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MARI TAGEL

Finding novel factors affecting the mutation frequency: a case study of tRNA modification enzymes TruA and RluA

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Finding novel factors affecting the mutation frequency: a case study of tRNA modification enzymes TruA and RluA

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

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LIST OF ORIGINAL PUBLICATIONS

I. Mikkel, K; **Tagel, M**; Ukkivi, K; Ilves, H; Kivisaar, M (2020). Integration Host Factor IHF facilitates homologous recombination and mutagenic processes in *Pseudomonas putida*. DNA Repair, 85, 102745. DOI: 10.1016/j.dnarep.2019.102745.

II. **Tagel, M**; Tavita, K; Hõrak, R; Kivisaar, M; Ilves, H (2016). A novel papillation assay for the identification of genes affecting mutation rate in *Pseudomonas putida* and other pseudomonads. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 790, 41–55. DOI: 10.1016/j.mrfmmm.2016.06.002.

III. **Tagel, M**; Ilves, H; Leppik, M; Jürgenstein, K; Remme, J; Kivisaar, M (2021). Pseudouridines of tRNA Anticodon Stem-Loop Have Unexpected Role in Mutagenesis in *Pseudomonas* sp. Microorganisms, 9(1), 25. DOI: 10.3390/microorganisms9010025.

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My contribution to the articles is as follows:

Ref I – participated in mutant frequency analyses experiments, provided the illustrations, conducted the statistical analysis, and took part in revision and editing of the manuscript.

Ref II – participated in planning the experiments, conducting the experiments, and writing and editing the manuscript.

Ref III – participated in planning the experiments, preformed experiments (except primer extension with tRNAs), analyzed data, provided the illustrations and statistical analysis, and wrote and edited the manuscript.

Ref IV – participated in planning the experiments, constructing the plasmids, and revision and editing of the manuscript.

ABBREVIATIONS

aa – amino acid
Amp – Ampicillin
ASL – anticodon stem loop
BER – base excision repair
CFU – colony forming unit
Cm – Chloramphenicol
D – dihydrouridine
dsDNA – double-stranded DNA
GGR – global genomic repair
Gm – Gentamycin
Hm – Hygromycin B
IHF – Integration Host Factor
MBR – mutagenic DNA break repair
MMC – Mitomycin C
MMR – mismatch repair
MMS – methylmethanesulfonate
MoTT – modification tunable transcript
NAP – nucleoid-associated proteins
NER – nucleotide excision repair
NQO – 4-Nitroquinoline 1-oxide
Phe – phenol
Pm – Puromycin
Pol – polymerase
PQ – Paraquat
Rif – Rifampicin
RNAP – RNA polymerase
ROS – reactive oxygen species
Sm – Streptomycin
ssDNA – single-stranded DNA
TAM – transcription-associated mutagenesis
TCR – transcription-coupled repair
Tet – Tetracycline
TLS – translesion synthesis
TSM – translational stress-induced mutagenesis
wt – wild type
Ψ – pseudouridine

INTRODUCTION

Bacteria have evolved to withstand even the harshest and seemingly unwelcoming environmental conditions on Earth. For inhabiting different environments and ecological niches, genetic versatility is necessary. The sources of genetic versatility are mutations and horizontal gene transfer. Due to the potential harmful effect of mutations, their occurrence in a cell is kept as low as possible and many error correction, removal, and tolerance mechanisms have coevolved with bacteria. To understand bacterial evolution, it is vital to understand the mechanisms behind the mutations. The first studies of mutational processes of DNA are as old as the discovery of DNA itself but are far from conclusive. Recent achievements in technology have led to many new findings in the field of DNA mutations, making it possible, for example, to measure the mutation frequency patterns across the chromosomes (Foster et al., 2013; Niccum et al., 2019), or to detect population heterogeneity by identifying subpopulations of cells with higher mutation frequency (Matic, 2019; Pribis et al., 2022, 2019). Also, studies of stress induced mutagenesis have revealed that the networks affecting the switch to mutagenic repair can be bigger than previously known and many nonobvious factors affect the mutational outcome (Al Mamun et al., 2012). Although the studies of mutational processes have been thorough, our present knowledge is biased towards the well-studied model organism *Escherichia coli*. Furthermore, in many aspects *E. coli* seems to be rather exceptional and not the best representation of the prokaryotic world.

In our research group, the genus *Pseudomonas* has been used as a model to study mutational processes. This genus contains a large group of bacteria with versatile habitats, broad metabolic capacity and great adaptive potential (Silby et al., 2011). *Pseudomonas aeruginosa*, although common in soil and water samples, is best known as an opportunistic human pathogen with an extraordinary ability to rapidly develop resistance to antimicrobial agents (Lister et al., 2009). *Pseudomonas putida* is a common soil bacterium which is often present in polluted areas. Its high tolerance to toxic compounds and ability to biodegrade versatile pollutants is the reason why *P. putida* is widely used in biotechnology (Kivisaar, 2020a).

The initial aim of my thesis was to find and characterize new mutation frequency affecting genes in the genus *Pseudomonas*. In the progress of this study, among others the most intriguing finding in *P. putida* was the significantly higher mutation frequency in strains lacking a specific tRNA modification enzyme. tRNAs are universal adaptor molecules that carry the building blocks of proteins from cytoplasm to ribosome and tie the amino acids with the mRNA sequence. Probably because the well conserved nature of a tRNA molecule and its wide variety of interaction partners, the tRNAs are abundantly covalently modified. To understand how the tRNA modification enzymes affect the cells physiology and potentially the formation of mutations, we investigated the mutation frequency, translation accuracy, stress tolerance, protein expression, and general fitness of

P. putida strains lacking tRNA modification enzymes TruA and RluA. Moreover, we compared the *P. putida* phenotypes with *P. aeruginosa* and *E. coli.* Intriguingly, while the substrate specificity of TruA and RluA is comparable in all studied strains, the phenotypes observed are markedly different. The finding, that a protein with the most obvious function in translation can affect the mutational outcome in cells of *P. putida* and *P. aeruginosa*, beautifully illustrates how a bacterial cell works as a whole, where all processes happen simultaneously, and can potentially affect one another.

1. REVIEW OF THE LITERATURE

1.1. Factors affecting mutation frequency

In broad terms, a mutation is an alteration in DNA molecule that can happen spontaneously during endogenic processes in a cell or is induced by exogenic factors. Mutations are vital for long-term adaption and evolution, but in short timescale are mostly neutral or deleterious. To avoid the harmful effects, there are numerous systems for removing, repairing, and tolerating the mutations in a cell. But on the other hand, there is no genotype which is optimally adapted for all of the potential present and future environments (Denamur and Matic, 2006). Therefore, for adapting to new conditions, there is constantly a need for genetic versatility, and in a bacterial cell, mutations are the main source of it. Next, I will describe the factors that can affect the mutational outcome of a bacterial population and, through this, mediate the evolution.

1.1.1. Chemical and physical factors

All biomolecules in a cell can potentially be damaged by different chemical and physical factors, e.g., the presence of extensive amount of reactive chemicals, extreme pH, extreme temperature, high pressure, UV-radiation (Friedberg et al., 2005). These factors usually originate from the environment. DNA is an intrinsically reactive molecule and because of it, it is susceptible to changes caused by chemical and physical factors. Furthermore, because the intactness of DNA is not only vital for cell's life but also for its offspring and in a broader sense for the entire population, therefore from the beginning of life the mechanism for repairing DNA damages have evolved.

Bacteria can inhabit all kind of environments and always there is something harmful in the environment. Both the ionizing radiation and UV radiation can cause damages in DNA. The direct absorption of the ionizing radiation energy by DNA can cause direct alterations in nucleobases and sugars, the latter can lead to single-stranded DNA (ssDNA) breaks. In addition, the ionizing radiation can also cause double-stranded DNA (dsDNA) breaks. Furthermore, the radiation can indirectly attack other molecules (e.g., water) and the resulting reactive species can react with DNA causing similar damage as the direct effect of radiation (Friedberg et al., 2005). The most common damage caused by UV radiation is the covalent linkages between neighboring pyrimidines which distort the DNA helix and require translesion synthesis polymerases (will be discussed later in detail) to replicate past them. Also, DNA and protein cross-linking and strand breaks can happen in response to extensive UV radiation. In addition to radiation, a wide range of harmful chemicals might be present in the environment: alkylating agents, aromatic compounds, and toxins from other lifeforms, etc. (Friedberg et al., 2005). However, not only the environmental factors affect the mutagenesis, many factors affecting mutations are simply byproducts of life.

For organisms living in oxygen-rich atmosphere, the reactive oxygen species (ROS) are an unavoidable byproduct of aerobic metabolism, usually as byproducts of electron transport chain of cellular respiration (Chatterjee and Walker, 2017; Friedberg et al., 2005). Furthermore, the exposure to radiation, and different synthetic and natural agents can induce the formation of ROS. For instance, the three major classes of bactericidal antibiotics with different targets of action, all induce the formation of hydroxyl radicals (•OH) which contribute to the cells death (Kohanski et al., 2007). Also, the innate immune system of a host produces ROS in response to colonization of bacteria. In addition to hydroxyl radical, other most abundant ROS are superoxide radicals ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) (Chatterjee and Walker, 2017). Oxidative damage is an important source of mutagenesis and ROS can damage DNA in many ways, e.g., by attacking the double bonds in nucleobase, attacking sugar base, causing breaks in DNA backbone (Chatterjee and Walker, 2017; Friedberg et al., 2005). To protect themselves against oxidative stress, numerous biochemical antioxidants and enzymes act to reduce the harmful effects of aerobic life. For example, many bacteria have specialized detoxifying enzymes like catalases and peroxidases for removing H_2O_2, and superoxide dismutases for eliminating $\bullet O_2^-$ (Borisov et al., 2021). In addition to damaging DNA, ROS are the main factor spontaneously damaging carbohydrates, lipids, RNA, and proteins as well. Moreover, it has been recently shown that oxidative stress directly affects aminoacyl-tRNA editing site leading to mistranslation, which in turn leads to accumulation of misfolded proteins (Ling and Söll, 2010). To conclude, although the aerobic life is advantageous in terms of energy production, the cells constantly need mechanisms to overcome the harmful effects of ROS.

1.1.2. DNA replication

Accurate DNA replication is the main mechanism maintaining genetic integrity. For consistency of life the cells need to replicate their whole genome and do it often and rapidly. It is unavoidable that a process so frequent makes mistakes. Also, these unavoidable mistakes are the source of genetic versatility. The overall DNA replication error rate is kept low at approximately 10^{-9}–10^{-11} mutations per base pair (Fijalkowska et al., 2012). The replicative DNA polymerase itself is much more erroneous but the action of DNA mismatch repair (MMR) pathway reduces the mutation rate two to three orders of magnitude (Fijalkowska et al., 2012; Schaaper, 1993).

In general, the accurate DNA replication is combination of correctly selected dNTPs from the nucleotide pool, DNA polymerase 3'–5' exonuclease activity, which is able to remove mis-incorporated nucleotides, and the action of MMR, which further improves the accuracy by removing errors that the polymerase missed (Fijalkowska et al., 2012). In *E. coli* the replication is carried out by a large replicase complex consisting usually of three copies of polymerase core, one for leading strand and two for lagging, coupled with other subunits (Kurth

and O'Donnell, 2013; Reyes-Lamothe et al., 2010). The core of replicative DNA polymerase III consists of three subunits: the DNA polymerase α subunit (*dnaE* gene), which is responsible for the correct nucleotide selection, the ε subunit (*dnaQ* gene) with proofreading activity, and the θ subunit (*holE* gene), which stabilizes the proofreading subunit interactions (Taft-Benz and Schaaper, 2004). Defects in both α (Maki et al., 1991) and ε (Taft-Benz and Schaaper, 1998) subunit cause mutator phenotypes, but in the case of α subunit mutant antimutator phenotypes have also been described (Fijalkowska et al., 1993). In addition, other subunits of polymerase contribute to the fidelity as well. For instance, *E. coli* strains lacking *holE* have a moderate mutator phenotype in MMR defective background (Taft-Benz and Schaaper, 2004) and the strains carrying mutations in the end of *dnaX* gene (in τ subunit) have enhanced transversions and frameshift mutations (Pham et al., 2006). The τ subunit of the clamp loader is responsible for connecting Pol III α subunits to replisome (Fijalkowska et al., 2012; Kurth and O'Donnell, 2013).

In addition to replicative DNA polymerase, the bacterial cells harbor numerous accessory polymerases, which can also contribute to the overall accuracy of the replication. Pol I (*polA* gene) is the most abundant polymerase in *E. coli* cells, and it is necessary for the replication of lagging strand where it removes the RNA primers and fills the gaps between Okazaki fragments (Patel et al., 2001). Another common example is usage of error-prone translesion synthesis (TLS) polymerases. The TLS polymerases are capable of filling a ssDNA gap at DNA replication blocking lesions sites, which are usually caused by exposure to exogenous DNA damaging agents. Because the TLS polymerases do not harbor proofreading activity and their action can be highly mutagenic, the gap filling by TLS polymerases is considered to be a DNA damage tolerance strategy, not a repair strategy (Fujii and Fuchs, 2020). The most common examples of TLS polymerases in *E. coli* are the Pol IV (*dinB* gene) and Pol V (*umuDC* genes). Although, usually the TSL polymerases contribute to mutagenesis, depending on the nature of DNA damage, the Pol IV can also be involved in error-free synthesis (Napolitano et al., 2000). The upregulation of TLS in stress response will be discussed in more detail in chapter 1.1.6. It is most likely that in addition to stress conditions, during normal replication the high-efficiency replicative polymerase with tight catalytic center encounters a disruption which causes temporary replicase stalling. It might be due to a mismatch or a DNA lesion or a secondary structure. In this case the DNA polymerase switching can occur. This is a prosses where the replicative polymerase is exchanged with another polymerase and at least partially this can be mediated through the τ subunit (Fijalkowska et al., 2012). In *E. coli* the Pol II is able to participate in chromosomal DNA replication and thus it has been speculated to act as a back-up polymerase and offer additional proofreading activity and error removal (Banach-Orlowska et al., 2005). Also, the mutation spectrum analysis indicate that there is a significant interplay between all five *E. coli* polymerases under certain conditions (Curti et al., 2009). Furthermore, recent studies have shown that most proteins in Pol III replisome are constantly exchanged during DNA replication, possibly providing additional flexibility for bypassing

obstacles (Beattie et al., 2017). Thus, the composition of replicative polymerase may be more heterogenous between cells in a population than previously believed (Vincent and Uphoff, 2020).

Although in *E. coli* both DNA strands are replicated by the same replicative polymerase, the error rate differs in leading and lagging strand replication. The DNA replication of lagging strand in *E. coli* is more accurate (Fijalkowska et al., 1998; Maslowska et al., 2018). In addition, in *P. aeruginosa* the mutation spectrum differs in the leading and lagging strand replication (Dettman et al., 2016). The nucleotide selection and proofreading are carried out by the same enzyme, but the overall replication strategy is different, while the leading strand is synthesized continuously, the lagging strand is synthesized in short Okazaki fragments. Thus, the differences in error rate are probably due to the different strategies and it has been proposed that the dissociability of polymerase on the lagging strand causes the higher fidelity phenotype (Maslowska et al., 2018). Thus, it can be concluded that both the composition of the enzyme replicating the genome and the strategy how the replication is carried out affect the mutational outcome.

