ribosomal subunits without one bridge associate although with reduced rate (Ref I, Figure 4, 5). The presence of 60S ribosomes in MGΔAB strain is not caused by incompletely assembled particles as demonstrated by the lack of extra peaks on

sucrose gradient profiles (Ref I, Figure 6). In addition, bL31-lacking ribosomes have the otherwise full composition of r-proteins determined by MS and correctly processed 5' end of the 23S rRNA as indicated by primer extension analysis (Ref I, Figure S1, S2). The expression of bL31A or bL31B in MGΔAB strain results in ribosome profiles similar to the wild type strain (Figure 9; Ref I, Figure 6) that can be explained by the finding that most exponential phase wild type ribosomes contain bL31A and stationary phase ribosomes have bL31B (Ref II, Figure 2, S3). This result suggests similar to the B-galactosidase time course assay that bL31 paralogs have a comparable effect on translation initiation.

Ribosomal subunit association *in vivo* is influenced by tRNAs and translation factors interacting with the ribosome (Kisly et al., 2016). Therefore, the importance of bL31 paralogs for subunit association was further analyzed by *in vitro* re-association assay. In this experiment wild type 30S subunits were mixed with wild type or mutant 50S subunits under various magnesium concentrations and their association was monitored by sucrose gradient centrifugation at corresponding magnesium concentrations (Ref I, Figure 7, Ref III, Figure S4). bL31-deficient 50S subunits associate at 12 mM Mg^{2+} whereas 50S subunits from the wild type and plasmid-borne bL31A or bL31B at all three concentrations (6, 8, 12 mM) (Figure 10; Ref I, Figure 7). The proportion of associated subunits reflected by 70S/50S ratios is similar in the bL31A and bL31B strain as compared to the wild type strain (Ref III, Figure S4b) indicating that bL31 paralogs have similar effect on subunit association. In addition to requirement for higher Mg^{2+} for association bL31-deficient ribosomes sediment slower than wild type ribosomes (Ref I, Figure 7). In general, results of *in vitro* re-association assay are in good agreement with ribosome profiles *in vivo* (Figure 10; Ref I, Figure 6) and a later study confirming the association phenotype of bL31-deficient ribosomes (Ueta et al., 2020).

Our results clearly demonstrate that the loss of bL31 in the bridge B1b leads to severely compromised translation initiation as deduced from reduced translation rate and 70S initiation complex formation in addition to problems with subunit association *in vivo* and *in vitro*. Interestingly, bL31A and bL31B seem to perform approximately equally in the context of translation initiation. It is important to note that all experiments addressing the impact of bL31 paralogs on translation initiation were done at 37 °C. Growth difference of the bL31A or bL31B expressing strains becomes evident under lower temperatures (Ref III, Figure 1). Therefore, it can be speculated that the possible different effect of bL31A and bL31B on translation initiation – if any – may be detected at lower temperatures. In support of this, the different contributions of bL31 paralogs in apparent processivity and frameshifting were demonstrated at 30 °C (Ref III, Figure 3, 4).

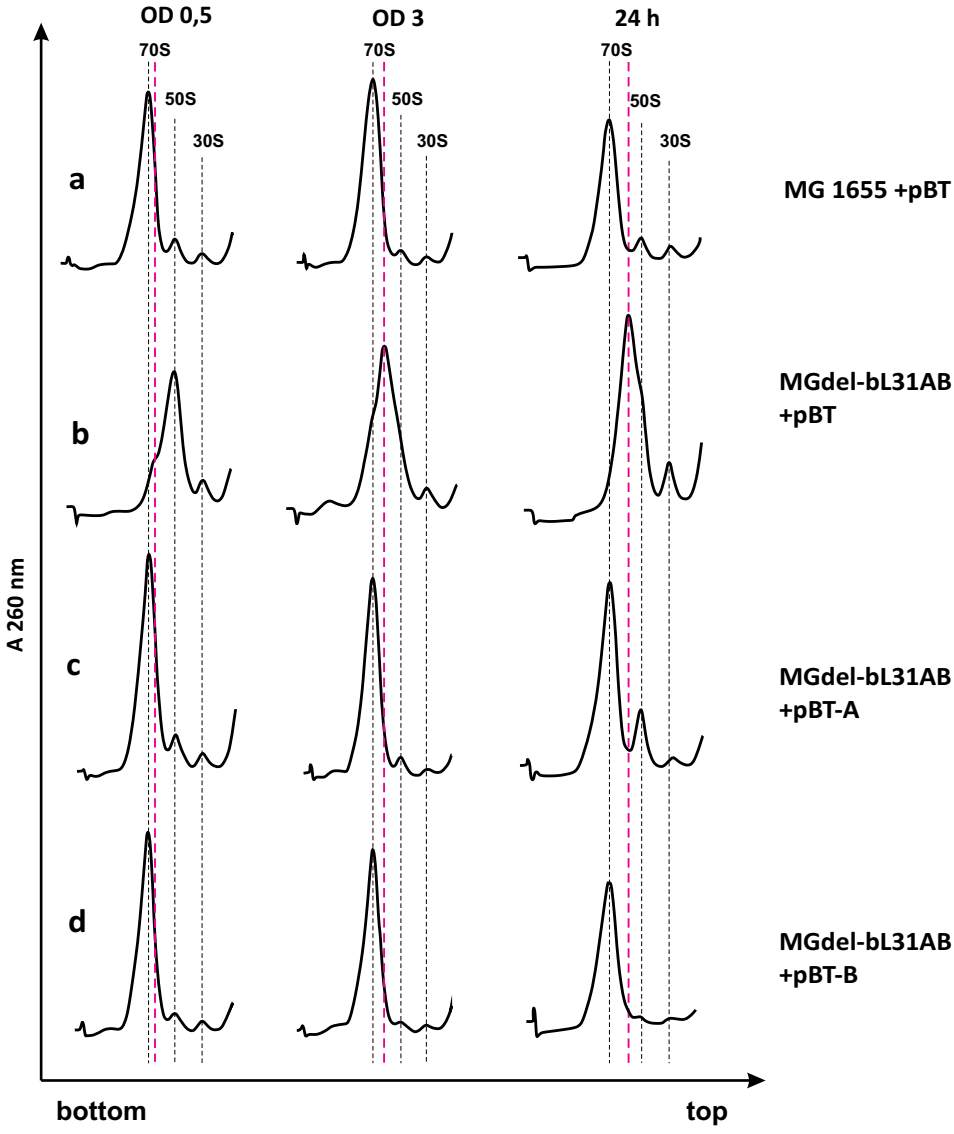

Figure 9. bL31A and bL31B are important for subunit association *in vivo*. Ribosome profiles of sucrose gradient analyses from (a) wild-type MG1655 cells, (b) MGΔbL31AB strain, and (c) MGΔbL31AB strain with pBT-bL31A, (d) MGΔbL31AB strain with pBT-bL31B. Cells were harvested from exponential (OD600 = ~0.5), late-exponential (OD600 = ~3), and stationary growth phase (24 h after inoculation); cell lysates were loaded onto 15–30% (wt/vol) sucrose gradients and analyzed by ultracentrifugation at 72000g for 16 h. The direction of sedimentation is from left to right. Red dashed lines indicate shifted 70S peak corresponding to ribosomes of the bL31 deletion strain. The profiles shown are representatives of at least three independent experiments. This figure is based on Figure 6, Ref I with additional data about MGΔbL31AB+pBT-bL31B.

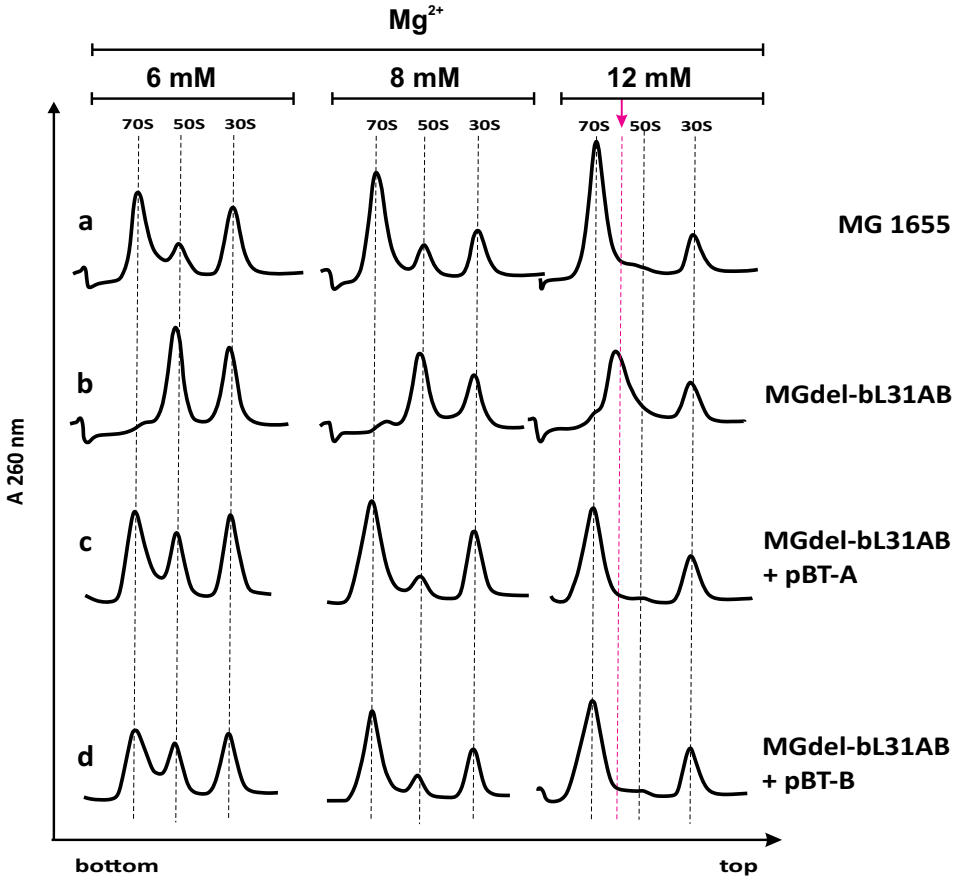

Figure 10. bL31 paralogs contribute to ribosomal subunit reassociation *in vitro*. 5 A_{260} units of bL31 variant 50S subunits were incubated with 5 A_{260} units of wild-type 30S subunits for 30 min at 37 °C. Reaction mixtures were loaded onto 15–25% sucrose density gradients with appropriate Mg2+ concentrations, followed by ultracentrifugation (72,000g for 16 h). Parallel reactions were performed at three different Mg2+ concentrations (6, 8, and 12 mM). The direction of sedimentation is from left to right. 50S subunits are from (a) wild-type MG1655 cells, (b) MGΔbL31AB strain, and (c) MGΔbL31AB strain with pBT-bL31A, (d) MGΔbL31AB strain with pBT-bL31B. A red dashed line indicates the shifted 70S peak corresponding to ribosomes lacking bL31. The profiles shown are representatives of at least three independent experiments. This figure is based on Figure 7, Ref I with additional data about MGΔbL31AB+pBT-bL31B.

3.2.2. bL31A confers slightly higher apparent translation processivity to ribosomes than bL31B *in vivo*

Our experimental data indicates that bL31 is important for rate of translation initiation and subunit association *in vivo* and *in vitro* and bL31A and bL31B seem to make approximately equivalent contribution under conditions tested. Given that bL31 is part of the intersubunit bridge B1b that is the most dynamic one during translocation (Zhang et al., 2009) connecting the central protuberance of the 50S subunit to the highly mobile head of 30S subunit, we next aimed to find out the role of bL31 in translation elongation step. Because they directly relate to ribosome activity rate of translation elongation and errors have been highlighted as important aspects to study when addressing functional importance of ribosome heterogeneity (Emmott et al., 2019).

First, the time and rate of translation elongation were assessed *in vivo* by the same β-galactosidase assay as for rate of translation initiation. The time of elongation (represented as the x-axis intercept) is defined as the time required to produce the first detectable reporter molecule (Ref I, Figure 4a). It takes about 3 minutes to produce the first detectable reporter molecule in the wild type strain, in the absence of bL31 and upon overexpression of bL31A or bL31B (Figure 8, Table 4; Ref I, Table 2). Accordingly, the rate of elongation is approximately the same in all strains (5.4 – 5.8 aa/s). In conclusion, the rate of elongation seems to be unaffected by the presence of bL31 in ribosome as opposed to the rate of translation initiation. However, when interpreting these results, it has to be kept in mind that in β-galactosidase assay "the time of translation elongation" includes several steps of LacZ protein expression (transcription, translation initiation, elongation, termination, protein folding and its first enzymatic reaction). Therefore, this method is rather unsuitable for making strong conclusions about the effect of bL31 paralogs specifically on translation elongation. It is possible that the differential contribution of bL31 paralogs may be masked by other processes.