1.1.3. DNA repair pathways

Obviously, the accuracy of replication depends upon the DNA itself and its integrity, but DNA is not only template for replication but also for transcription, recombination and repair processes, and all these processes can affect DNA integrity. For maintaining the overall DNA fidelity, there are several different repair pathways in the bacterial cell for repairing specific types of mutations caused by different stressors. Inactivation of common repair pathways leaves cells extremely vulnerable to DNA damage. Broadly, the DNA repair mechanisms can be divided into six different strategies (reviewed in (Friedberg et al., 2005)). Firstly, the direct reversal of DNA damage also, referred to as “direct reversal”, combines many unrelated processes directly eliminating a lesion in DNA and not including excision and re-synthesizing a part of DNA. This is usually highly specific to a mutation, error-free and is carried out as a single-step reaction by only one specific enzyme, e.g., photolyases, methyltransferases (Friedberg et al., 2005). Secondly, dsDNA breaks can be repaired by non-homologous end-joining (Shuman and Glickman, 2007) or by homologous recombination repair. The last one can also correct ssDNA gaps that are not resolved by other repair pathways, and homologous recombination is also important for the recovery of collapsed replication forks (Li and Heyer, 2008; Wyman et al., 2004). Usually, if there is a damage in one strand of a dsDNA, it is repaired by one of the three repair pathways: base excision repair (BER), nucleotide excision repair (NER) or mismatch repair (MMR). In BER, once the damage is recognized, the DNA glycosylase cleaves the N-glycosyl bond linking the nucleobase to deoxyribose-phosphate backbone creating an apurinic/apyrimidinic (AP) site. This AP site is further removed from DNA leaving a single stranded gap, which can either be a single nucleotide long or a longer patch of DNA, that is filled with correct

nucleotide(s) (Zharkov, 2008). Next, I will briefly describe the two major DNA repair pathways – MMR and NER pathways.

Firstly, all repair pathways need to recognize the DNA damage. Already this task could be challenging, since the copy number of repair enzymes can be rather low in bacteria. The MMR pathway functions as an error correction mechanism for replication and it is tightly coupled with replication (Hasan and Leach, 2015). MMR repairs single base pair misincorporations and up to 3–4 base pairs long insertion/deletion loops that bypass the DNA polymerase. In *E. coli* MMR, the errors are detected by the enzyme MutS. The homodimer of MutS recognizes the mutation in dsDNA, undergoes a conformational change and initiates the MMR machinery (Jiricny, 2013; Jun et al., 2006; Li, 2008). The MutL protein interacts with MutS and recruits and activates the endonuclease activity of MutH (Hall and Matson, 1999). While DNA is replicating, the newly synthesized strand is unmethylated and this allows the MMR's strand specificity to target and eliminate the mismatch from the daughter DNA strand only. The endonuclease MutH cleaves the unmethylated DNA strand at hemimethylated Dam methylase sites for removal of mismatch-containing strand. The mismatch is then removed. For this, first, the helicase UvrD unwinds the nascent strand, whereas the interaction of UvrD is also facilitated by MutL. Then exonuclease removes the new strand from the nick to the mismatch and the single-strand binding protein SSB, DNA polymerase III and DNA ligase carry out the repair (Jiricny, 2013; Jun et al., 2006; Li, 2008; Yang, 2000).

Although the *E. coli* MMR system is the well-known model, the MMR pathway can be rather different in other bacteria. The presence of *mutH* gene in the genomes of major pathogenic bacterial species is rather exception than a norm (Ambur et al., 2009). For example, *P. aeruginosa*, like other Pseudomonads, does not have Dam methylase nor MutH (Kivisaar, 2010; On and Welch, 2021). In brief, the roles of MutS, MutL and UvrD in *P. aeruginosa* MMR are similar to the ones in *E. coli.* Additionally, MutL has endonuclease activity and is responsible for nicking the nascent DNA strand carrying the mutation (On and Welch, 2021). But there are still many gaps in knowledge about this methylation-independent MMR pathway. For instance, it is still unclear how the discrimination between daughter and parental strand is carried out.

While the main role of MMR is improvement of the DNA replication fidelity (postreplicative repair), other repair pathways are important for repairing damage caused by exogenous mutagenic agents. NER is a unique repair pathway because of its diverse substrate specificity. It can repair a diverse set of structurally different damages, e.g., UV-induced photoproducts (Van Houten et al., 2005) or even DNA-peptide cross-links (Minko et al., 2005). The basic mechanisms of NER are similar to other excision repair pathways: the damage is detected, verified, damaged DNA strand is nicked at both sides of the damaged site, damage is removed, and the gap is filled by DNA polymerase and the new strand is ligated (Kisker et al., 2013). Depending on the damage detection mechanism, the NER can be divided into global genomic repair (GGR) and transcription-coupled repair (TCR). In GGR the $UvrA_2$-$UvrB_2$ complex scans the DNA, and the damage is

detected by UvrA. After initial recognition the DNA is passed from UvrA to UvrB. UvrB verifies the lesion and separates DNA strands. This is followed by the incision by endonuclease UvrC from both sides of the lesion. This damaged oligonucleotide is then removed from the DNA by UvrD helicase and the single-stranded gap is filled by DNA polymerase I and ligated by DNA ligase I (Kisker et al., 2013). In TCR, the steps of repair are the same as in GGR, except the damage recognition differs. The TCR is triggered by transcription blockage when RNA polymerase (RNAP) in unable to transcribe the template DNA due to a blocking lesion. It causes the RNAP stalling and the RNAP blocks the lesion. A translocase Mfd binds to stalled RNAP and pushes it forward away from the lesion to allow NER enzymes to gain access to damage, and at the same time Mfd recruits UvrA (Spivak, 2016). Also, a Mfd-independent TCR has been described, where the helicase UvrD is able to slide RNA polymerase backward away from the DNA lesion and together with regulatory protein NusA recruit the NER complex to the damage (Epshtein et al., 2014). In conclusion, NER in cells is important for mending a wide variety of DNA lesions which are detected either by the NER enzymes independently scanning the DNA or by transcription machinery.

1.1.4. Transcription

In TCR the transcription can trigger the repair, enabling faster detection and removal of lesions, but at the same time transcription can contribute to genetic instability or so-called transcription-associated mutagenesis (TAM) (reviewed in (Jinks-Robertson and Bhagwat, 2014)). Thus, transcription can induce mutagenesis as well as repair. While the DNA is transcribed into RNA, the dsDNA is unwound, and the non-transcribed strand is exposed and more susceptible to damage by different chemicals. For instance, in *E. coli* there is an increase in C to T mutations in the non-transcribed strand (Beletskii and Bhagwat, 1996). In addition, the transcription occurs simultaneously with replication and the genomic stability can be hindered if the DNA polymerase and RNAP collide, causing TAM. Since in prokaryotes the DNA polymerase is approximately an order of magnitude faster than RNAP, depending on a gene's orientation, the replication can face RNAP head-on or co-directionally. The head-on collision has a greater negative effect on DNA replication than co-directional, leading to replication stalling and disassembly, breaks in DNA, and mutagenesis (Paul et al., 2013). In prokaryotes almost all the highly transcribed rRNA genes are co-oriented with replication (Rocha and Danchin, 2003), and it has been proposed that this composition of genome has been at least partly shaped to prevent the head-on collision of replication and transcription (Merrikh et al., 2012). Based on these observations, it has been proposed that the gene's strand-specific orientation can be a mechanism for cells to gene-specifically and temporally accelerate the evolution of specific genes (Merrikh, 2017).

However, it is hard to describe the exact cumulative effect of transcription to mutagenesis. Based on comparative genome analyses of 34 *E. coli* genomes, it was

shown that the highly expressed genes have lower rate of mutations, suggesting that the mutation frequency has been optimized to reduce the risk of obtaining deleterious mutations in highly expressed genes (Martincorena et al., 2012). On the other hand, in more recent work with *E. coli* mutation frequency throughout the genome, there was no negative or positive correlation between mutation density and gene expression (Foster et al., 2013). Furthermore, in budding yeast and in human germline, there is an elevation of mutation rate in highly expressed genes and on the genomic scale in eukaryotes, the TAM exceeds TCR (Park et al., 2012). However, there are many fundamental differences in eukaryotic and prokaryotic transcription, and therefore one cannot transmit these observations to prokaryotes. Yet, although in prokaryotes the net impact of transcription to mutations may not be easily calculated, it is evident that a process using DNA as a template can hinder its integrity.

1.1.5. Mutation rate dependence on chromosome location

In addition to gene's orientation, its chromosomal location can affect the mutations. Since the mutation rate is kept at an extremely low level in cells, it has been historically challenging to study it, especially the overall mutation distribution in a genome. Almost all early studies of mutation rate were dependent on a reporter gene and were usually limited to few specific mutations. But already these experiments indicate that the mutation rate is not constant throughout the genome and is chromosome position-dependent (reviewed in (Kivisaar, 2020b)). With the aid of new fast and cheaper sequencing possibilities, the whole-genome sequencing has improved our knowledge about mutation rate and factors affecting it (Lynch et al., 2016). With mutation-accumulation experiments followed by the whole-genome sequencing in *E. coli* MMR defective strains, it has been shown that not only the mutation rate varies across the genome, but it does so in a spatial wave-like manner. Interestingly, from the replication origin the large-scale pattern of mutations follows symmetrically two replichores (Foster et al., 2013). Similar pattern is also evident in other bacteria, e.g., *P. aeruginosa* (Dettman et al., 2016), *B. subtilis* (Niccum et al., 2019), and in the larger chromosome of *Vibrio cholera* and *V. fischeri* (Dillon et al., 2017). The further studies showed that the mutation rate across the genome is dependent on nucleoid-associated proteins (NAPs) (HU and Fis) and replication initiation, progression, and termination. The DNA polymerase appeared to be more accurate close to the replication origin and then the accuracy declines and after around 1/3 the accuracy rises again. This fluctuation can be at least partly explained by the fluctuations of dNTP pool (Niccum et al., 2019).

The dNTPs are the precursors of the DNA, and an optimal and undamaged dNTP pool is highly important for the accurate DNA replication. If the correct cellular dNTP levels are changed, the replication errors are induced, whereas the change in dNTP levels enhances also mutational specificity (Schaaper and Mathews, 2013). But not only the imbalance of dNTP ratios affects frequency of

mutations, but also if the amount of all dNTPs is increased the mutation rate is higher in *E. coli* (Gon et al., 2006). Generalizing, it can be said that everything that affects the DNA replication, can also affect the occurrence of mutations.

1.1.6. Stress-induced mutagenesis

Although most mutational studies have been carried out in laboratory under controlled and nutrient-rich environments, in natural environments bacteria constantly encounter different stressors and optimal growth conditions are rarely met. Encountering different stresses, the bacterial cell can vastly reshape their gene expression. In addition to phenotypic changes, often for adapting new environments genetic changes are necessary. When the available genetic versatility is not sufficient for adapting to new conditions, bacteria can somewhat accelerate the evolution by transiently increasing the mutation rate (Denamur and Matic, 2006; Fitzgerald and Rosenberg, 2019; Matic, 2019; Rosenberg, 2001). Furthermore, endogenously stressed cells have higher DNA replication error rate (Woo et al., 2018).

The most obvious stress that triggers genetic changes is the stress caused by DNA damaging agents. In response to extensive DNA damage in bacteria the SOS response is induced. When cells encounter DNA replication blocking lesions, the emerging ssDNA fragment is first bound to ssDNA binding protein SSB which then is replaced by recombinase RecA. This nucleoprotein filament stimulates the self-cleavage of repressor LexA and the SOS response is triggered (Foster, 2007). In *E. coli* the upregulation of at least 43 genes is caused by LexA cleavage, among them DNA damage tolerance and repair enzymes (Courcelle et al., 2001). In *E. coli*, three DNA polymerases are upregulated in SOS response – Pol II, Pol IV, and Pol V – the last two of these are low processivity and low fidelity TSL polymerases. While the Pol II and Pol IV are detectable under nonstress conditions as well, the Pol V is undetectable without the SOS induction (Fujii and Fuchs, 2020). In *E. coli*, if the SOS response is constitutively activated, both the Pol IV and Pol V increase the mutation frequency even without the DNA damage (Kuban et al., 2006). Regardless of the highly mutagenic effect of TLS polymerases, the presence of TLS polymerases is widespread from prokaryotes to higher eukaryotes (Ohmori et al., 2001) indicating that TLSs are likely to be beneficial in terms of evolution. Moreover, even without external DNA damaging agents, all three SOS polymerases contribute to competitive long-term survival and evolutionary fitness (Yeiser et al., 2002).

Again, the well-described *E. coli* SOS response serves as a good model, but the SOS response in other bacteria can be noticeably different. The distribution of TLS polymerases differs and most studied genomes, including those of different *Pseudomonas* species, do not harbor the genes for TLS Pol V (*umuDC* operon in *E. coli*) (Ambur et al., 2009; Cirz et al., 2006; Erill et al., 2006). In both *P. aeruginosa* (Cirz et al., 2006) and *P. putida* (Abella et al., 2007) the number of genes belonging to LexA-regulated SOS regulon is markedly smaller than in

E. coli. Furthermore, in *P. putida*, two different LexA regulators have been described, both have their own binding site and both are regulating a separate set of transcriptional units, whereas only one gene is regulated by both LexA1 and LexA2 (Abella et al., 2007). The LexA2 of *P. putida* regulates a damage inducible mutagenic gene cassette of *lexA2-imuA-imuB-dnaE2*, where DnaE2 (or ImuC) is a protein homologous to DNA pol III α subunit. This *dnaE2* gene carrying operon is widespread among different bacteria (Abella et al., 2004). It has also been suggested that genomes carrying the aforementioned gene cassette do not have TLS Pol V genes, indicating that these genes could functionally replace the role of Pol V (Erill et al., 2006). In addition, in *P. putida*, the basal level of *dinB* transcription is much higher than in *E. coli*, whereas the level of SOS induction of *dinB* promoter is markedly smaller (Tegova et al., 2004). However, the overall induction of SOS response due to DNA lesions and its role in overcoming the DNA damage stress seems to be conserved among prokaryotes.

Another targeted approach overcoming stress conditions is the general stress response. It is usually triggered by nutrition limitation and starvation, e.g., when cells enter the stationary phase, but also other stresses like extreme temperature shifts, low pH and high osmotic pressure can induce it (Battesti et al., 2011; Foster, 2007). Furthermore, even subinhibitory concentrations of antibiotics can induce the general stress response (Gutierrez et al., 2013). The general stress response is controlled by sigma factor σ^S (RpoS) and the RpoS is required for stress-induced mutagenesis in carbon starved cells (Lombardo et al., 2004). Encountering stress conditions, the bacterial cells extensively reshape their gene expression pattern by replacing the housekeeping sigma factor RpoD in RNAP with RpoS, and this stationary phase sigma factor alters the RNAP promoter preferences. In *E. coli* almost 10% of the genes can be regulated by RpoS (Weber et al., 2005) and in *P. aeruginosa* the percentage of RpoS regulated genes is similar (about 14%) (Schuster et al., 2004). In the general stress response, a wide variety of different cellular pathways are affected and among them some can increase spontaneous mutation rate. In *E. coli*, in addition to SOS regulation, the induction of TLS polymerase Pol IV (dinB) is RpoS-dependent, and the levels of Pol IV increase in stationary phase (Layton and Foster, 2003). In *P. putida* PaW85, the Pol IV facilitates the occurrence of 1-bp deletions in stationary phase (Tegova et al., 2004). Furthermore, in *E. coli*, the MMR pathway enzymes MutS and MutH are RpoS-dependently downregulated in stationary phase or carbon starved cells (Feng et al., 1996; Tsui et al., 1997). The action of Pol IV and the RpoS-dependent downregulation of MutS are also the causes of mutagenesis in the presence of subinhibitory concentrations of β-lactam antibiotics (Gutierrez et al., 2013). Also, the general stress response induces the movement of mobile genetic elements and increases genetic versatility through it (Foster, 2007; Ilves et al., 2001; Lamrani et al., 1999).