An important parameter of consecutive enzyme's activity is its processivity. Therefore, the contribution of bL31 paralogs on ribosome apparent processivity was addressed *in vivo* by dual luciferase assay. In short, *Renilla*-luciferase and Firefly-luciferase were expressed as a fusion protein and both activities were measured from cell lysates prepared from exponentially growing cells and expressed as Fluc/Rluc ratio. In contrast to previous experiments addressing translation initiation (37 °C) this experiment was conducted under 30 °C because bL31-specific differences in growth phenotype have been detected at lower temperatures (Ref I, Table 1, Ref III, Figure 1).

In the wild type strain, the median Fluc/Rluc ratio is 7.75 (Ref III, Figure 3a) whereas the absence of bL31 results in about 3.2 times decreased Fluc/Rluc ratio (2.42). This result indicates that significantly less bL31-deficient ribosomes complete the synthesis of the second protein (Fluc) in the fusion protein suggesting that bL31 is important for apparent translation processivity. Reduced processivity correlates with slower growth as compared to the wild type in liquid and on solid media at 30 °C (Ref I, Table 1, Ref III, Figure 1a) that can be explained

by the inability of bL31-deficient ribosomes in synthesizing functional proteins in sufficient amount.

Interestingly, the expression of bL31A in the ΔAB background (A-strain) leads to restoration of the wild type phenotype whereas the expression of bL31B (B-strain) does not (media Fluc/Rluc ratios 8.66 and 4.39, respectively) (Ref III, Figure 3a). Control experiments with bL31A overexpression in the B-strain result in Fluc/Rluc ratio higher than in the wild type strain (10.27 and 7.75, respectively) indicating that the lack of bL31A in the B-strain causes lower apparent processivity (Ref III, Figure 3b). At the same time, bL31B overexpression in the A-strain leads to decrease in Fluc/Rluc ratio as compared to the wild type strain (5.88 and 7.75, respectively) (Ref III, Figure 3b). This supports that bL31B containing ribosomes have lower apparent processivity than bL31A containing ribosomes (r-protein composition of the A- and B-strain was quantified with MS, Ref III, Figure S2). Altogether, A-ribosomes seem to be slightly more processive than B-ribosomes.

These results are supported by the finding that bL31 paralogs seem to differently affect intrinsic ribosome stabilization – a situation when due to certain amino acid sequence growing peptide may destabilize the translating ribosome leading to premature translation abortion (Chadani et al., 2017). The deletion of bL31B leads to wild type level of complete reporter protein suggesting that these ribosomes (that probably contain bL31A) can manage translation of destabilizing amino acid sequences maintaining translation processivity (Chadani et al., 2017). In contrast, bL31A deletion results in accumulation of incomplete reporter protein indicating problems with ribosome stability in response to destabilizing amino acid sequences leading to reduced translation processivity (Chadani et al., 2017).

3.2.3. bL31 paralogs are important but not equivalent for maintaining correct reading frame *in vivo*

Computational and structural studies have suggested the involvement of bL31A in regulating tRNA selection to the ribosomal A-site and translocation based on its position in ribosomes. More specifically, bL31A has been proposed to belong to rRNA and r-protein network responsible for proofreading step of tRNA selection to the A-site during translation elongation (chapter 1.1.2) (Jenner et al., 2010a). In addition, computer simulations led to speculations that bL31A may define the maximum amplitude of the ratcheting movement during translocation (Shasmal et al., 2010). This speculation is supported by a cryo-electron microscopy structure of *E. coli* ribosome in complex with EF-Tu demonstrating that during ratcheting the linker region of bL31A switches from an extended to a kinked conformation while still bound to both subunits (Fischer et al., 2015). In line with this, bL31A has been proposed to modulate the head swiveling of the 30S subunit during translocation based on X-ray structures of 70S from *Thermus thermophilus* in complex with tRNAs in the A- and P-sites (Jenner et al., 2010a). How could the presence of bL31B in ribosomes affect translocation, for example via

its extra loop (Ref II, Figure 5d)? Altogether, these considerations motivated the study of bL31 paralogs with respect to translation fidelity focusing on frame-shifting.

First, the contribution of bL31 paralogs in translation fidelity was assessed in exponentially growing cells at 37 °C by β-galactosidase assay. Reporter constructs contain a programmed ribosomal frameshift (FS) signal or a premature stop codon in the 5' end of the *lacZ* gene (Ref I Materials and Methods). Translation errors lead to expression of the functional LacZ protein that is detected in reaction with its synthetic substrate. In the absence of bL31 ribosomes are significantly more error prone in comparison to the wild type strain as shown by their strikingly higher –1 and +1 frameshifting (about 20x and 3.7x) and stop codon read through (about 7.4 – 9.9x) (Ref I, Figure 2). bL31A or bL31B expression in the ΔAB background leads to the level of stop codon readthrough similar to the wild type whereas –1 and +1 frameshifting are reduced in comparison to the MGΔAB strain but they remain higher than in the wild type strain (Figure 11).

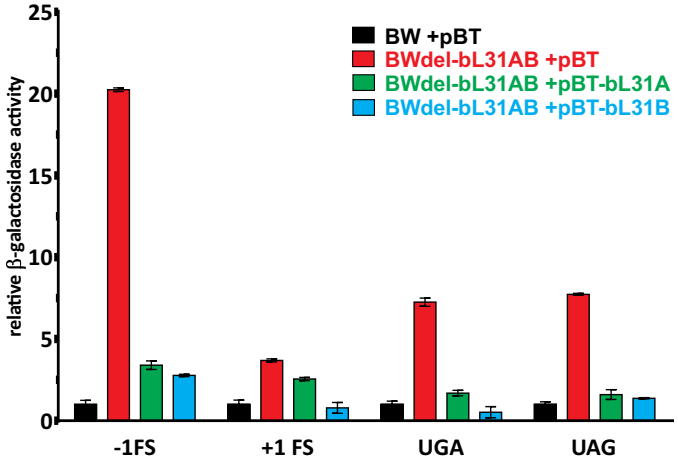

Figure 11. The loss of bL31 reduces the accuracy of decoding *in vivo*. Wild-type (BW +pBT; black), bL31 deletion strain (BWdel-bL31AB +pBT; red) and bL31 deletion strain harboring bL31A expressing plasmid (BWdel-bL31AB+pBT-bL31A; green) or bL31B expressing plasmid (BWdel-bL31AB+pBT-bL31B; blue). BW strains were transformed with plasmids encoding a lacZ gene with N-terminal frameshifts [pSG12DP (–1 FS), pSGlac7 (+1 FS)] or premature nonsense codons [pSG3/4 (UGA), pSG163 (UAG)] and were assayed for β-galactosidase activity. The levels of UAG or UGA read-through and –1 or +1 frameshifting were measured from exponentially growing cells (A600 = ~0.5) and normalized to cell amount. Results are presented relative to the respective values of the wild-type strain. Means of at least three independent experiments are shown with standard errors. This figure is based on Figure 2, Ref I with additional data about BWΔbL31AB+pBT-bL31B.