Comparing the mutation frequency of one day old and 7 days old *E. coli* colonies from almost 800 different environmental isolates, on average, the mutation frequency elevated sevenfold and in 13% of the strains even more than 100-fold. The observed phenomena was called mutagenesis in aging colonies (Bjedov

et al., 2003). Based on example of one representative isolate tested, the mutagenesis in aging colonies phenotype depends upon RpoS, downregulation of MMR pathway and SOS response-inducible *polB* (Pol II), but is SOS regulator LexA-independent (Bjedov et al., 2003). The results of this study well illustrate how the frequency of mutations can be increased in response of environmental conditions, which in turn could enhance the adaptive evolution.

In unstressed conditions, the dsDNA breaks are repaired by non-mutagenic homologous recombination, but under stress conditions the repair is switched to mutagenic DNA break repair (MBR) which causes base substitutions and indels (reviewed in (Fitzgerald and Rosenberg, 2019; Pribis et al., 2022)). In *E. coli,* for the switch to homologous recombination-directed MBR, both the activation of SOS-response and the RpoS-mediated general stress response are needed (Ponder et al., 2005). The MBR of dsDNA breaks is also dependent upon TLS polymerase Pol IV (*dinB*) (Ponder et al., 2005; Shee et al., 2011), which is regulated by both above-mentioned responses. Also, just the stress response itself is sufficient to induce the MBR and the actual starvation is not necessary (Shee et al., 2011). Furthermore, the membrane protein stress regulator σ^E contributes to the MBR (Al Mamun et al., 2012; Gibson et al., 2010), indicating that the cells double (or triple) check the stressful environmental conditions before switching to the mutagenic repair in starving cells (Fitzgerald and Rosenberg, 2019). In studies of *E. coli*, a network of at least 93 proteins functioning in MBR was identified. Most of the proteins detected function in sensing the stress and transducing the signal to stress response regulators (Al Mamun et al., 2012). The vast majority of those proteins were not obvious to function in mutagenesis but additional functional studies showed that more than half of those affected mutation frequency (Al Mamun et al., 2012). The complexity of MBR network illustrates the tight regulation of mutational processes in response to stress conditions, but also indicates that networks regulating mutagenesis might be more complex than previously expected.

1.1.7. Translation

Although seemingly translation and replication are two autonomous processes, they do affect each other. In some conditions increased mistranslation leads to translational stress-induced mutagenesis (TSM) (Humayun, 1998). A well-studied examples of this are the defective tRNAs genes, which cause a mutator phenotype in *E.* coli (Dorazi et al., 2002; Slupska et al., 1996). The mutator tRNAs (*mutA* and *mutC*) encode a glycine tRNA which normally decodes GGU and GGC codons, but due to a mutation in the anticodon it reads aspartic acid codons GAU and GAC instead (Slupska et al., 1996). These mistranslating cells bear a recombination-dependent (*recA* and *recB*) but SOS-response independent (*umuD*, *umuC*, *dinB*, *lexA*) mutator phenotype (Murphy and Humayun, 1997; Ren et al., 1999). The mutator phenotype is not only Asp → Gly mistranslation-specific, but also the general streptomycin-induced mistranslation (Balashov and Humayun, 2002),

ribosomes with increased mistranslation (Balashov and Humayun, 2003), and mistranslating alanine tRNAs (X → Ala mistranslation) (Dorazi et al., 2002) increase mutations. Comparable to *mutA* phenotype, the streptomycin-induced mutagenesis effect is also recombination-dependent and SOS-response-independent (Balashov and Humayun, 2002). This leads to the speculation that a tRNA modification enzyme MiaA (discussed more in chapter 1.2.1.2) deficiency-induced *recA*-dependent mutator phenotype (Connolly and Winkler, 1991, 1989; Zhao et al., 2001) is also caused by mistranslation. Although it is in good correlation with the result showing that lack of *miaA* causes rise in translational frameshifting (Urbonavičius et al., 2001), it does not take into consideration that *miaB*-deficiency which also increases translational frameshifting (Urbonavičius et al., 2001), does not cause a mutator phenotype (Zhao et al., 2001). Furthermore, the TSM is not always dependent of recombination, as the cells carrying ribosomes with mutant S4 protein (Balashov and Humayun, 2003) or mistranslating alanine tRNAs (Dorazi et al., 2002) possess a *recA*-independent mutator phenotype.

It is proposed that the TSM phenotype is rather induced by faulty DNA replication than by defective repair pathways. This is demonstrated by *in vitro* replication fidelity analyzes with purified polymerase III complex from the *mutA* cells (Al Mamun et al., 2002). The purified polymerase III complex from TSM phenotype-carrying cells is more error-prone compared to the control, but the effect is noticeably milder than the *in vivo* mutator phenotype (Al Mamun et al., 2002; Slupska et al., 1996). It is suggested that along with the mutator phenotype in the *mutA* cells, the replication fork collapses more often and for the restoration of replication recombination is needed (Al Mamun et al., 2006). This would explain the recombination dependence of the phenotype. Recent studies of *E. coli* population heterogeneity revealed that the subpopulation of cells with increased translation errors had also higher rate of replication errors (Woo et al., 2018). These results could also indicate that mistranslation leads to defects in DNA replication machinery.

Although mistranslation has been connected with DNA damage and stress response in several other cases, the outcome of mistranslation is not always increased mutation frequency. In *E. coli* measurements of mistranslating mutant due to genetically depleted initiator tRNA content showed higher survival of mutant cells under various kinds of DNA damage (UV radiation, H_2O_2 and ciprofloxacin stress) and elevated temperature stress. Different experiments proved that in the case of this mistranslating mutant the threshold for SOS response induction was lower (RecA level was higher and LexA degradation was faster) but the overall mutation rate was comparable to wild type. The authors speculate that due to the mistranslation the Lon protease levels are increased, which in turn triggers the earlier induction of SOS response and this enables the cells to survive different stresses including DNA damage stress (Samhita et al., 2020). Similarly, *E. coli* cells with mistranslating ribosome have higher survival under oxidative stress that is caused by activation of general stress response by increasing the RpoS protein level (Fan et al., 2015).

The mistranslating mutants with an editing-defective aminoacyl-tRNA ($ileS_{Ala}$) had significantly higher mutation frequency in aging colonies (day 7) compared to wild type (Bacher and Schimmel, 2007) but in growing cells had no effect to the mutation frequency (Bacher et al., 2005). The effect on mutagenesis in aging colonies was via the induction of SOS response because the cells unable to induce the SOS response did not have higher mutation frequency (Bacher and Schimmel, 2007).

In addition to the general mistranslation, the overall well-being of the proteome is important for the cells. The induced oxidative protein damage triggers the rise in spontaneous mutation rate, whereas reducing the level of oxidative protein damages produces an anti-mutator phenotype (Krisko and Radman, 2013). All this clearly demonstrates how different processes in cells could affect each other and both the mistranslation and general defects in proteome can lead to increased mutation rate. To conclude, all these examples of factors affecting mutagenesis are illustrating perfectly how a bacterial cell works as a whole, and imbalances in central processes can lead to mutations.

1.2. RNA modifications

RNA carries the central roles in translation. In addition to the four standard nucleotides – C, G, U, and A – the nucleotides in RNA are highly modified with a wide variety of different covalent modifications. Modifications can be chemically very simple, e.g., an addition of a methyl group to a various position of nucleobase or ribose, or complex additions of a set of chemically diverse groups (Jackman and Alfonzo, 2013; Ontiveros et al., 2019). In the last case, a nucleotide can reach its fully modified state by the action of numerous different enzymes acting in a defined order. Modified nucleotides are found in all types of stable RNA species and in all kingdoms of life. In rRNAs the modifications are clustered around catalytically important regions (Decatur and Fournier, 2002). tRNAs, being the universal adapter molecules, have been found to harbor the most versatile repertoire of modifications and are also the nucleic acid molecules with the highest percentage of modified nucleotides (Fig. 1). Gram negative bacterial tRNAs have about 10% of nucleotides modified and in eukaryotes an average of 16% (up to 25%) of nucleotides are modified (Björk and Hagervall, 2014; Machnicka et al., 2014), whereas in bacterial rRNA there are approximately 1% of nucleotides modified. There is also a growing evidence of modifications in mRNA coding sequences, mostly in eukaryotes but also in prokaryotes (Gilbert et al., 2016). The knowledge about mRNA modification and their relevance in translation is still limited.

1.2.1. tRNA modifications

tRNAs carry the building blocks of proteins from cytoplasm to ribosome and tie amino acid sequence with genetic code, but even in addition to this obviously important role they have additional tasks. To name some of the noncanonical roles of tRNAs, the regulation of nucleotide alarmone synthesis, synthesis of small metabolites, and modification of macromolecules, e.g., lipids and peptidoglycan, etc., have been reported (Katz et al., 2016). The overall stability, structural stability and the functionality of tRNAs can be modulated through the posttranscriptional modifications (Björk and Hagervall, 2014; El Yacoubi et al., 2012; Shepherd and Ibba, 2015). The modifications can reshape the structural, thermodynamical, and chemical properties of nucleotides and through that contribute to the functioning of tRNA.

Although studies of unmodified *E. coli* $tRNA^{Phe}$ revealed that the overall L-structure of mature tRNA can be achieved without modifications (Byrne et al., 2010; Harrington et al., 1993), nevertheless, there are several structural differences compared to a fully modified one (Byrne et al., 2010). Also the translation accuracy of unmodified $tRNA^{Phe}$ *in vivo* is significantly affected (Harrington et al., 1993). Albeit addition of a single modification to an otherwise unmodified $tRNA^{Phe}$ anticodon stem loop (ASL) at 32 or 37 position did not significantly alter the structure (Cabello-Villegas et al., 2002; Cabello-Villegas and Nikonowicz, 2005), the modifications affected the ASL in other ways, e.g., by increasing the mobility of nucleotides in the loop (Cabello-Villegas et al., 2002) or by increased the thermal stability (Cabello-Villegas and Nikonowicz, 2005). At the same time, the unmodified ASLs of $tRNA^{Lys}$ and $tRNA^{Gln}$ were not able to bind to ribosome and addition of a single modification s^2U34 restored the ribosomal binding of $ASL^{Lys(UUU)}$ (Ashraf et al., 1999; von Ahsen et al., 1997). It could be concluded that the tRNA modifications can help tRNAs to attain their functional optimum. It is also interesting to note that the addition of magnesium ions can partially compensate the absence of modifications (Motorin and Helm, 2010).

In bacteria, almost all the modifications are synthesized at polynucleotide level by specialized site-specific enzymes (Björk and Hagervall, 2014). The pattern of modifications can vary greatly among different tRNA species. For instance, the modifications m^5U54 and Ψ55 are present in all elongator tRNAs of Gram negative bacteria (Machnicka et al., 2014). However, several specific modifications are found only in one tRNA species, meaning that there are tRNA-modifying enzymes for making only one modification (usually for modifications at the positions 34 or 37, Fig. 1) (Björk and Hagervall, 2014; Machnicka et al., 2014). Some of the modifications are found in all three kingdoms of life, and even more, they locate in a comparable set of tRNA species at comparable positions (e. g., m^1G37, Ψ39, m^7G46, m^5U54, Ψ55, Fig. 1) (Machnicka et al., 2014).

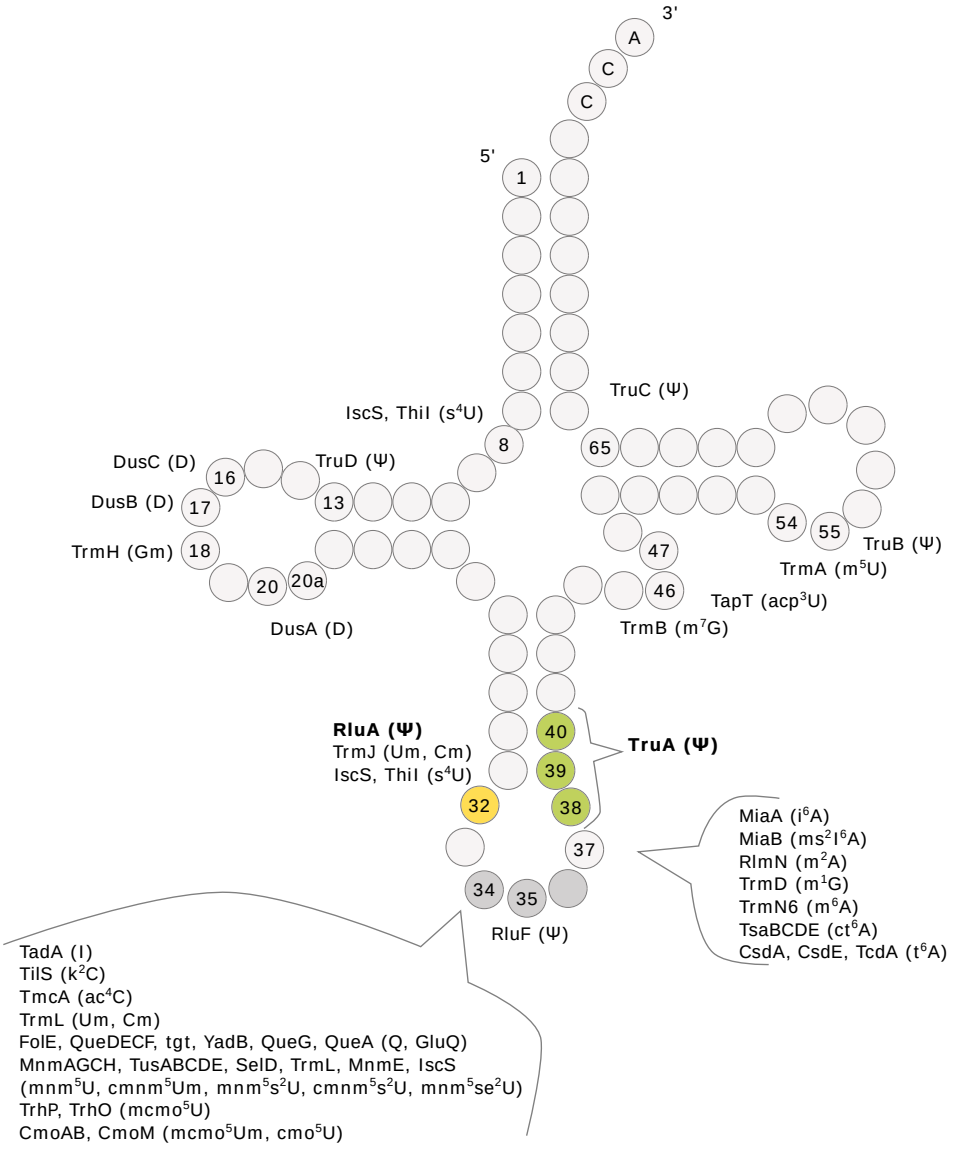

Figure 1. Scheme of a tRNA and its modifications. All *E. coli* tRNA modifications known so far are presented. The number indicates the modified position, the responsible enzyme(s) and the resulting modifications (in brackets) are listed adjacently. Target positions of TruA are shown in green and target position of RluA is yellow, the anticodon is shaded dark grey (based on (Björk and Hagervall, 2014; de Crécy-Lagard and Jaroch, 2021; El Yacoubi et al., 2012)).

Generally, the modifications are located in single-stranded regions of tRNA, and by the locational clustering tRNA modifications can be divided roughly into two groups, in the ASL domain and in the structural core of the 3D L-shape structure (Helm, 2006). Modifications in the core are usually considered to play primarily the structural role and help to stabilize the overall L-shape structure of tRNA (Shepherd and Ibba, 2015). Some locations in the core are so frequently modified with conserved modifications that these modifications have begun to be a part of the tRNA structure nomenclature (e.g., D-loop and T-loop) (Agris, 2004). Interestingly, the majority of these modifications are biosynthetically simple (Agris et al., 2017; Helm, 2006). At the same time, the modifications in the ASL can be very versatile and contribute directly to decoding of mRNA and translation efficiency and accuracy (Agris et al., 2017). Non-anticodon ASL nucleotides and their modifications play altogether such an important role in anticodon-codon interactions that the concept of "extended anticodon" has been proposed to describe the codon-binding of tRNAs (Grosjean and Westhof, 2016; Yarus, 1982).

Positions 34 and 37 at ASL are modified most frequently (this varies between species but approximately 45% and 78%, respectively (Björk and Hagervall, 2014; Machnicka et al., 2014)), and these positions have the highest diversity among modifications (Fig. 1). With the exception of few modifications in Archaea, all the complex modifications which require more than one enzyme for their synthesis, are located either at the position 34 or 37 (El Yacoubi et al., 2012). Both positions are important for translation. The nucleotide modifications at the position 37, which is located next to the 3' end of anticodon (Fig. 2), is considered to improve both the efficiency and fidelity of translation, and it helps to maintain reading frame (Björk and Hagervall, 2014; Shepherd and Ibba, 2015; Urbonavičius et al., 2003, 2001). The nucleotide modification at the position 34, which is the first nucleotide of anticodon and pairs with third nucleotide of codon (Fig. 2), directly improves codon-anticodon recognition (Agris, 2004; Agris et al., 2007; Ranjan and Rodnina, 2016) and through this modification the translation fidelity is improved (Urbonavičius et al., 2003, 2001). The fact that out of the only few tRNA modifications which absence is lethal to bacteria all are located at the positions 34 or 37, illustrates the importance of the modifications at these positions (de Crécy-Lagard and Jaroch, 2021; El Yacoubi et al., 2012), e.g., in *E. coli* the modifications I34 (Wolf et al., 2002) and t^6A37 (El Yacoubi et al., 2009).

In addition to the positions 34 and 37, the ASLs of *E. coli* are also modified at the positions 38–40 (Ψ, catalyzed by the enzyme TruA (Kammen et al., 1988)), 32 (different modifications s^2C, Cm, Um, and Ψ, catalyzed by the corresponding enzymes (de Crécy-Lagard and Jaroch, 2021; Machnicka et al., 2014)) and in one tRNA species also at the position 35 (Ψ in $tRNA^{Tyr}$, catalyzed by RluF (Addepalli and Limbach, 2016)) (Fig. 1). Based on the "extended anticodon" concept, all these modifications can participate in codon-anticodon interaction and through that in translation.

Although in bacterial genome up to 10% of genes encode enzymes that are involved in modifying tRNA (Anantharaman et al., 2002; El Yacoubi et al.,

2012), most of the modifications are not essential. Even today the role of many modifications is not well understood. The full repertoire of tRNA modification enzymes is known only for a couple of model organisms (de Crécy-Lagard and Jaroch, 2021), meaning that there is still a lot to learn about the repertoire and the functions of tRNA modifications. In addition to the above-mentioned involvement in achieving and stabilizing the correct 3D structure, the tRNA modifications can be important for overall stability of tRNA molecules. For example, the lack of m^7G46 in combination with other seemingly nonessential modifications outside ASL leads to rapid decay of $tRNA^{Val(AAC)}$ in yeast (Alexandrov et al., 2006). tRNAs use posttranscriptional modification to achieve both structural stability and uniformity to be recognized by ribosome and the originality for recognizing specific codons (Agris, 2004; Agris et al., 2007). Also, the tRNA modifications can act as identity determinants or antideterminants for specific aminoacyl-tRNA synthases (Agris, 2004; El Yacoubi et al., 2012; Sylvers et al., 1993). In addition, the availability and improvements of analytic tools have made it possible to study new aspects of tRNA modifications, and the list of tRNA modifications having a role in gene regulation is growing (de Crécy-Lagard and Jaroch, 2021; Endres et al., 2015; Pollo-Oliveira and de Crécy-Lagard, 2019). However, it is likely that the main and the initial role of tRNA modifications is to participate in fine-tuning of translation.

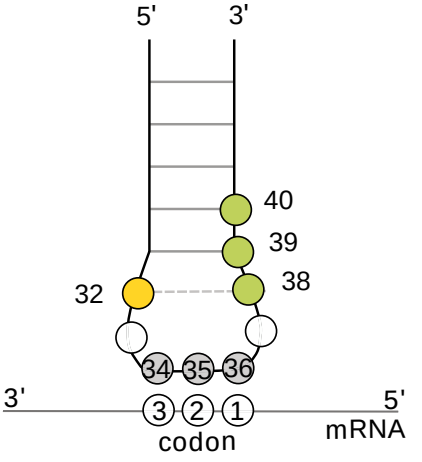

Figure 2. Scheme of an ASL of tRNA pairing with mRNA. TruA target positions are highlighted in green and RluA target position is highlighted in yellow, the anticodon is highlighted in grey. The lines indicate hydrogen bonds in the anticodon stem and the dotted line represents non-canonical bifurcated hydrogen bonds between nucleotides 32 and 38.

1.2.1.1. The role of tRNA modifications in translation

Although there are 61 different amino acid-coding codons, not all corresponding tRNA genes are represented in a genome of one organism. Usually, in a bacterial genome there are around 40–50 different tRNA genes (e.g., in *E. coli* K12 49, in *P. putida* KT2440 42, in *P. aeruginosa* PAO1 44 (Jühling et al., 2009; Winsor et al., 2016)). The posttranscriptional modifications significantly improve the coding capacity, and this allows the organism to have less different tRNAs and still properly decode all the codons (Agris, 2004; Agris et al., 2007). The importance of tRNA modifications in decoding is also illustrated by the fact that in *E. coli* only 7 out of 61 codons are decoded by tRNAs without modifications at the positions 34 or 37 (Agris, 2004).

It appears that the function of ASL modifications may vary greatly depending on the coding strategies of a tRNA. In the case of amino acids with many codons (four or six), modifications enable the cells to use fewer isoacceptor tRNAs for coding. Pro, Thr, Ala, Val and Gly all belong to a completely 4-fold degenerate codon box and only the tRNAs reading 4-fold degenerate codons carry the cmo^5U34 modification (Yarian et al., 2002). In *Salmonella* Typhimurium out of the three proline tRNAs only one has a cmo^5U34 modification. The cells carrying only the tRNA with cmo^5U34 modification are viable, meaning that cmo^5U34 can form a base pair with all four nucleotides at the third position of codon (Näsvall et al., 2004). Similar mechanisms have been identified in the case of other four codon box tRNAs; for example, in *E. coli*, there are two alanine tRNAs and *in vitro* the tRNA carrying cmo^5U34 modification is capable to form codon-anticodon interaction with all four alanine codons (Kothe and Rodnina, 2007).

In the case of tRNA species translating twofold degenerate codons, the modifications are proposed to be necessary for their cognate codon recognition and for discriminating near-cognate codons (Agris, 2004; Agris et al., 2007). Modifications are especially important for tRNAs with pyrimidine rich anticodon loops, which without modifications are often not able to bind to their cognate codons (e.g., $tRNA^{Lys}$, $tRNA^{Gln}$, $tRNA^{Glu}$) (Agris, 2004; Ashraf et al., 1999; von Ahsen et al., 1997). For instance, the addition of only s^2U34 already restores the ability of *E. coli* $tRNA^{Lys(UUU)}$ ASL to decode Lys codons AAA and AAG (Ashraf et al., 1999), and discriminate the near-cognate Asn codons AAC and AAU (Yarian et al., 2002). While in the case of human $tRNA^{Lys(UUU)}$ the single incorporation of mnm^5U34 or t^6A37 restored ribosome binding to AAA codon, but for binding to alternative AAG codon, both modifications at 37 and 34 position are necessary (Yarian et al., 2000).

As seen from above-discussed examples, not only the modification at the position 34 but also at the position 37 are important for decoding. There is also a good correlation that the weak anticodon-codon interaction (A1–U36 or U1–A36) is often followed by a strongly stabilizing modification at the position 37 (t^6A or ms^2i^6A in *E. coli*) (Grosjean and Westhof, 2016). For instance, the t^6A37 in $tRNA^{Lys(UUU)}$ has an important role in preventing the base pairing of U33–A37 and improving the stacking of nucleotides 37 and 38, and thus by helping to

maintain the right structure of ASL (Fig. 2), this modification plays a part in codon discrimination and also helps to improve the translation fidelity (Murphy et al., 2004; Stuart et al., 2000).

Improving translation fidelity seems to be a common function of structurally different modifications, especially helping to maintain the reading frame. Although the effect of modifications is tRNA and codon specific, several modifications in *E. coli* and *S.* Typhimurium at the positions 34 and 37 help to prevent +1 frameshift (Urbonavičius et al., 2001), whereas most of the measured modifications do not affect –1 frameshifting (with the exception of mnm^5s^2U34) (Urbonavičius et al., 2003).

In addition to codon recognition and discrimination, the modifications can be important for other steps of protein synthesis. In *S.* Typhimurium, different modifications in ASL at 3' side of anticodon stimulate the selection of aa-tRNAs (m^1G37, ms^2io^6A37, Ψ38) but these modifications can have very tRNA-dependent effects (Li et al., 1997). Studies of translocation revealed that addition of just mnm^5U34 or t^6A37 modification to *E. coli* ASL^{Lys} did not restore the translocation of tRNA from A-site to P-site, but the doubly modified ASL^{Lys} translocated effectively (Phelps et al., 2004).

The tRNA modifications can also act as identity elements for aminoacyl synthases in the aminoacylation of tRNA. For example, the *in vitro* aminoacylation of $tRNA^{Glu}$ by *E. coli* glutamyl-tRNA synthase is less efficient when the modification mnm^5s^2U34 is missing (Sylvers et al., 1993).

In conclusion, although the tRNA modifications have very specific effects depending on the tRNA nucleotide sequence, codon specificity, and interplay between different modifications, the modifications can affect every step of translation. It is most probable that the modifications provide moderate compensatory effects, enabling the cells to grow normally even without multiple modifications under nonstress conditions. Nonetheless, translation is such an essential process in cells and therefore it cannot be excluded that even modifications with moderate effects can turn out to be crucial in changing and stressful environments (Jackman and Alfonzo, 2013).

1.2.1.2. tRNA modifications in stress response

There is a growing number of evidence showing that tRNA modifications can modulate the stress response and take part in reprogramming the gene expression. Here it is important to note that the modification levels are often not static and may vary depending on different physiological conditions. It is proposed that cells modulate the stress response by dynamically adjusting the tRNA modification patterns, which in turn affects translation fidelity and selective protein expression (Agris et al., 2017; Chan et al., 2010; Dedon and Begley, 2014; Endres et al., 2015; Ranjan and Rodnina, 2016). For example, in *Saccharomyces cerevisiae* different stresses (exposure to H_2O_2, MMS, $NaAsO_2$ or, NaOCl) cause non-random changes in tRNA modification levels, and the amount of specific tRNA modifications change dynamically in response to a growing stress (Chan et al., 2010). Although

there is less evidence for this in prokaryotes, there are proofs that the level of specific tRNA modifications in bacteria can be affected by salt stress (Fleming et al., 2022), growth phase (Emilsson et al., 1992), mild antibiotic stress and starvation (Galvanin et al., 2020), oxidative stress (Thongdee et al., 2019), and temperature (Shigi et al., 2006).

tRNA modifications may also play a role in fine-tuning the optimal translation of particular genes under specific conditions by exploiting codon bias. The usage of synonymous codons varies between different organisms as well as between different genes within one organism (reviewed in (Quax et al., 2015)). tRNAs with modifications might favor some synonymous codons over others, differently modified tRNAs do not necessarily exist in a cell in equal amounts, and the decay of modified and unmodified tRNAs can be different. Through all this, modifications can alter tRNA codon usage bias. Furthermore, it is important to note that the optimal translation of transcripts achieved by fully modified tRNAs may not be the optimal choice in particular stressful conditions and environments (Agris et al., 2017).

Studies in yeast have revealed how a specific tRNA modification reshapes the gene expression in response to stress conditions. In *S. cerevisiae* Trm4 methylates C34 nucleotide in $tRNA^{Leu(\underline{C}AA)}$ and cells lacking Trm4 are hypersensitive to oxidative stress (Chan et al., 2010, 2012). In these studies, the oxidative stress increased the amount of m^5C34 modified $tRNA^{Leu(\underline{C}AA)}$, which in turn led to increased translation efficiency of transcripts enriched with UUG codon. Among the other changes this translational reprogramming leads to increased protein expression of ribosomal protein Rpl22A, which, unlike its paralogue Rpl22B, is encoded by a TTG-rich gene. The lack of ribosomal protein paralogue Rpl22A caused sensitivity to oxidative stress and thus it is hypothesized to be an example of how cells reprogram their translation through tRNA modifications to survive stress (Chan et al., 2012).

Similar reprogramming has been seen with other yeast tRNA modifications at the position 34. Trm9 modifies U nucleotide at the position 34 in $tRNA^{Arg(\underline{U}CU)}$ and $tRNA^{Glu(\underline{U}UC)}$, and this modification solidifies the codon-anticodon pairing. From gene- and codon-clustering data it is evident that there is a clustered group of 425 genes with AGA and GAA codons overrepresented (compared to relative genome averages). This group is overrepresented in the functional categories of protein synthesis, energy and metabolism, and stress and damage responses (Begley et al., 2007). In further analyses, it was proven that the expression of proteins, which genes are enriched in AGA and GAA codons, was downregulated in cells lacking Trm9 under normal and stress conditions. Although the lack of Trm9 caused a significant shift in the expression of AGA- and GAA-enriched genes, the regulation was not "on-off" type. Among the proteins affected by Trm9, a remarkable proportion of proteins in translation machinery were downregulated in *trm9Δ* cells. Interestingly, in yeast the AGA and GAA codons cluster together more than they would be expected in the case of random distribution and such clustering can affect local translation rate (Deng et al., 2015). This strongly

supports the idea that modifications at Wobble position can dynamically alter the codon-based translation and reprogram translation machinery.

The effect of tRNA modification at 34th position on translational reprogramming has also been seen in prokaryotes. Under stressful conditions while infecting the host, the pathogenic *Mycobacteriun bovis* BCG cells enter a quiescent hypoxic persistence state and during different stages of this state and re-aeration the pattern of 40 different tRNA modifications changes, which can lead to selective translation (Chionh et al., 2016). More specifically, it was shown that the reading of Thr codons is considerably altered under hypoxia, where there was a remarkable shift in Wobble position modification of tRNA^{Thr}. The resulting change in codon preferences contributed to increased translation from less abundant ACG codon and reduced the translation from most abundant ACC codon. By reprogramming tRNAs to favor some codons over others, *M. bovis* could favor critical genes like the master regulator of hypoxic bacteriostasis *dosR* which gene displays significant ACG/ACC bias. Again, it was noteworthy that there was no uniform up- or downregulation of codon-biased transcripts as the expression of many proteins with ACG-enriched genes remained the same (Chionh et al., 2016).