However, expression of bL31B restores wild type level of +1 FS. The biological impact of frameshifting is potentially larger as compared to the stop codon read through. Frameshifting may easily result in shorter proteins due to pre-mature stop codons or to proteins with altered primary sequence, i.e. to potentially non-functional proteins whereas C-terminally extended proteins are synthesized when a stop codon is not recognized (Caliskan et al., 2015; Tinoco et al., 2013). Therefore, following experiments were focused on frameshifting.

Next, the effect of bL31 paralogs on frameshifting levels was assessed independently by dual luciferase assay. For that Rluc-Fluc constructs used in the translation processivity measurements (Ref III, Figure 3) were modified with five different FS signals in the intergenic region of the Rluc-Fluc reporter construct (Ref I, Figure 3a). In the absence of bL31 both –1 and +1 FS frequency are raised as compared to the wild type (Ref I, Figure 3b) with the greatest increase of –1 FS (15x, 9x or 2x depending on the specific signal in the construct) (Ref I, Figure 3b). This is in agreement with frameshifting level measurements by β-galactosidase assay along with the expression of bL31A *in trans* restoring wild type phenotype (Ref I, Figure 3b).

In β-galactosidase assay BL31A or bL31B was overexpressed *in trans* from a high copy plasmid meaning that their levels have probably been markedly higher than in the wild type strain where one copy of the bL31A or bL31B encoding gene is expressed from chromosome. For bL31A or bL31B expression level reflecting more adequately real-life situation than overexpression from plasmid and to be able to compare the effects of both paralogs we expressed their genes in the same location in the chromosome under *tac* promoter using conditional-replication, integration and modular (CRIM) plasmids (Haldimann and Wanner, 2001). In addition, frameshifting levels were assessed at 30 °C based on cold sensitive growth phenotype of bL31-deficiant strain and different effect of bL31A and bL31B on growth at lower temperatures (Ref III, Figure 1).

Consistent with our previous results at 37 °C the absence of bL31 leads to profoundly increased –1 and +1 frameshifting levels at 30 °C as compared to the wild type (–1 FS: 3.8 – 5.9x increase; +1 FS 2.4 – 3.5x increase depending on the concrete signal) (Ref III, Figure 4, S5). Also, the expression of the bL31A leads to frameshifting levels comparable to the wild type strain readily explained by the dominant presence of bL31A in ribosomes of the exponentially growing *E. coli* (Ref II, Figure 2). By contrast, the levels of FS in the B-strain are slightly higher (1.5 – 2.0x) than in the wild type strain (in four constructs out of five tested) indicating that B-ribosomes are more prone to frameshifting than A-ribosomes. Therefore, it can be concluded that bL31 paralogs are important but not equivalent for maintaining correct reading frame during translation. Biological meaning of this phenomenon needs closer investigation in the future.

Significantly increased frameshifting levels in bL31-deficient strain lead to the conclusion that bL31 is important for maintaining reading frame during translation. It can be speculated that without bL31 the head of the 30S subunit can move with a greater amplitude that in the presence of this r-protein in the B1b bridge. Movement of the 30S head is important for translocating tRNAs and

mRNA through the ribosome by on codon and change in the 30S head dynamics has been proposed to be the primary way for increasing frameshifting (Tinoco et al., 2013). If this movement is not adequately controlled, for example with the help of bL31 in the bridge B1b, mRNA may move more or less than by one codon during translocation resulting in ribosome in +1 or –1 reading frame as compared to the initial one. Importantly, detailed mechanisms of +1 and –1 frameshifting appear to be different but they are not known (Caliskan et al., 2015; Chen et al., 2014; Dunkle and Cate, 2010).

Changes in reading frame alter sequence of the growing peptide deviating from the mRNA encoded variant and may reduce processivity due to premature stop codons. If translation still continues cells invest resources (time, energy, amino acids) into synthesizing nonfunctional proteins. This scenario could explain the significantly slower growth of the bL31-deficient strain. However, the contribution of frameshifting in creating variability in proteome *in vivo* has remained enigmatic, at least in eukaryotic cells (Sonneveld et al., 2020). In addition, it is tempting to speculate that slightly higher frameshifting levels in the bL31B expressing strain than in the wild type or bL31A expressing strain may point to the less restricted 30S head swiveling. It would be interesting to see whether bL31B, or its extra loop could have a role in determining the range of the 30S movement during translocation. Increased frameshifting correlates with reduced processivity similar to the bL31-deficient strain.

The effect of bL31A on translation activity has been evaluated in *E. coli* by three *in vitro* translation systems. First, synthesis of dihydrofolate reductase in a pure transcription/translation system indicates that in the presence of bL31A-deficient ribosomes protein synthesis is reduced by approximately 40% as compared to the bL31A containing ribosomes (Ueta et al., 2017) whereas ribosomes having bL31B exhibited the same translational activity as those with bL31A (Ueta et al., 2020).

In the next two assays, the effect of 8 amino acids from the C-terminus of bL31A on translation elongation and initiation was intended to evaluate. This peptide has been shown to be cleaved during ribosome purification by the outer membrane protease 7 (OmpT) yielding in short bL31 (62 aa) (Ueta et al., 2017). It is important to note that this experimental design does not allow to address this question adequately. More specifically, ribosomes containing intact bL31A were compared with heterogeneous ribosomes having a mixture of intact and short bL31A. Instead, utilization of ribosomes containing only short bL31A would be necessary to test along with appropriate controls like bL31A deficient ribosomes with those from the complementation strain where bL31A is expressed in bL31A deletion background. Therefore, the result that in the presence of heterogeneous ribosomes translation activity (measured by incorporation of poly(U) dependent Phe or MS2 dependent Leu) is decreased by 20% (Ueta et al., 2017) needs to be validated with controls suggested above. Since the former assay measures translation elongation activity and the latter translation initiation as well as elongation the authors conclude that bL31 modification (cleavage of the last 8 amino acids

by outer membrane protease OmpT) affects rather the elongation and not initiation of translation.

The discovery that bL31A is cleaved during ribosome purification by OmpT (Ueta et al., 2017) raises the question whether our *in vitro* experiments addressing subunit association could have been affected by the presence of short and intact bL31A in ribosomes. Importantly, bL31B does not contain a potential the cleavage site for OmpT (Ref II, Figure 6). The possibility of our results being confounded by the presence of short bL31A seems to be rather small for the following reasons. First, bL31A has been shown to be barely sensitive to OmpT in purified 70S but not in free 50S *in vitro* (Ueta et al., 2017). Second, 50S subunits having short bL31A have been demonstrated to not to stably associate with 30S subunits in *in vitro* association experiments (Ueta et al., 2017). In our subunit re-association experiment 50S subunits dissociated from 70S particles were used and they reassociated with similar to wild type efficiency (Figure 10; Ref III, Figure S4). According to MS analysis our ribosomes contain intact bL31A isoform.

In general, our results indicate that bL31 paralogs are equivalent for contributing to translation initiation rate and subunit association (chapter 3.2.1). However, bL31A containing ribosomes have higher processivity and lower frameshifting levels as compared to ribosomes with bL31B. This raises the question why would *E. coli* maintain two genes and incorporate two r-proteins (bL31A, bL31B) with low sequence identity mutually exclusively to ribosomes. We speculate that the importance of bL31B for translation and bacterial survival may become evident under stress conditions. This speculation is based on our results indicating that (1) bL31A is less stably associated with the ribosome at acidic pH *in vitro* and (2) bL31B is the dominant paralog in wild type stationary phase ribosomes. Indeed, under stress conditions bacteria may benefit from more error-prone, bL31B containing ribosomes that by frameshifting potentially create higher variation in proteome, for example moderate levels of mistranslation have been shown to enhance oxidative stress tolerance in *E. coli* (Fan et al., 2015).

CONCLUSIONS

In general, ribosomes are widely considered as macromolecular complexes with homogeneous and stable composition implying that all ribosomes are functionally equivalent, and their structure is relatively insensitive to environmental changes. This view is challenged by the concept of ribosome heterogeneity that has motivated the study of ribosomes from the perspective of potential translation regulators rather than regarding them as passive protein factories. The current study presents evidence for bacterial ribosome heterogeneity from the perspective of r-protein paralogs in *E. coli*. The main results of this thesis can be summarized as follows:

Ribosome heterogeneity

- *E. coli* ribosomes are heterogeneous with respect to r-protein paralog content *in vivo*.
- bL31A and bL36A are prevalent in ribosomes of exponentially growing bacteria, whereas in the stationary phase, most ribosomes contain bL31B and bL36B as quantified by MS.
- The change in r-protein paralog composition takes place as bacterial cells transition from exponential to stationary growth phase, likely via ribosome remodeling.

Growth phenotype

- The loss of both bL31 paralogs results in a cold-sensitive growth phenotype in liquid, and on solid media, the effect increases profoundly as incubation temperature lowers.
- bL31A and bL31B are important but not equivalent for optimal growth at lower temperatures. bL31A confers a growth advantage over bL31B during the exponential phase.
- In growth competition, bL31A confers higher fitness to *E. coli* than bL31B during cyclic growth but not in the stationary phase.

Protein synthesis

- bL31 is needed for optimal translation initiation as shown by a considerably reduced rate of translation initiation and 70S initiation complex formation along with loosely bound ribosomal subunits *in vivo* and *in vitro* in the absence of both bL31 paralogs.
- bL31A and bL31B contribute to a similar extent to ribosomal subunit association
- Both bL31 paralogs are equal regarding translation elongation rate *in vivo* according to the β-galactosidase assay.

- bL31 is important for determining apparent translation processivity with bL31A containing ribosomes being apparently slightly more processive than ribosomes with bL31B *in vivo*, as shown by dual luciferase assay.
- bL31-deficient ribosomes display profoundly increased miscoding and frameshifting *in vivo*. bL31B containing ribosomes are slightly more prone to shift the reading frame than ribosomes with bL31A indicating that higher frameshifting correlates with lower apparent processivity.

Altogether, our data demonstrate ribosome heterogeneity with respect to r-protein paralogs in *E. coli* and its functional importance for bacterial growth and protein synthesis. These results show that bL31 paralogs are not functionally equivalent in translation, and it can be speculated that they may participate in ribosome-mediated translation regulation affecting translation processivity and fidelity.

SUMMARY IN ESTONIAN

Ribosoomide heterogeensus bakterites *Escherichia coli* bL31 paraloogide näitel

Selleks, et ellu jääda, kasvada ja paljuneda, vajavad organismid valke, mis toimivad struktuursete komponentide, ensüümide, signaalivahendajate, transpordi- ja säilitusmolekulidena. Lisaks sellele on elutähtis, et valgud oleksid funktsionaalsed sobivas koguses, õigel ajal ja vajalikus kohas – seetõttu on valgusüntees ja selle regulatsioon kesksemaid/ universaalsemaid eluprotsesse. Kõiki valke sünteesivad ribosoomid, makromolekulaarsed RNA-valk kompleksid. Bakteri ribosoom, selle doktoritöö uurimisobjekt, koosneb kolmest ribosoomi RNAst (rRNA) ja rohkem kui 50 ribosoomi valgust (r-valgud), mis omavahel interakteerudes jagunevad kahe subühiku vahel. *E. coli* väikeses subühikus (30S) on 16S rRNA ja 21 r-valku (joonis 1) ning suures subühikus (50S) 5S ja 23S rRNA koos 33 r-valguga (joonis 2). Väike ja suur subühik assotsieeruvad omavahel translatsiooni initsiatsioonietapis, moodustades ribosoomi (70S). Subühikute vahelisi molekulaarseid kontakte – ribosoomi sildu – on bakteri ribosoomis 12 ja nende ülesanne on hoida subühikuid koos, ent ka võimaldada neil valgusünteesi käigus liikuda (joonis 3). Ribosoomi 3D struktuuri määrab ja funktsioonis domineerib rRNA: selle teatud nukleotiidid osalevad nii mRNA koodoni ja tRNA antikoodoni vahelise aluspaardumise kontrollil väikese subühiku dekodeerivas tsentris kui ka peptiidsideme moodustamise reaktsioonil kasvava peptiidi kahe aminohappe vahele suure subühiku peptidüültransferaasses tsentris. Valgud on translatsioonil assisteerivas rollis, ent ribosoomi komplekssuse ja kooperatiivsuse tõttu on igale valgule ainuomase funktsiooni määramine keeruline.