In *P. aeruginosa* several tRNA modifications are linked with oxidative stress. In *P. aeruginosa* PA14 the lack of TrmJ, which catalyzes the 2'-O methylation at position 32, causes sensitivity to H_2O_2. The overall catalase activity was remarkably lover in *trmJ* mutant and under H_2O_2 stress the transcription of *katB* and *katE* (but not *katA*) was lower in the *trmJ* mutant (Jaroensuk et al., 2016). In *P. aeruginosa* PAO1 the lack of TtcA, which thiolates C32, also caused a ROS sensitive phenotype. In the *ttcA* deletion mutant the total catalase activity in exponentially growing and in stationary phase cells was decreased, and the activity of KatA was also decreased. In addition, the mutants lacking *ttcA* were less capable of infecting *Drosophila melanogaster* than wild-type bacteria (Romsang et al., 2018). In *P. aeruginosa* PA14, the lack of *trmB* also causes a H_2O_2 hypersensitive phenotype. Unlike the previously described modifications, TrmB modifies G nucleotide at position 46, which locates in an extra loop and not in the ASL (Fig. 1). Although in this *P. aeruginosa* PA14 stain there are 23 tRNAs which are TrmB substrates, only the translation efficiency of repeated Phe and Asp codons was significantly decreased in *trmB*-deficient mutant (Thongdee et al., 2019). Interestingly, the TrmB made modifications is one of the few examples of tRNA modifications not located in the ASL but still affecting the translation accuracy. The overall catalase activity and KatA activity was decreased in the *trmB*-deficient mutant, but the transcription level of KatA and KatB remained *trmB*-independent, suggesting that TrmB mediates the stress response at translation level. In addition, analyses of *katA* and *katB* sequence revealed the genes to be enriched with clustered Phe and Asp codons, supporting the idea of TrmB-mediated translation regulation (Thongdee et al., 2019).

The tRNA modification enzyme MiaA catalyzes the first modification i^{6}A (addition of isopentenyl group to N6) of two-step modification at the position 37 and the additional roles of MiaA have been actively studied for decades. Usually,

the MiaA made modification is further modified by enzyme MiaB. In *E. coli* K12, the lack of MiaA increased the rate of spontaneous mutations (Connolly and Winkler, 1989), whereas the increase in mutation frequency was due to GC–AT transversions (Connolly and Winkler, 1991) and recombination dependent (Zhao et al., 2001). On the other hand, the iron limitation increased mutation frequency in MiaA-proficient strain but not in *miaA* mutants (Connolly and Winkler, 1989). Also, MiaA could play a part in regulating gene expression, as MiaA was needed for the full expression of the highly regulated stress response sigma factor RpoS in *E. coli* (Thompson and Gottesman, 2014) and for the expression of *vir* genes in *Agrobacterium tumefaciens* (Gray et al., 1992). In the case of RpoS, its coding sequence is significantly enriched with UUN-Leu codons, which are read by MiaA-modified tRNAs (Thompson and Gottesman, 2014). The MiaA requirement for the *rpoS* translation was shown to be due to promoting the decoding of UUN-Leu codons (Aubee et al., 2016). In extraintestinal pathogenic *E. coli* (ExPEC), the lack of MiaA reduced fitness and virulence of bacteria (Fleming et al., 2022). Importantly, it has been shown already earlier that the lack of *miaA* causes an increase in +1 frameshifts in *E. coli* and in *Salmonella* strains (Urbonavičius et al., 2003, 2001). Now, in addition to increasing frameshifting in ExPEC strain, it was demonstrated that both the lack of MiaA and overexpression of MiaA changed the proteome, and the changes in proteome were directed at least partially by UNN codon prevalence (Fleming et al., 2022). This indicates that tRNAs modified by MiaA can take part in regulating gene expression.

Similarly to MiaA, in *E. coli* the enzymes TrmL and TusA are also necessary for the full expression of RpoS (Aubee et al., 2017). TrmL (methylates 2'-O) and TusA (2-thiolation) both modify the position 34 in the leucine tRNAs which are also substrates for MiaA and at least the effect of TrmL catalyzed modification on RpoS is UUN-Leu codon dependent (Aubee et al., 2017).

The regulation of stress response is not always operated through the upregulation of specific modifications but also via the stability and degradation of tRNAs, meaning that the modifications pattern can be changed because of the altered tRNA pool. Interestingly, it has been proposed that altering tRNA pool itself can add another regulatory level to protein synthesis in response to different stress conditions (Torrent et al., 2018). It is noteworthy that many modifications which appear to have no apparent role, could be crucial at extreme conditions, e.g., m^6A37 in valine tRNA affects cell survival under temperature, osmotic and oxidative stress conditions in *E. coli* (Golovina et al., 2009). Furthermore, the tRNA modifications play important role for thermophilic bacteria living at extreme temperatures (reviewed in (Lorenz et al., 2017)). Thus, both the inducible and so-called housekeeping modifications, which levels are constant throughout the lifecycle of a cell, can be important in overcoming stressful conditions.

It was proposed already decades ago that tRNA modifications can take part in regulatory processes in a cell (Persson, 1993) but with the aid of new quantitative methods, it has been possible to investigate the roles of tRNA modifications from new angles. The combination of genome-wide analyzes of codon usage, quantitative measurements of tRNA modification level, ribosome profiling, and

proteomics analyzes has greatly improved the understanding of different roles of tRNA modifications (Pollo-Oliveira and de Crécy-Lagard, 2019). This new knowledge has led to the use of a term "modification tunable transcripts" (MoTTs) (Dedon and Begley, 2014; Endres et al., 2015). Most of the examples discussed in this chapter can be classified as MoTTs. Although there are more and more examples of MoTTs in connection of stress response, there are many gaps in understanding the whole regulatory pathway of MoTTs. The tRNA modification-mediated regulation adds another level on the top of other known regulatory mechanisms. Furthermore, it is usually not "on-off" type regulation and this makes understanding the precise regulatory role of tRNA modifications extra challenging.

1.2.1.3. TruA and pseudouridines at the positions 38, 39, and 40 in tRNA

Pseudouridine (Ψ) is one of the most common nucleotide modification and also the first one described in RNA already in the 1950s (Cohn, 1959; Davis and Allen, 1957; Yu and Allen, 1959). It is a uridine isomer where the nucleobase and sugar are connected with a C–C glycosyl bond instead of a N–C glycosyl bond as in other nucleotides (Fig. 3). This uncommon bond affords greater rotational freedom to Ψ. Also, due to the free N1-H, Ψ has the potential to form an additional hydrogen bond (Fig. 3, reviewed in (Gray and Charette, 2000)). In *E.coli* tRNAs, in addition to universally conserved Ψ55 the Ψs are also found at the positions 13, 32, 35, 38–40, and 65 (Fig. 1) (de Crécy-Lagard and Jaroch, 2021). In prokaryotic cells, the formation of Ψ is catalyzed by stand-alone enzymes Ψ synthases, which based on structural similarities belong to five distinctively different families (Hamma and Ferré-D'Amaré, 2006).

E. coli TruA is one of the first Ψ synthase enzymes described and it belongs to the TruA enzyme family (Hamma and Ferré-D'Amaré, 2006). It carries out the isomerization reaction in tRNA at the positions 38, 39, and 40 (Fig. 1 and 2) (Kammen et al., 1988). A little less than half of the tRNAs in *E. coli* are substrates for TruA (Boccaletto et al., 2022). Most of the enzymes modifying RNA are highly specific to a substrate and its position, and not many enzymes are multi-site-acting. TruA is one of the most common example of a multisite acting enzyme, as the same enzyme is able to modify U nucleotides in different sequence contexts and the distance between target nucleotides can be up to 15 Å (Hur and Stroud, 2007).

Another uncommon feature of TruA is that the enzyme acts as a homodimer and recognizes not just the ASL but the whole specific shape of tRNA (Foster et al., 2000), while most of bacterial Ψ synthases act as monomers (Hamma and Ferré-D'Amaré, 2006). However, the pseudouridylation reaction is carried out by universally conserved aspartic acid residue, Asp60 in *E. coli* TruA (Foster et al., 2000; Huang et al., 1998), and the overall reaction is similar in all Ψ synthases (reviewed in (Hamma and Ferré-D'Amaré, 2006)).

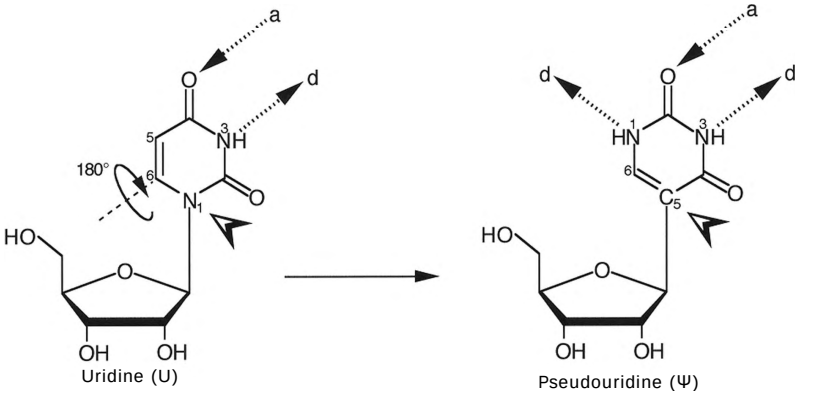

Figure 3. The differences between uridine (U) and pseudouridines (Ψ). The big arrow shows the molecule connected with ribose and the black arrows show hydrogen bond acceptors (a) and donors (d) (Gray and Charette, 2000).

Compared to U, Ψ harbors improved stacking ability (Davis, 1995), which can contribute to structural stability. Ψ located in a single-stranded region next to a RNA duplex has a double-strand stabilizing ability, as in case of Ψ38 (Davis et al., 1998). Analyzes of yeast $tRNA^{Phe}$ ASL revealed that Ψ at the position 39 increases the thermal stability of the ASL rather by increasing stacking ability and not by an extra hydrogen bond. Also, the Ψ39 does not significantly affect ribosome binding, but if the 31–39 base pair is disrupted, the thermal stability and the affinity for the ribosome are significantly lowered (Yarian et al., 1999). Analyzes of *E. coli* $tRNA^{Lys}$ (without modifications at 34 and 37 position) revealed that the Ψ39 increases base-stacking within the loop and stabilizes the 31–39 base pair. Also, at lower pH the Ψ39 stabilizes both the 31–39 base pair and non-canonical C32–A38 base-pair (Fig. 2) (Durant and Davis, 1999). The incorporation of Ψ39 to otherwise unmodified human ASL^{Lys} significantly increased the thermostability (more than other modifications in Lys ASL), but did not restore ribosome biding while modifications at the positions 34 and 37 contributed to ribosomal binding (Yarian et al., 2000).

In *S.* Typhimurium, the lack of Ψ38–Ψ39 reduced the efficiency of amino acid tRNA selection of $tRNA^{Leu}$s, but with the example of $tRNA^{Pro(GGG)}$, it appeared that the Ψ40 had no effect on the selection (Li et al., 1997). Although the P-site slippage has been measured with different TruA substrate tRNAs ($tRNA^{His(AUG)}$, $tRNA^{Pro(GGG)}$ and 4 Leu tRNAs), only in case of $tRNA^{Leu(UAG)}$ the lack of Ψ38 increased translational +1 frameshifting in *S.* Typhimurium (Urbonavičius et al., 2001) and the Ψ39 did not affect –1 frameshift in *E. coli* and *S.* Typhimurium (Urbonavičius et al., 2003).

In *S. cerevisiae*, the enzyme Pus3p pseudouridylates the positions 38 and 39 but not 40 in both cytoplasmic and mitochondrial tRNAs. The lack of these modifications caused remarkable slow growth phenotype especially at higher temperatures (Lecointe et al., 1998). Although Pus3p modifies 19 different tRNA molecules, it has been proven that the slow growth phenotype is due to the lack of Ψ38

in $tRNA^{Gln(UUC)}$. It was hypothesized that the effect of Ψ38 might be caused by reduced coding ability of $tRNA^{Gln(UUC)}$ (Han et al., 2015). Unlike in prokaryotes, in yeast the lack of Ψ38 and Ψ39 reduced +1 frameshift and stop codon read-through (Lecointe et al., 2002). In addition, analyzes of natural viral –1 frameshift sequences showed that in the case of sequences with higher frameshifting frequency there was a tRNA with Ψ39 at the E-site. Measuring few of high frequency constructs in yeast showed that the lack of Pus3p reduced –1 frameshifting, indicating that higher frameshifting efficiency could be correlated with the presence of the tRNA with Ψ39 at the E-site at the time of slipping (Bekaert and Rousset, 2005).

Among yeast cytoplasmic tRNAs, with some exceptions, there appeared to be a biased distribution of Ψ39 – the tRNAs with an otherwise less stable anticodon stem had a Ψ39 more frequently (Han et al., 2015). This finding is in good correlation with the idea that the role of Ψ39 is to stabilize the ASL structure.

In addition to the growth effect in yeast (Han et al., 2015; Lecointe et al., 1998), the absence of Ψ38–40 also affects growth, fitness and virulence in prokaryotes (Ahn et al., 2004; Tsui et al., 1991; Yang et al., 2019). In *E. coli*, the absence of TruA (HisT at that time) prolonged the lag phase and remarkably reduced the growth rate and exhibited abnormalities in cell division which lead to filament formation in minimal medium (Tsui et al., 1991). In *S. enterica* Typhimurium infection, the lack of *truA* caused a remarkably higher survival rate of mice, and the expression of *truA* was higher when the samples were treated with H_2O_2 (Yang et al., 2019). In *P. aeruginosa* clinical isolate PAK, the lack of TruA did not affect growth of bacteria but TruA modulated the regulation of expression of type III secretion genes, and through that the virulence of the pathogen (Ahn et al., 2004).

Overall, it appears that although the Ψs in the ASL participate in stabilization of tRNA structure, physiologically the Ψ38–40 can have different roles and effects, depending on the tRNA carrying the modification. Also, the role can most likely be affected by other nucleotides and nucleotide modifications nearby. This is also illustrated by the fact that in the consensus where about 400 elongator tRNAs were compared from all domains of life, at the position 40, there is very rarely a U nucleotide (less than 10% of cases) and at the position 39 almost half of the sequences carry U nucleotide (Grosjean and Westhof, 2016). Also, in the analyzed genomes of Gram positive bacteria, there was no evidence of Ψ at the positions 38 nor 40 (Machnicka et al., 2014), suggesting that the presence of Ψ in ASL is not uniform. However, the conserved nature of Ψ38 and Ψ39 in all three kingdoms of life indicates that these modifications may have a conserved function in tRNAs. As described above, the Ψ improves both the base stacking and rotational freedom of nucleobase (due to C–C glycosyl bond), and considering the different phenotypic effects of TruA, it is possible that in different organisms, distinctive features of Ψ have greater overall impact.

1.2.1.4. RluA and pseudouridine at the position 32 in tRNA

RluA is a RluA family Ψ synthase with dual-specificity, it is able to make Ψ at the position 746 in 23S rRNA and at the position 32 in tRNA (Fig. 1 and 2) (Wrzesinski et al., 1995). In *E. coli*, RluA is modifying 4 tRNAs: $tRNA^{Phe}$, $tRNA^{Cys}$, $tRNA^{Leu(UAA)}$, $tRNA^{Leu(CAA)}$ (Hoang et al., 2006; Raychaudhuri et al., 1999; Wrzesinski et al., 1995). The overall structure of RluA is similar to the other Ψ synthases (Hoang et al., 2006), and the pseudouridylation reaction is carried out by universally conserved Asp, Asp64 in *E. coli* (Ramamurthy et al., 1999; Raychaudhuri et al., 1999).