Eksperimentaalselt on kindlaks tehtud, et nii eukarüootsed kui prokarüootsed organismid toodavad veidi erineva struktuuriga/ ülesehitusega ribosoome. Selle nähtuse – ribosoomide heterogeensuse – all mõistetakse olukorda, kus organismis esinevad samaaegselt erineva koostisega ribosoomid. On näidatud, et ribosoomide heterogeensus võib tekkida nii erisustest rRNA nukleotiides järjestuses, keemilistes modifikatsioonides kui r-valkude stöhhiomeetrias, posttranslatsioonilistes modifikatsioonides ja paraloogses koostises. Ribosoomide heterogeensuse bioloogilise olulisuse kohta on püstitatud alljärgnev hüpotees:

ribosoomide heterogeensus → muutused translatsioonis →
muutused proteoomis → teistsugune fenotüüp

Ribosoomide heterogeensust on senini peamiselt uuritud eukarüootsetes organismides ning selle nähtuse kohta bakterites on veel vähe andmeid/ teada. Seniste uurimuste põhjal on püstitatud hüpotees ribosoomide heterogeensuse vajalikkusest bakterite stressivastuse kontekstis. Samas ei ole teada, kas ja mis ulatuses realiseerub ribosoomide heterogeensuse geneetiline potentsiaal eri keskkonnatingimustel ega ka see, millal ja kuidas eri tüüpi ribosoomide heterogeensus tekib.

Peamine küsimus on, kas ribosoomide struktuurne mitmekesisus võiks tingida/ põhjustada muutusi nende funktsioonis.

Selle doktoritöö fookuses on r-valgu bL31 paraloogide roll bakterite ribosoomide heterogeensuses *E. coli* näitel. Eri kasvufaasidest eraldatud ribosoomide mass-spektromeetriline analüüs näitas, et *E. coli* ribosoomide valguline koostis on eri kasvufaasides üldiselt püsiv. Erandi moodustavad r-valkude bL31 ja bL36 paraloogid: kiire kasvu faasis sisaldab valdav osa ribosoome bL31A-d ja bL36A-d, ent statsionaarses kasvufaasis bL31B-d ja bL36B-d. Kirjeldatud muutused ribosoomide koostises toimuvad rakukultuuri üleminekul kiire kasvu faasist statsionaarsesse kasvufaasi. Samaaegselt esinevad nii A või B paraloogi sisaldavate ribosoomide populatsioonid – seega on tegemist ribosoomide heterogeensusega *in vivo*.

Bakteri ribosoomi struktuurianalüüsid on tuvastanud, et bL31A paikneb ribosoomi sillas B1b, ühendades suure subühiku keskset kühmukest väikese subühiku dünaamilise peadomeeniga (joonis 8). Võttes arvesse bL31A translatsiooni regulatsiooni seisukohast tähelepanuväärset positsiooni, on pakutud, et see r-valk võiks osaleda translatsiooni täpsuse tagamisel. A ja B paraloogide aminohappeline järjestus erineb oluliselt (järjestuse identsusmäär alla 40%), mis tõstatab küsimuse nende funktsionaalsest samaväärsusest ribosoomis/ valgusünteesil ja laiemas plaanis bakterite kasvus. Selle väljaselgitamiseks kasutati käesolevas doktoritöös nii topeltdeletsioonitüve (delAB) kui ainult bL31A-d või bL31B-d ekspresseerivat tüve (A-tüvi ja B-tüvi), kus vastav geen oli paigutatud samasse kohta bakteri kromosoomis CRIM meetodi abil. Selline lähenemine võimaldab sarnasel tasemel ekspressiooni, et võrrelda paraloogide rolli *in vivo*.

Kasvukatsed näitasid, et mõlema bL31 paraloogi puudumisel esineb bakterirakkudel nii vedel- kui tardsöötmel külmatundlik kasvufenotüüp, mis süveneb temperatuuri alanemisel ja mida leevendab erineval määral kas bL31A või bL31B ekspressioon. See tähendab, et bL31A ja bL31B on olulised, ent mitte samaväärsed kasvamiseks madalamatel temperatuuridel, kuivõrd bL31A olemasolu annab bakterirakkudele kiire kasvu faasis kasvueelise võrreldes bL31B-ga. A- ja B-tüve kasvatamine segakultuuris näitas, et bL31A tüvi saavutab ülekaalu bL31B tüve suhtes tsüklilise kasvu tingimustes, ent mitte statsionaarse kasvu tingimustes.

Lisaks kasvule selgitati bL31 paraloogide rolli valgusünteesi initsiatsioonil ja elongatsioonil ning translatsioonitäpsuse tagamisel, keskendudes teaduskirjandusele tuginedes lugemisraami hoidmisvõimekusele. Translatsiooni initsiatsiooni hindavad eksperimendid (initsiatsiooni kiiruse mõõtmine β-galaktosidaasi reaktsioonil *in vivo*, funktsionaalse 70S initsiatsioonikompleksi kineetika mõõtmine *in vitro*, ribosoomi subühikute assotsiatsioonivõime hindamine *in vivo* ja *in vitro*) näitasid 37 °C juures, et mõlema paraloogi puudumisel on (võrdluses metsiktüüpi tüvega) initsiatsiooni kiirus vähenenud ligi 38% võrra, funktsionaalse 70S initsiatsioonikompleksi tekkimiskiirus on ligi 50% võrra väiksem lisaks nõrgemini seotud ribosoomi subühikutele. Samas piisab optimaalseks translatsiooni initsiatsiooniks ükskõik kumma paraloogi olemasolust ribosoomis. Erinevalt initsiatsioonist ei mõjuta bL31 olemasolu ribosoomis valgusünteesi elongatsiooni kiirust *in vivo*.