In addition to RluA in *E. coli*, so far, there is only one other Ψ synthase and one RNA methylase described with the ability to modify both rRNA and tRNA. The enzyme RluF catalyzes the formation of Ψ in 23S rRNA at the position 2604 and in $tRNA^{Tyr(Q\underline{\Psi}A)}$ anticodon at the position 35 (Addepalli and Limbach, 2016). RlmN methylates A nucleotide at the position 2503 in 23S rRNA and at the position 37 of six tRNAs. In 23S rRNA, the local structure of the helices 89 and 90–92 resembles the L-shape structure of tRNA and there are also some sequence similarities in all of the substrates of RlmN (Benítez-Páez et al., 2012). Similarly, all 5 RluA substrates share a common sequence surrounding the modification site – 5' ΨUXXAAA 3' (Hoang et al., 2006; Raychaudhuri et al., 1999). In addition, stem-loop RNAs were shown to be good substrates for RluA, and the truncated ASL was almost as good substrate as the full size tRNA (Hamilton et al., 2006), illustrating that in addition to sequence similarities, the stem-loop region of ASL and 23S rRNA helix 35 structure can be important for RluA recognition.

In general, the nucleotide 32 is important for the discriminatory ability of the tRNA in translation. For example, if the C32 in $tRNA^{Gly(UCC)}$ was mutated to U in *Mycoplasma mycoides*, the tRNA's ability to decode non-cognate Gly codons significantly diminished, while in wild type the $tRNA^{Gly(UCC)}$ was able to read all Gly codons (Claesson et al., 1995). In *E. coli*, the mutation of U32 to C increased the ability to read near-cognate codon GGA and this also increased frameshifting at near-cognate codon GGA (O'Connor, 1998). Less is known about the role of Ψ32. Relative to unmodified molecule, the Ψ32 does not affect the overall structure of the ASL but it increases the stability at higher temperature (Cabello-Villegas and Nikonowicz, 2005).

Although usually the nucleotides at the positions 32 and 38 cannot form a Watson-Crick base pair, they do, in most of the cases, form a non-canonical base pair (with bifurcated hydrogen bond, Fig. 2) (Auffinger and Westhof, 1999). It was proposed that the 32–38 base pair helps to regulate the aa-tRNA binding to the ribosome A site (Olejniczak and Uhlenbeck, 2006) and in the case of synthetic tRNA, with an additional nucleotide between positions 37 and 38, the formation of 32–38 base pair is hindered, which in turn leads to +1 frameshift events (Maehigashi et al., 2014). Based on the consensus sequence, all RluA tRNA substrates carry A nucleotide at the position 38 which is able to form a base pair with Ψ32 (Auffinger and Westhof, 1999; Cabello-Villegas and Nikonowicz, 2005). There is a possibility that via this additional base pair, the Ψ32 can play a role in

translation accuracy. Also, formation of a Watson-Crick base pair between 32 and 38 would require a conformational change in the loop structure leading to a less optimal anticodon conformation for pairing with codon, being thus less favorable (Grosjean and Westhof, 2016).

In contrast to TruA the lack of RluA does not have many described phenotypes. In *E. coli*, the lack of RluA did not affect growth rate but the cells lacking RluA were outcompeted by wild-type cells (Raychaudhuri et al., 1999). In yeast, Ψ32 is unusually synthesized by two different enzymes: Rib2/Pus8p in the cytoplasm and Pus9p in the mitochondria. The lack of pseudouridylation activity of these enzymes does not affect the growth rate of *S. cerevisiae* (Behm-Ansmant et al., 2004).

In conclusion, although TruA and RluA both make Ψs in the ASL, the role of modifications Ψ38–40 seems to be more prevalent than the role of Ψ32. While the lack of *truA* can affect translation fidelity (Urbonavičius et al., 2001), growth rate (Tsui et al., 1991), virulence (Ahn et al., 2004; Yang et al., 2019) and tRNA selection in translation (Li et al., 1997), the only described effect of the lack of *rluA* is the outcompeting by the wild type (Raychaudhuri et al., 1999). Nevertheless, both enzymes modify the nucleotides in the ASL, and as described above, the modifications in ASL can affect all the steps of translation and could also became especially important under particular stress conditions.

2. THE AIM OF THE THESIS

Without genetic variability, there is no material for evolution. Mutations are the main source of genetic diversity in bacteria, and therefore for understanding evolution, it is vital to understand the mutational processes. Obviously, the faithful DNA replication and the functionality of repair pathways are the key elements for genetic integrity. But one cannot overlook all the other processes involving DNA – transcription, recombination, nucleoid structure formation – that also affect its intactness. Furthermore, bacteria constantly face stress, induced by both endogenic and exogenic factors, which in turn evokes numerous changes in gene expression and some of these changes contribute to adaptation by increasing mutations.

Our present-day knowledge about mutational processes relies mainly on research carried out with *E. coli*, which may not always be a best representative of bacteria. For instance, the methylation-based mismatch repair pathway (On and Welch, 2021) and the SOS regulated TLS polymerase Pol V (Ambur et al., 2009), are not so widespread as thought previously. To shed light on mutational processes in non-enteric bacteria, the aim of this study was to find and characterize new factors affecting the mutation frequency in the genus *Pseudomonas*. The genus *Pseudomonas* is outstanding for comprising bacteria with broad metabolic abilities and diverse habitats. For instance human, plant, and insect pathogens, but also bacteria which can promote plant growth or be used for bioremediation (Silby et al., 2011).

First, as the previously obtained results with *P. putida* have indicated a correlation between recombination rate and the presence of nucleoid-associated protein (NAPs) biding sites (Tavita et al., 2012), one of the aims of my thesis was to elucidate the role of one of these NAPs, Integration Host Factor (IHF), in mutagenesis.

To reveal more new factors affecting the mutation frequency, we first needed to establish mutation frequency-monitoring assay for *Pseudomonas* species. Hence, the following aim of my study was to create, describe and verify a test system and to evaluate its application in different *Pseudomonas* species. With the help of newly created test system, we found potential mutation rate-affecting genes in *P. putida* PaW85. Among numerous genes functioning in DNA replication and repair, we also found genes previously not known to be associated with mutations. The most intriguing of those was the gene of pseudouridine synthase *truA*. Thus, the subsequent part of my dissertation concentrated on the tRNA modification enzymes, pseudouridines synthase enzymes TruA and RluA by characterizing their role in mutagenesis and in cells physiology of *P. putida* PaW85.

3. RESULTS AND DISCUSSION

3.1. The nucleoid-associated protein IHF influences mutation frequency in *P. putida* (Ref. I)

Different nucleoid-associated proteins (NAPs) bind to DNA and affect the nucleotide structure dynamically by binding, bending, and wrapping DNA. By changing DNA accessibility, the NAPs contribute to the gene expression regulation and in some extent it is hard to distinguish NAPs from conventional transcriptional regulators (Dillon and Dorman, 2010). Also, by affecting the DNA accessibility to different repair and recombination enzymes, NAPs can interfere efficient repair, but on the other hand, binding DNA can shield it, and in some cases, rather protects it from mutagenesis. Some of the most common NAPs in Gram negative bacteria are Fis, Hu, H-NS, Dps, and IHF (Dillon and Dorman, 2010). The studies of *E. coli* mutation frequency across the chromosome have revealed that the mutation density is higher at the areas with HU- and Fis-responsive genes expression, but the H-NS and Dps does not affect mutation frequency (Foster et al., 2013; Niccum et al., 2019). Although the NAPs can affect the mutation frequency in many ways, it has been speculated that the DNA replication becomes inaccurate in DNA regions with high superhelical density (Niccum et al., 2019).

The previous studies published in our laboratory have predicted *in silico* a strong correlation between homologous recombination frequency and the presence of NAP biding sites (for at least Fis, IHF and MvaT/MvaU) in *P. putida* (Tavita et al., 2012). In subsequent studies, our goal was to study the role of NAPs in mutational processes in *Pseudomonas*. We investigated the role of IHF in mutagenic processes in *P. putida* strain PaW85 (Ref. I), which is isogenic to *P putida* KT2440 (Nelson et al., 2002). Our results revealed that IHF affects the occurrence of point mutations in *P. putida*. However, the effects of IHF varied depending on the test system (Ref. I, Fig. 4 and 5, Table S6 and S7). We used phenol (Phe) utilization-based test systems and the rifampicin (Rif) resistance acquisition-based test system. In the Phe-based test systems the cells carried a phenol monooxygenase gene *pheA* with premature stop codon (stop instead of 22. Leu codon) either in the plasmid (Ref. I, Fig. 4) or in the chromosome (Ref. I, Fig 5A) or a chromosomal *pheA* gene with deleted C nucleotide at position 221 (Ref. I, Fig 5B). The cells were plated onto the minimal plates supplemented with Phe as the sole carbon source and the emergency of phenol utilizing mutants (Phe^+ phenotype) was monitored. We observed that in the plasmidial test system, the lack of IHF reduced the base substitution in exponentially growing population but did not affect the mutant frequency in stationary phase cells. On the other hand, the overexpression of IHF increased the mutant frequency only in stationary phase population (Ref. I, Fig 4). The opposite effects of IHF expression on mutation frequency at different growth phases indicates that the cellular levels of IHF have significant impact on mutation frequency: both the overexpression and

downregulation can cause imbalances in mutational processes. The growth phase-dependent effect of IHF is not surprising since the expression of different NAPs is dynamic and highly dependent on growth phase of bacteria. In *E. coli* IHF reaches it maximum level at transition from exponential phase to stationary phase, but unlike many other NAPs, IHF is abundant throughout the growth curve (Ali Azam et al., 1999). In *P. putida*, the IHF content is almost an order of magnitude higher in stationary phase than in exponential phase (Valls et al., 2002). Also, the experiments with *P. putida* indicate that the IHF is necessary for the physiological changes in cell that take place before entering to the stationary phase. For example, IHF downregulates RNAP alpha subunit and many ribosomal proteins (Silva-Rocha et al., 2013).

In the chromosomal test systems, the lack of IHF also reduced the appearance of Phe^+ mutants, but did not affect the appearance of Rif^R mutants (Ref. I, Fig. 5). This clearly illustrates how distinct test system can reveal different effects. While the Phe^+ assay measures only certain mutations at the defined location in the *pheA* gene, the Rif^R assay is based on the detection of broader spectrum of mutations at different locations in the *rpoB* gene, which decrease the affinity of rifampicin binding to the enzyme (Jatsenko et al., 2010). In addition, not only the spectrum of mutations measured was different, but also the Rif assay is based on lethal selection and the Phe^+ assay is nonlethal. Also, as mentioned in the literature, mutation frequency can be very different depending on the chromosomal location of the target (Kivisaar, 2020b; Niccum et al., 2019). Since the IHF is DNA-binding protein, its effect on mutation frequency could be especially dependent on the genetic context and the presence of IHF binding sites in the flanking DNA regions.

Although we did not observe the effect of IHF on the spectrum of Phe^+ mutants, it is interesting to note that the mutation spectrum was significantly different if the same tester gene *pheA* was either in the chromosome or in the plasmid (Ref. I, Table 1). The different spectra of the chromosomal and plasmidial assays can be due to the fact that the chromosomal Phe^+ assay allows mainly the detection of exponential phase mutations but the plasmidial assay enables also the detection of mutations occurring in stationary phase. The mutational spectrum of Phe^+ mutants appeared to be more heterogeneous in stationary phase population (Ref. I, Table 1), indicating that the mutational processes are growth-phase dependent. These results are supported by our previous findings that the spectrum of TAG stop codon reversion mutations is more heterogeneous in the populations of stationary-phase bacteria than that in growing bacteria (Saumaa et al., 2006). Moreover, it has been shown that in *P. putida* carbon-starved cells the chromosomal DNA content decreases significantly compared to exponential phase cells, whereas no changes in the copy number of pKTpheA22TAG plasmid were detected (Ukkivi and Kivisaar, 2018). Thus, the decline of chromosomal DNA content could be one explanation why it was not possible to measure stationary phase mutation in the chromosomal Phe^+ test system. In addition, the replication of plasmid is independent of chromosomal replication and different replication strategies and replication timing can affect mutations as well (Agier and Fischer,

2012). For instance, in bacteria with multiple chromosomes (*Burkholderia* and *Vibrio*) the replication from the smaller chromosome occurs later and also those genes tend to evolve faster (Cooper et al., 2010).

In conclusion, we were able to determine a new mutation frequency affecting factor in *P. putida* – IHF. Its effect on mutational processes is both growth phase- and chromosomal location-dependent. Based on our present knowledge it is hard to determine whether IHF directly affects mutational processes or is the effect the consequence of different DNA accessibility due to IHF's action.

3.2. A new test system for identifying mutation rate-affecting genes in *Pseudomonas* species (Ref. II)

Different test systems have historically greatly improved the knowledge about mutational process, but many of them are designed for a specific model organism and are difficult to transfer to other bacteria. For instance, numerous *E. coli* test systems are based on lactose utilization (Foster and Rosche, 1999; Rosenberg, 2001), which are not applicable to organisms unable to metabolize lactose. Since our aim was to screen for new mutation rate-affecting genes in *Pseudomonas* species, we were in need for a new assay. The ability to form secondary micro-colonies or so called papillae on the surface of the main colony has been a useful tool for studying mutational processes (Al Mamun et al., 2012; Miller et al., 1999; Yang et al., 2011). We developed a new *in vivo* papillation-based mutational assay lac-lsc, which enables the detection of mutations in *lacI* gene encoding the transcriptional repressor of the *lac* operon, LacI (Ref. II). The pervious works in our laboratory have proven the chromosomal *lacI*-P_{tac} gene cassette to be a good target for determining a diverse set of mutations in *P. putida* (Juurik et al., 2012). In the lac-lsc assay the levansucrase gene *lsc-3* of *Pseudomonas syringae* pv. tomato DC3000 and the β-galactosidase gene *lacZ* of *E. coli* are placed under the control of P_{tac} promoter and its repressor *lacI* (Ref. II, Fig. 1A). This gene cassette was inserted into the chromosome of a strain of interest either as a part of mini-transposon Tn*5*, which inserts into chromosome randomly, or as a part of Tn*7*, which inserts into an intergenic region downstream the *glmS* gene. Levansucrase is an extracellular enzyme which carries out sucrose hydrolysis into glucose and fructose and at the same time transfructosylates fructosyl units to a fructan polymer levan. When sucrose is added to the medium, the activity of levansucrase can be easily visually detected by the production of mucous levan. β-galactosidase is an enzyme that hydrolysis the glycosidic bond of β-galactose, but it can also hydrolyze the bond in chemical X-gal which product 5-bromo-4-chloro-3-hydroxyindole is oxidized into easily detectable blue compound.

Under normal conditions, the P_{tac} promoter is repressed by its repressor LacI, but if a spontaneous mutation inactivates the *lacI* gene or alters its operator area, the transcription of *lsc-3* and *lacZ* is permitted. On indicator media containing sucrose and X-gal this expression is visually detectable as a transparent or a blueish mucous papilla (Ref. II, Fig. 1C–D).