Et erinevused bL31 paraloogidest põhjustatud kasvufenotüübis avaldusid madalamatel temperatuuridel, otsustati uuringuid jätkata 30 °C juures. Kahe lutsiferaasi reportersüsteemi kasutades selgus, et *in vivo* on mõlema bL31 paraloogi puudumisel näiv translatsiooni protsessiivsus võrreldes metsiktüüpi tüvega vähenenud ligi 3,2 korda. Huvitaval kombel näivad bL31A-d sisaldavad ribosoomid olevat veidi protsessiivsemad kui bL31B-d siduvad ribosoomid. Need tulemused korreleeruvad bL31 paraloogidest sõltuvate muutustega translatsiooni lugemisraami hoidmisel. Nimelt ilma bL31-ta bakteritüves esineb 2,4 – 5,8 korda rohkem raaminihkeid (olenevalt konkreetsest signaalist) kui metsiktüüpi tüves. bL31B-d sisaldavad ribosoomid/ B-tüves tuvastati nii metsiktüüpi tüvega võrreldes kõrgem raaminihete tase kui alanenud translatsiooni protsessiivsus. Seega mida rohkem raaminihkeid, seda väiksem protsessiivsus.

Kokkuvõtvalt võib väita, et *E. coli* ribosoomid on heterogeensed r-valkude paraloogide suhtes *in vivo*. bL31A ja bL31B on samaväärsed translatsiooni initsiatsioonil, kus selle optimaalseks toimumiseks on tarvis üht, ükskõik kumba paraloogi. Erinevalt initsiatsioonist selgus, et translatsiooni protsessiivsuse ja täpsuse tagamisel lugemisraami hoidmisel ei ole bL31A ja bL31B üksteisega samaväärsed, sest bL31A-d sisaldavad ribosoomid on protsessiivsemad ja täpsemad kui bL31B-d sisaldavad ribosoomid. Seega võivad bL31B-d sisaldavad ribosoomid sünteesida vähem funktsionaalseid ja rohkem muutunud järjestusega valke. Mis võiks olla sellise fenomeni bioloogiline tähtsus, näiteks statsionaarses faasis või ka stressivastuses, jääb tulevaste uuringute selgitada. Selle doktoritöö tulemused avardavad teadmisi bakteri ribosoomide heterogeensusest ning r-valgu bL31 funktsionaalsest olulisusest valgusünteesil.

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PUBLICATIONS

CURRICULUM VITAE

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Education

2014– University of Tartu, PhD studies, Molecular and Cell Biology
2014 University of Tartu, MSc *cum laude*, Biology (Molecular and Cell Biology)
2012 University of Tartu, BA, German Language and Literature
2007 Tartu Raatuse Secondary School, gold medal

Professional career

2021– **Junior research fellow**, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu
2015– **Laboratory Assistant**, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu

Research and development work

Main fields of research: Biochemistry; CERCS SPECIALTY: P320 Nucleic acids, protein synthesis

Publications

Lilleorg, S., Reier, K., Volõnkin, P., Remme, J., Liiv, A. (2020). Phenotypic effects of paralogous ribosomal proteins bL31A and bL31B in E. coli. Sci Rep 10, 11682.

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. * shared first authorship

Lilleorg, S; Reier, K; Remme, J; Liiv, A (2017). The Intersubunit Bridge B1b of the Bacterial Ribosome Facilitates Initiation of Protein Synthesis and Maintenance of Translational Fidelity. Journal of molecular biology, 429 (7), 1067–1080. 10.1016/j.jmb.2017.02.015.

Research grants and scholarships

2021 Tiit Talpsep Graduate Student scholarship, The Estonian National Culture Foundation
2018 The Scholarship of Tartu Raefond for participation at the EMBO conference *Protein Synthesis and Translational Control 2019* (Heidelberg, Germany), the University of Tartu Foundation

2018 Young Scientists' Forum (YSF) grant for participation at the YSF and the FEBS Congress 2018 (Prag, Czech Republic), The Federation of European Biochemical Societies (FEBS)

2018, 2016 Scholarship for talented students, Deutschbaltische Studienstiftung, Germany

2017 The Olev and Talvi Maimets Scholarship for participation at the EMBO conference *Protein Synthesis and Translational Control 2017* (Heidelberg, Germany), The University of Tartu Foundation, Estonia

2017, 2013 The Helle Kiis Scholarship for PhD studies

2016 Kristjan Jaak Scholarship for participation at the EMBO conference *Ribosome Structure and Function 2016*, Archimedes Foundation, Estonia

2015 Scholarship for participation at the EMBO conference *Ribosome Synthesis 2015* (Brussels, Belgium), Graduate School in Biomedicine and Biotechnology, University of Tartu

2012 Diploma for Bachelor's thesis in the field of social sciences and culture at the National Student Research Competition, Estonia

2009 Scholarship from the German Academic Exchange Service (DAAD) for an exchange semester at Georg-August-Universität Göttingen, Germany

Other administrative and professional activities

2018– Student member of the Estonian Biochemical Society

2016 Organizing committee member of the Annual Conference of the Institute of Molecular and Cell Biology, Estonian Genome Centre and Estonian Biocentre, Estonia

2015–2019 A sole internal examiner of BSc and MSc theses, Institute of Molecular and Cell Biology, University of Tartu

Teaching and supervision at the University of Tartu

2018 Laboratory Projects (in English), Laboratory Course (both BSc level)

2018 Co-lecturer in Protein Biosynthesis (3 ECTS, MSc level), lecture *Translation Regulation in Bacteria*

2017–2019 Co-lecturer in molecular biology and genetics of Seminar in Biology and Biodiversity Conservation, (3 ECTS, BSc level), problem-based learning methodology course

2015–2018 Co-instructor of the laboratory work in Chemistry of Nucleic Acids (3 ECTS, MSc level)

2019 supervisor of Annete Sikora (BSc)

2018 co-supervisor of Pavel Volõnkin (MSc)

Professional development

2022 How to review a scientific paper (8 hours), EMBO Solutions GmbH, Germany

2019 Lecturer's Voice and Self-Expression (16 h), Human Resources Office, University of Tartu

2017–2018 Seminars for Supervisors of Students Research Papers (28 h), Human Resources Office, University of Tartu

2017 Peer-review: Scholarship of Teaching and Learning (52 h), Human Resources Office, University of Tartu

2015 Summer school *Professional English. A Programme of Academic English for postgraduate students and newly qualified academic staff* (2 ECT)*,* Utrecht Network, University of Hull, United Kingdom.

Public and social activities

Science communication

2019 Lilleorg, S. Ribosoom – mis ja milleks? In: Teadus kolme minutiga 2. 2019, Argo Publishers; popular science article „Why Ribosomes?" (in Estonian), lay summary of my research field and PhD project

2019 Finalist of the Estonian Academy of Sciences public outreach and research communication campaign „The Science in Three Minutes" My presentation: https://www.youtube.com/watch?v=MEKr_Y85xE0

2014–2016 Instructor of the Traveling Biology Classroom programme (University of Tartu & Thermo Fisher Scientific outreach activity for high school pupils)

Translations from German

2019 Reichholf, Josef H (2019). Liblikad. Miks nad kaovad ja mida see meie jaoks tähendab. Hea Lugu.

2018 Vegesack, Siegfried von. Jaschka ja Janne (katkend). Akadeemia, 30 (10), 1807–1825. Literary translation competition, I place, Estonian Goethe-Society.

2015 Jochmann, Carl Gustav. Ajakohatu tõde (valitud palu). Akadeemia, 27 (5), 862–869. Literary translation competition, I place, Estonian Goethe-Society.

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Haridus

2014 – Tartu ülikool, doktorantuur molekulaar- ja rakubioloogia erialal
2014 Tartu ülikool, MSc *cum laude*, bioloogia (molekulaar- ja rakubioloogia)
2012 Tartu ülikool, BA, saksa keel ja kirjandus
2007 Tartu Raatuse Gümnaasium, kuldmedal

Teenistuskäik

2021– biomeditsiini ja biotehnoloogia nooremteadur 0,5 k, molekulaarbioloogia õppetool, molekulaar- ja rakubioloogia instituut, Tartu Ülikool
2015– laborant 0,5 k samas üksuses kus nooremteadur

Teaduslik ja arendustegevus

Peamised uurimisvaldkonnad
ETIS KLASSIFIKAATOR: 1. Bio- ja keskkonnateadused; 1.1. Biokeemia
CERCS KLASSIFIKAATOR: P320 Nukleiinhappesüntees, proteiinisüntees

Publikatsioonid

Lilleorg, S., Reier, K., Volõnkin, P., Remme, J., Liiv, A. (2020). Phenotypic effects of paralogous ribosomal proteins bL31A and bL31B in E. coli. Sci Rep 10, 11682.

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.

Lilleorg, S; Reier, K; Remme, J; Liiv, A (2017). The Intersubunit Bridge B1b of the Bacterial Ribosome Facilitates Initiation of Protein Synthesis and Maintenance of Translational Fidelity. Journal of molecular biology, 429 (7), 1067–1080.10.1016/j.jmb.2017.02.015.

Uurimistoetused ja stipendiumid

2021 Tiit Talpsepa nimeline doktorandi stipendium, Eesti Rahvuskultuuri Fond
2018 Tartu Raefondi stipendium osalemiseks EMBO konverentsil *Protein Synthesis and Translational Control 2019* (Heidelberg, Saksamaa), Tartu Ülikooli sihtasutus

2018 stipendium osalemiseks Noorte teadlaste foorumil ja FEBS'i aastakongressil, Euroopa Biokeemia Seltside Föderatsioon (FEBS)
2018, 2016 stipendium, Deutschbaltische Studienstiftung, Saksamaa
2017 Olev ja Talvi Maimetsa stipendium osalemiseks EMBO konverentsil *Protein Synthesis and Translational Control 2017* (Heidelberg, Saksamaa), Tartu Ülikooli sihtasutus
2017, 2013 Helle Kiis'i stipendium doktoriõpinguteks
2016 Kristjan Jaagu välissõidu stipendium osalemiseks EMBO konverentsil *Ribosome Structure and Function 2016*, sihtasutus Archimedes
2015 välislähetustoetus osalemiseks EMBO konverentsil *Ribosome Synthesis 2015*, Biomeditsiini ja biotehnoloogia doktorikool, Tartu Ülikool
2012 tänukiri, üliõpilaste teadustööde riiklik konkurss, ühiskonnateaduste ja kultuuri valdkond, bakalaureuseõppe üliõpilaste astmes
2009 GIP-stipendium vahetussemestriks Göttingeni Ülikoolis, *Deutscher Akademischer Austauschdienst*, Saksamaa

Muu erialane tegevus

2018– Eesti Biokeemia Seltsi üliõpilasliige
2016 TÜ molekulaar- ja rakubioloogia instituudi, Eesti geenivaramu ja Eesti Biokeskuse aastakonverentsi korralduskomitee liige
2015–2019 bakalaureuse- ja magistritööde oponent, molekulaar- ja rakubioloogia instituut

Õppetöö ja juhendamine TÜ molekulaar- ja rakubioloogia instituudis

2018 Laboratoorsed projektid (inglisekeelne), Laboritöö praktika (mõlemad BSc tase) juhendaja
2018 Valgu biosüntees (MSc tase), loeng Translatsiooni regulatsioon bakterites
2017–2019 Bioloogia ning elustiku kaitse erialaseminar (BSc tase), molekulaarbioloogia ja geneetika osa üks läbiviijatest
2015–2018 Nukleiinhapete keemia (MSc tase), praktikumi kaasjuhendaja
2019 Annete Sikora bakalaureusetöö juhendaja
2018 Pavel Volõnkini magistritöö kaasjuhendaja

Erialane enesetäiendus

2022 *How to review a scientific paper* (8 tundi), EMBO Solutions GmbH, Saksamaa
2019 Õppejõu häälekultuur ja eneseväljendus (16 tundi), personaliosakond, Tartu ülikool
2017–2018 Üliõpilaste uurimistööde juhendajate koolitus (28 tundi), personaliosakond, Tartu ülikool

2017 Kollegiaalne tagasiside: oma õpetamise arendamine (52 tundi), personaliosakond, Tartu ülikool

2015 suvekool *Professional English. A Programme of Academic English for postgraduate students and newly qualified academic staff* (52tundi), Utrecht Network, University of Hull, Ühendkuningriik

Ühiskondlik ja publitsistlik tegevus

Teaduskommunikatsioon

2019 Lilleorg, S. Ribosoom – mis ja milleks?. Teadus kolme minutiga 2. 2019, Argo.

2019 finalist, Teadus kolme minutiga. Eesti Teaduste Akadeemia. Minu ettekanne: https://www.youtube.com/watch?v=MEKr_Y85xE0

2014–2016 instruktor, Rändav Bioklass. Tartu ülikooli ja Thermo Fisher Scientific'u koostööprojekt eluteaduste tutvustamiseks gümnaasiumiõpilastele

Tõlked saksa keelest

2019 Josef H. Reichholf. „Liblikad. Miks nad kaovad ja mida see meie jaoks tähendab". Hea Lugu. 255 lk.

2018 Siegfried von Vegesack "Jaschka ja Janne" (katkend). Akadeemia, 30 (10), 1807–1825.

2015 Carl Gustav Jochmann "Ajakohatu tõde" (valitud palu). Akadeemia, 27 (5), 862–869.

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