We monitored the papillae formation in the *P. putida* wildtype cells, in the DNA mismatch repair (MMR)-deficient ΔmutS background, and in the presence of DNA damaging agent MMC (Ref. II, Fig. 1C–D, Table 1). The papillae formation was somewhat dependent upon the colony size as the colonies growing more densely formed less papillae than the ones growing more sparsely (Ref. II, Table 1). Possibly, if a colony is able to grow larger, it can undergo more cell-divisions and therefore, have more chance to obtain a beneficial mutation. Also, it should be mentioned that the papillae on a larger colony are easier to visually detect. In the developed assay, when the occurrence of mutations was exogenously induced with a mutagen MMC or endogenously elevated due to the lack of MMR, the formation of papillae significantly increased (Ref. II, Fig. 1B–D, Table 1). These colonies of bacteria either exposed to the mutagen MMC or lacked the functionality of major DNA repair pathway MMR often carried numerous papillae, which almost never happened in the wild-type background (Ref. II, Fig. 1B–D). These results demonstrated that the assay developed could be a promising tool for screening mutation frequency-affecting genes in *P. putida.*

We wanted to know whether this papillation-based assay is also applicable in other non-levan-producing bacteria. For this, we selected a set of *Pseudomonas* non-levan-producing strains from the Collection of Environmental and Laboratory Microbial Strains (CELMS; available in the Estonian Electronic Microbial database (EEMB) website http://eemb.ut.ee/eng/) and transferred the gene cassette *lacI*-P_{tac}-*lsc3lacZ* as a part of mini-Tn*7* into the chromosome of these environmental strains. We constructed the tester strains of the following species: *P. corrugata* 7228, *P. stutzeri* 2C63, *P. mendocina* PC1, *P. aeruginosa* D10, *P. migulae* D67, *P. guineae* 2C3, *P. anguilliseptica* 2Bnah2, and *P. thivervalensis* N7. Although for some of the strains the insertion of the gene cassette into the chromosome changed the colony morphology on sucrose-containing medium, the mucous papillae were observable in all environmental strains investigated (Ref. II, Fig. 5 and Fig. S1). Interestingly, when the gene cassette was inserted into the chromosome of the laboratory strain *P. aeruginosa* PAO1, we were not able to detect the formation of papillae in the obtained tester strain PAO1lsc-lac7 (Ref. II, Fig. 6A). Nevertheless, when the mutation rate in the tester strain PAO1lsc-lac7 was elevated by the deletion of *uvrD*, the papillae formation was detected (Ref. II, Fig. 6B). Taking together, although the test system needs optimization in some Pseudomonas strains, it is adaptable to a wide variety of non-levan-producing *Pseudmonas* species.

Overall, in *P. putida*, the first papillae were detectable on the third day of incubation. Because of the leaky transcription of the reporter genes, after seventh day the papillae formation was hard to notice. The appearance of papillae was lower in the tester strain PaWlac-lsc5 than in PaWlac-lsc7 (Ref. II, Table 1) and therefore we chose the tester strain PaWlac-lsc5 to conduct the transposon mutagenesis experiment for the detection of mutation frequency-affecting genes.

3.3. Search for mutation-affecting genes in *P. putida* (Ref. II)

To detect mutation frequency-affecting genes, we conducted transposon mutagenesis experiment with transposon mini-Tn*5* and *P. putida* tester strain PaWlac-lsc5. Approximately 27 000 transposon mutants from seven independent transposon mutagenesis experiments were obtained, and the appearance of papillae was monitored for six days. The approximate number of colonies of transposon mutants per plate was about 400–900. By the sixth day 918 colonies formed at least one papilla and all those colonies were further analyzed in secondary screening on rifampicin and sucrose plates. From these transposon mutants 351 passed the secondary screening and were subjected to the identification of the chromosomal position of mini-Tn*5*. The chromosomal location of mini-Tn*5* was determined for 327 transposon mutants. We identified 34 different genes and one intergenic region that were repeatedly targeted and were further considered as potential mutation frequency-affecting genes or regulatory regions (Ref. II, Table 2).

Most frequently the transposon had disrupted genes belonging to the functional class of DNA replication, recombination, and repair (Ref. II, Table 3). The fact that we were able to monitor the frequent transposon insertion into the genes already known to affect mutation frequency (*mutS*, *mutL*, *uvrABC*, *uvrD*) proved this assay to be applicable for the detection of mutation frequency-affecting genes in *P. putida* under the established experimental conditions. The experimental setup mimicked the natural conditions where readily available nutrients are exhausted during colony aging and through genetic adaptations some mutants can achieve growth advantages over other cells in population. We expected that under these circumstances we could identify genes affecting stress-induced mutagenesis and mutagenesis in aging colonies. For example, we detected transposon insertions into *rpoS* gene (Ref. II, Table 2). RpoS is a stationary phase sigma factor of the RNA polymerase, and in addition to regulating the general stress response, it regulates numerous other processes in a cell (e.g., biofilm formation and virulence) (Schellhorn, 2014). Our research group has previously shown that the RpoS-deficiency in *P. putida* elevates the occurrence of base substitutions during starvation due to enhanced sensitivity to oxidative damage (Tarassova et al., 2009). This finding confirmed that the lac-lsc assay could enable to find factors affecting mutation frequency in starving population of bacteria.

We further analyzed the transposon mutants previously not known to affect mutation frequency with the Rif^R assay and the Phe^+ assay. The analysis revealed that although numerous mutants exhibited mutant frequency comparable to that of the wild-type strain, there were many mutants with significantly higher mutant frequency in either exponential or stationary growth phase (Ref. II, Fig. 2 and Fig 3). The stationary phase mutagenesis was promptly increased in transposon mutant carrying transposon insertions in *gacS*. GacS is a sensor kinase of a two-component regulatory system GacS-GacA, which positively controls production of secondary metabolites, extracellular enzymes, and some carbon storage compounds *via* the expression of noncoding small RNAs (Lapouge et al., 2008). In further work in our laboratory, it has been verified that the deficiency of both

gacS and *gacA* causes a remarkable increase in base substitution mutations in stationary phase but not in exponential phase (Uusaar, 2022).

The highest Rif^R mutant frequency in exponential phase cells was observed in transposon mutants carrying insertions in the *truA* gene (Ref. II, Fig. 2). We further focused on understanding the mechanisms behind the elevated mutation frequency in the absence of TruA and describing the phenotypes of *truA*-deficient mutant.

3.4. Characterization of TruA and RluA (Ref. III and IV)

3.4.1. The substrates of TruA and RluA

TruA is a tRNA pseudourdine synthase reviewed in chapter 1.2.1.3. The involvement of tRNA modification enzyme in mutagenesis is surprising. According to our knowledge, only the tRNA modification enzyme MiaA have been shown to affect mutation frequency in *E. coli* (Connolly and Winkler, 1991, 1989; Zhao et al., 2001). However, to our knowledge, there is no previous record of pseudouridines affecting mutation frequency. To investigate the role of pseudouridines in the ASL, we also included RluA to our further experiments. RluA is another pseudouridines synthase, which also modifies U nucleotide in the ASL of tRNA (reviewed in chapter 1.2.1.4). Since the substrate specificity of TruA and RluA in *P. putida* has not been studied before, we analyzed the substrates of TruA and RluA in *P. putida* PaW85. We verified with CMCT/alkali treatment and primer extension analyzes that the TruA modifies U nucleotide at the positions 38–40 in all the studied tRNAs ($tRNA^{Ser(CGA)}$, $tRNA^{Cys(GCA)}$, $tRNA^{Leu(CAA)}$, $tRNA^{Leu(CAG)}$ and $tRNA^{Tyr(GUA)}$) (Ref. III, Fig. 1B, Fig. S2). These tRNAs are previously known to be modified by TruA in *E. coli* (except $tRNA^{Ser(CGA)}$) (Boccaletto et al., 2022). The pseudouridylation reaction is carried out by conserved aspartic acid residue; in *E. coli* the Asp residue at the position 60 in TruA (Huang et al., 1998). We verified that the catalytically vital Asp residue in *P. putida* is located at the position 70 of TruA and when the Asp70 was mutated, we detected no pseudouridines at the positions 38–40 (Ref. III, Fig. 1B, Fig. S2A). Analyzing tRNA sequences of *P. putida*, we concluded that in total there are 19 different potential TruA substrate tRNA molecules in *P. putida* (Table 1; Ref. III, Table S2). Out of the measured tRNAs, RluA modifies U32 in three tRNAs ($tRNA^{Ser(CGA)}$, $tRNA^{Cys(GCA)}$, $tRNA^{Leu(CAA)}$), and based on RluA's consensus sequence analyses it also modifies $tRNA^{Phe(GAA)}$ in *P. putida* (Table 1; Ref. III, Fig. 1B, Fig. S2 and Table S2). We further confirmed that the catalytic amino acid of *P. putida* RluA is Asp at the position 57 (Ref. III, Fig. 1B, Fig. S2A). We also analyzed the sequences of tRNA genes in *P. aeruginosa* PAO1 and predicted the substrates of TruA and RluA in this organism and compared potential substrates of these enzymes in *P. putida*, *P. aeruginosa* with those identified in *E. coli* (Table 1). We found that the potential substrate pattern of TruA and RluA is very similar in *P. putida*, *P. aeruginosa* and *E. coli*, and that there are only minor differences between these three species (Table 1).

Table 1. Comparison of TruA and RluA substrates in *P. putida* KT2440 (P.p.), *P. aeruginosa* PAO1 (P.a.), and *E. coli* K12-MG1655 (E.c.). The codons and corresponding amino acids (aa) are followed by the presence of the tRNAs with corresponding anticodon in the respective strain (marked with *). If the cell is highlighted green, the tRNA with corresponding anticodon is a potential substrate for TruA (carries U nucleotide at position 38–40) and if the cell is highlighted yellow, the tRNA with corresponding anticodon is a potential substrate for TruA and RluA (carries U nucleotide at position 38–40 and RluA consensus sequence). *P. putida* and *P. aeruginosa* results are based on the analysis of all tRNA gene sequences in the respective genomes (Winsor et al., 2016), and *E. coli* results are based on transfer RNA database (tRNAdb, used 20.06.2022) (Jühling et al., 2009).

1st	2nd: U					2nd: C					2nd: A					2nd: G					3rd
	codon	aa	P.p.	P.a.	E.c.	codon	aa	P.p.	P.a.	E.c.	codon	aa	P.p.	P.a.	E.c.	codon	aa	P.p.	P.a.	E.c.	
U	UUU	F				UCU	S				UAU	Y				UGU	C				U
	UUC	F	*	*	*	UCC	S	*	*	*	UAC	Y	*	*	*[b]	UGC	C	*	*	*	C
	UUA	L	*	*	*	UCA	S	*	*	*	UAA	*				UGA	*	*[se]	*[se]	*[se]	A
	UUG	L	*	*	*	UCG	S	*	*	*	UAG	*				UGG	W	*	*	*	G
C	CUU	L				CCU	P				CAU	H				CGU	R	*	*		U
	CUC	L	*	*	*	CCC	P	*	*	*	CAC	H	*	*	*	CGC	R				C
	CUA	L	*	*	*	CCA	P	*	*	*	CAA	Q	*	*	*	CGA	R			*	A
	CUG	L	*	*	*[b]	CCG	P	*	*	*	CAG	Q			*	CGG	R	*	*	*	G
A	AUU	I				ACU	T				AAU	N				AGU	S				U
	AUC	I	*	*	*	ACC	T	*	*	*	AAC	N	*	*	*	AGC	S	*	*	*	C
	AUA	I				ACA	T	*	*	*	AAA	K	*	*	*	AGA	R	*	*	*	A
	AUG	M[a]	*[b]	*[b]	*[b]	ACG	T	*	*	*[b]	AAG	K				AGG	R	*	*	*	G
G	GUU	V				GCU	A				GAU	D				GGU	G				U
	GUC	V	*	*	*[b]	GCC	A	*	*	*	GAC	D	*	*	*	GGC	G	*	*	*	C
	GUA	V	*	*	*	GCA	A	*	*	*	GAA	E	*	*	*	GGA	G	*	*	*	A
	GUG	V				GCG	A				GAG	E				GGG	G	*	*	*	G

[a] – TruA modifies elongator methionine tRNAs not initiator methionine tRNAs
[b] – TruA modifies at least two tRNAs with same anticodon but with differences in sequence
[se] – tRNA of selenocysteine

3.4.2. The lack of Ψ38–40 and Ψ32 increases mutation frequency in *P. putida*

To investigate the role of TruA and RluA in mutagenesis, we measured mutant frequency of Rif^R colonies by fluctuation assay in *P. putida* strains lacking *truA* and/or *rluA*. The lack of *truA* caused 5-fold increase in mutant frequency (Ref. III, Fig. 2A), which is in good accordance with the initial results obtained with *truA* transposon mutants (Ref. II, Fig 2). As the *P. putida* strain with catalytically inactive TruA (D70A) still had 5-fold elevated mutant frequency, it illustrates that the increased mutant frequency was caused by the lack of pseudouridylation

activity of TruA and not by some yet unknown secondary function of it (Ref. III, Fig 2A). The lack of *rluA* also increased the mutant frequency but the effect was lower than that in *truA*-deficient cells. Both the lack of *rluA* and the lack of its pseudouriylation activity caused approximately 3-fold increase in mutant frequency (Ref. III, Fig 2A). The double mutant strain carried a comparable mutator phenotype to that of ΔtruA strain (Ref. III, Fig 2A). To our knowledge, it is the first recorded case of pseudouridines in tRNA affecting mutant frequency. To investigate the prevalence of this effect, we measured the Rif^R mutant frequency in *P. aeruginosa truA*- and *rluA*-deficient mutants as well. The ΔtruA strain of *P. aeruginosa* had significantly increased mutant frequency, but the effect was markedly lower than in *P. putida* (Ref. III, Fig. 2B). The lack of *rluA* did not affect mutant frequency in *P. aeruginosa* (Ref. III, Fig. 2B). Previously, the effect of TruA to the frequency of spontaneous mutations has been measured in *E. coli* where the *truA*-deficiency did not cause a mutator phenotype (Connolly and Winkler, 1989). We have also attempted to measure the Rif^R mutant frequency in *E. coli* cells lacking *truA*, but since this strain grows very poorly, it was hard to obtain reliable results under comparable growth conditions of the mutant and the wild-type *E. coli* strain or under comparable growth conditions with *Pseudomonas* species. This indicates that although the TruA's effect on mutation frequency could be a phenomenon possibly widespread among *Pseudomonas* species, it is not common for all the bacterial species carrying the Ψ at positions 38–40 in tRNAs. Furthermore, it is interesting to note that although the enzymes TruA and RluA of these three bacterial species have such a similar substrate pattern, the mutation frequency phenotype is noticeably different.

As mentioned above, previously it has been described that the deficiency of tRNA modification enzyme MiaA causes a moderate mutator phenotype in *E. coli* (Connolly and Winkler, 1991, 1989; Zhao et al., 2001). MiaA modifies A nucleotide in the ASL of tRNA at the position 37 by adding isopentenyl group to the N6 nitrogen and creating i^6A37. The MiaA-made modification is often found in UNN codon-recognizing tRNA molecules and this modification is usually further methylthiolated into ms^2i^6A37 by MiaB. The lack of MiaB does not cause a mutator phenotype (Connolly and Winkler, 1991). It has been shown that the effect of *miaA*-deficiency is abolished in strains lacking *recA*, *recB* and *recD*; thus, the phenotype is recombination-dependent (Zhao et al., 2001). Also, in *E. coli* the mistranslating glycine tRNAs induces *recA*- and *recB*-dependent mutator phenotype (Murphy and Humayun, 1997; Ren et al., 1999; Slupska et al., 1996). However, not always is the mistranslation induced mutator phenotype dependent on recombination (Balashov and Humayun, 2003; Dorazi et al., 2002). In *P. putida*, it has been previously shown that the lack of *recA* alone does not affect spontaneous mutations in the Phe^+ phenotype-based assay (Tegova et al., 2004). We measured the Rif^R mutant frequency in *P. putida* ΔtruAΔrecA double mutant cells with Rif^R-based fluctuation assay. The mutants lacking both *truA* and *recA* genes still harbored a mutator phenotype, while cells carrying only *recA* deletion have mutant frequency comparable to wild type (Fig. 4). This indicates that the mutator phenotype caused by the *truA*-deficiency in *P. putida* is recombination-independent.

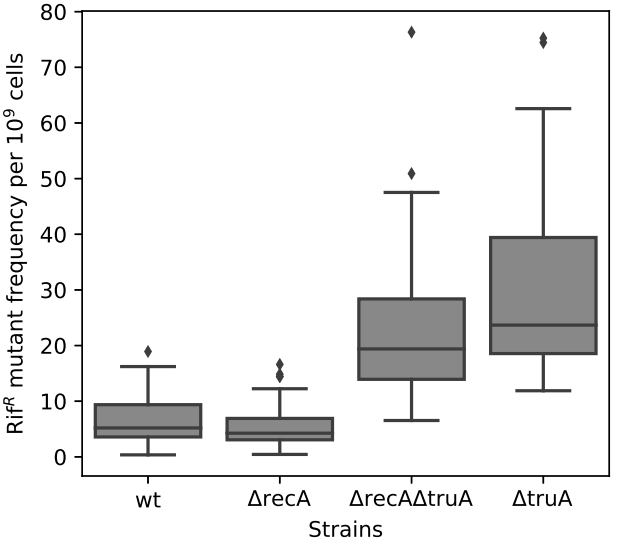

Figure 4. Comparison of Rif^R mutant frequencies of wild-type *P. putida*, *recA* deletion strain and *recA truA* double deletion strain. The mean values (line in the box) of Rif^R mutant frequencies per 10^9 cells are presented. The upper and lower borders of box represent third and first quartile, respectively, the whiskers represent non-outlier range and diamonds indicate outliers. The experiment has been performed as described in Ref. III chapter 2.7.

3.4.3. The role of SOS response and the DNA repair pathways in *P. putida* mutator phenotype

In addition to recombination, RecA also participates in the regulation of SOS response. In response to extensive DNA damage the SOS-response can transiently induce mutagenic processes. The SOS-response is also induced in different *E. coli* mutants with increased mistranslation (Bacher and Schimmel, 2007; Samhita et al., 2020). Although the SOS-response in *E. coli* is a well-studied regulatory network and a good example of stress induced mutagenesis, it is rather exceptional compared to other bacteria. For instance, in *P. putida* the number of genes regulated by SOS response is markedly smaller than in *E. coli*, the SOS response in *P. putida* is regulated by two LexA regulators, and the basal level of transcription of Pol IV gene (*dinB*) under normal conditions is higher than that in *E. coli* (Abella et al., 2007; Tegova et al., 2004). Nevertheless, the action of "error-prone" TLS polymerases can increase the spontaneous mutation rate in cells (Kuban et al., 2006; Tegova et al., 2004). We measured the Rif^R mutant frequency in *P. putida* strains lacking the inducible "error-prone" DNA polymerases in addition to *truA* or *rluA*. The constructed Δpol strain had *polB* (Pol II), *dinB* (Pol IV) and *imuABC* (where *imuC* also known as *dnaE2* codes for a DNA polymerase DnaE2) genes deleted. The mutator phenotype of *truA*- and *rluA*-deficiency was still present in the DNA polymerases Pol II, Pol IV and DnaE2-

deficient background (Ref. III, Fig. 3A) indicating that the TruA and RluA effect on mutation frequency in *P. putida* is SOS response- and TLS polymerases-independent.

If a mutation frequency increases, it always raises a question whether the DNA repair pathways are working correctly? In bacteria, there are many specialized DNA repair pathways for recognizing and mending damage in DNA. The main DNA repair pathways participating in maintaining genetic integrity are MMR, which removes errors in newly synthesized DNA, and NER, which repairs a wide repertoire of structurally unrelated lesions. Both these repair pathways need a DNA helicase UvrD to work properly. We analyzed whether the malfunction of these repair pathways could cause the elevated mutant frequency in ΔtruA and ΔrluA strains. For this we constructed mutant *P. putida* strains where in addition to *truA* or *rluA* the *uvrD* gene was also deleted. Measuring the Rif^R mutant frequency, it was apparent that compared to the *uvrD* single deletion, double mutants with deleted *uvrD* and *truA* or *rluA* still harbored higher mutant frequency in *P. putida* (Ref. III, Fig. 3A). In addition, the action of specialized polymerases (Sidorenko et al., 2011) or malfunction of repair pathways (Lee et al., 2012; Long et al., 2015) usually affects the spectrum of mutations. We have sequenced a set of Rif^R mutants picked up in wild-type, ΔtruA and ΔrluA strains and observed no remarkable difference in mutation spectrum (Ref. III, Table S6). This further proves that the observed mutator phenotype is not the outcome of malfunctioning repair pathways or action of TLS DNA polymerases.

3.4.4. The effect of Ψ38–40 and Ψ32 on translation in *P. putida*, *P. aeruginosa*, and *E. coli*

Different tRNA modifications in ASL have been shown to affect translation fidelity (Agris, 2004; Urbonavičius et al., 2001; Yarian et al., 2002). Also in many cases mistranslation leads to translational stress-induced mutagenesis (TSM) (Balashov and Humayun, 2003, 2002; Dorazi et al., 2002; Murphy and Humayun, 1997; Slupska et al., 1996), indicating that tRNA modifications affecting translation accuracy could affect mutagenesis via mistranslation. It has been hypothesized that *miaA*-deficient mutator phenotype is also caused by mistranslation (Humayun, 1998; Zhao et al., 2001). However, this hypothesis has some inconsistencies because in *S.* Typhimurium the lack of both *miaA* and *miaB* increased the translational frameshifting (Urbonavičius et al., 2001) but the mutator phenotype in *E. coli* was only present in the case of *miaA* deficiency but not in *miaB*-deficient strain (Zhao et al., 2001). Nevertheless, it raises a question whether the absence of *truA* and/or *rluA* could increase mistranslation, which can in turn lead to a mutator phenotype.

Based on the studied examples, the tRNA modification effects on translation can be very context- and tRNA-specific. In *S.* Typhimurium, the lack of Ψ38 increased the +1 frameshift frequency when $tRNA^{Leu(CUA)}$ was in the P-site, but not when other leucin tRNAs were studied (Urbonavičius et al., 2001), whereas

in the measured sequences the –1 frameshifting was not affected by TruA (Urbonavičius et al., 2003). To study the effect of pseudouridines in the ASL on translation accuracy in different bacteria, we inserted a dual-luciferase based reporter assays into a broad host range plasmid pSEVA. The assay uses a fusion protein of two luciferases, Rluc (renilla luciferase) and Fluc (firefly luciferase), and a test sequence inserted between them. The Rluc works as an internal control and the Fluc is synthesized only after frameshift event or stop codon readthrough. We measured the frequency of frameshift (5 different sequences, three –1 and two +1 frameshift sequences) and the stop codon readthrough (UAG and UGA stop codons) in the absence of *truA* or *rluA* in *P. putida* PaW85, *P. aeruginosa* PAO1 and *E. coli* MG1655 (Ref. IV, Fig. 1, 3, 5, and 6). To our surprise, the effect of TruA and RluA on translation errors significantly varied between different bacteria and different reporter sequencies (Ref. IV, Fig. 3, 5, and 6). In *P. putida*, the lack of *truA* increased significantly –1 frameshift frequency in one of the studied frameshift sequences but had no effect in the other two –1 frameshift constructs, and it did not affect +1 frameshifting (Ref. IV, Fig. 3). These results are opposite to that obtained in *S.* Typhimurium (Urbonavičius et al., 2003, 2001). However, the measured context differed, and as it can be seen from our result, the role of tRNA modification is context specific. For example, the *truA*-deficiency in *P. putida* increased UAG stop codon readthrough but had no effect on UGA stop codon readthrough (Ref. IV, Fig. 3). Contrarily to TruA, the lack of RluA had no effect on translation errors in *P. putida* in any of the measured reporters (Ref. IV, Fig. 3). In *P. aeruginosa* both the *truA*- and *rluA*-deficiency did not have any remarkable effects on translational accuracy (Ref. IV, Fig. 5). Out of the studied strains TruA had the greatest effect on translational accuracy in *E. coli*, affecting almost all the studied reporters, but again RluA had almost no effect on translational errors (Ref. IV, Fig. 6). Although from pervious results it can be seen that the tRNA modifications can have tRNA-specific effects (Li et al., 1997; Urbonavičius et al., 2003), it is still surprising that in three related bacteria with comparable set of TruA and RluA modified tRNAs (Table 1), the effect of pseudouridines to mistranslation is vastly different. Still, it should be noted that while the TruA and RluA substrates are almost the same, different codon usage in different bacteria could affect the importance of TruA and RluA in respective strain.

Although it can be concluded that Ψ38–40 in the ASL do affect the translation accuracy, we do not believe that this explains the mutator phenotype in *P. putida* and in *P. aeruginosa*. First, the lack of Ψ32 does not markedly affect the translation accuracy in any of the studied strains and especially not in *P. putida*, where the *rluA*-deficiency causes a mutator phenotype. Secondly, the results obtained with *E. coli* are opposite to the TSM hypothesis – the lack of *truA* increases mistranslation (Ref. IV, Fig. 6) but does not affect mutation frequency (Connolly and Winkler, 1989). Thus, we concluded that the increase in mutation frequency in *P. putida* and in *P. aeruginosa* is not caused by TSM. Nevertheless, we cannot exclude the possibility that the absence TruA (and RluA) causes extreme mistranslation at some very specific sequence which in turn leads to mutagenesis.

3.4.5. The effect of TruA and RluA on stress tolerance in *P. putida*, *P. aeruginosa*, and *E. coli*

To determine whether the elevated mutant frequency could be caused by changed sensitivity of *truA*- and *rluA*-deficient strains to different stress conditions, we tested the growth of *truA*- and *rluA*-deficient strains of *P. putida*, *P. aeruginosa*, and *E. coli* at different temperatures or on agar plates containing different stressors – e.g., ROS producing chemicals (NQO, PQ), translation affecting antibiotics (Sm, Km, Tet, Gm, Pm), and cell wall synthesis inhibiting antibiotics (Amp) (Table 2; Ref. III, Fig 2D-E). We observed that not only the TruA's and RluA's effect on mutagenesis and mistranslation were different in *P. putida*, *P. aeruginosa* and *E. coli*, but other phenotypes differed as well. While the *truA*-deficiency caused reduced tolerance to almost all chemicals tested in *P. putida* and *E. coli*, it did not change the tolerance to most chemicals studied in *P. aeruginosa* (Table 2; Ref. III, Fig 2D-E). Interestingly, this effect was comparable to the translation accuracy results where the lack of *truA* had almost no effect on mistranslation in *P. aeruginosa* (Ref. IV, Fig. 5). It is even more surprising that the increased tolerance to NQO and Sm were the only visible effects in *P. aeruginosa* strain lacking *truA*, which is opposite to the effects observed in *P. putida* or *E. coli* (Table 2).

In both *P. putida* and *E. coli*, the lack of *truA* remarkably decreased tolerance to many chemicals causing different type of stress (Table 2). Furthermore, as stated above the *E. coli* ΔtruA strain has remarkable growth retardation, what could nonspecifically contribute to the susceptibility of different stresses. As discussed in chapter 1.2.1.2, the importance of tRNA modifications could be revealed in stress conditions and tRNA modifications may participate in different stress responses. The results of our stress tolerance experiments indicated that the Ψ38–40 in the ASL could take part in the stress response to different stressors in *P. putida* and *E. coli*.

For instance, the tRNA modifications can participate in ROS response in both prokaryotes (Jaroensuk et al., 2016; Romsang et al., 2018; Thongdee et al., 2019) and eukaryotes (Chan et al., 2012). Although adding ROS-scavenging agent thiourea to growth medium had no decreasing effect on mutant frequency in *P. putida* ΔtruA and ΔrluA strains (Ref. III, Fig. 3C), we observed that the *P. putida* strains lacking Ψ38–40 had increased sensitivity to ROS-producing chemicals NQO and PQ (Table 2; Ref. III, Fig. 3D). This is in good accordance with the results obtained from proteome analyzes indicating that the catalase KatE levels are downregulated in ΔtruA strain if compared to wild-type *P. putida* (Ref. III, Fig. 4B, Table 1). The downregulation of catalases causes reduced tolerance to ROS. There are four different catalase genes in *P. putida* (Kim and Park, 2014; Nelson et al., 2002), and the protein levels of the others were either not changed (KatA and KatG) or not detected (PP2887) (data is available in proteome database ProteomeXchange with identifier PXD022353). In *P. aeruginosa,* it has also been shown that the catalase activity is decreased in different tRNA modification-deficient mutant strains, and that also only a subset of

catalases can be affected (Jaroensuk et al., 2016; Romsang et al., 2018; Thongdee et al., 2019). For instance, in *P. aeruginosa* lacking TrmJ, the transcription of *katB* and *katE* was reduced (Jaroensuk et al., 2016) and in *P. aeruginosa trmB*-mutant the protein levels of KatA and KatB were reduced but the transcription of *katA* and *katB* was *trmB*-independent (Thongdee et al., 2019). Based on the results of our proteome analysis we cannot say whether the KatE is down-regulated already at transcriptional level or at translational level but based on *P. aeruginosa* analogues we could speculate that TruA modulates partially the ROS stress response via KatE in *P. putida* cells.

Table 2. The stress tolerance of *P. putida* PaW85, *P. aeruginosa* PAO1, and *E. coli* MG1655 *truA*- and *rluA*-deficient strains. The stress tolerance assay was done as described in Ref. III Materials and Methods. *P. putida* strains were grown at 30 °C and *P. aeruginosa* and *E. coli* strains at temperature 37 °C. The overnight cultures were serially diluted and spotted on agar plates containing different stressors and incubated under different stress conditions. The growth of mutant strains was compared to respective strain under nonstress conditions. "0" no effect on growth compared to the same strain under nonstress conditions. "+" (if not stated otherwise) decreased growth compared to the same strain under nonstress conditions. "++" greatly decreased growth compared to the same strain under nonstress conditions. "nt" not tested on this stress condition.

	P. putida			*P. aeruginosa*			*E. coli*		
Stressors	Conts.	ΔtruA	ΔrluA	Conts.	ΔtruA	ΔrluA	Conts.	ΔtruA	ΔrluA
NQO	300 µM	+	0	300 µM	+[a]	0	20 µM	+	0
PQ	0.05 mM	++	0		nt	nt		nt	nt
MMC	5 µg/ml	0	0	1 µg/ml	0	0	1 µg/ml	++	0
Sm	25 µg/ml	+	0	10 µg/ml	+[a]	0	2.5 µg/ml	++	0
Km	2 µg/ml	+[a]	0	15 µg/ml	0	0	1.5 µg/ml	0[b]	0
Cm	35 µg/ml	+	0		nt	nt		nt	nt
Tet	1 µg/ml	++	0	2 µg/ml	0[b]	0	0.25 µg/ml	0	0
Gm	2 µg/ml	0	0	2 µg/ml	0	0	2 µg/ml	++	0
Amp	250 µg/ml	++	0	100 µg/ml	0[c]	0[c]	20 µg/ml	+	0
Pm	600 µg/ml	++	0		nt	nt		nt	nt
Hm	250 µg/ml	+	0		nt	nt		nt	nt
Temp. 42 °C		nt	nt		0	0		0	0
Temp. 37 °C		0	0		0	0		0	0
Temp. 30 °C		0	0		0	0		0	+
Temp. 20 °C		0	0		0	0		0	++

[a] – increased stress tolerance compared to the same strain under nonstress conditions
[b] – does not affect the viability but the colony size is smaller than wild type
[c] – the lack of *truA* nor *rluA* does not affect the viability but the double mutant has decreased viability

Unlike the absence of TruA, RluA deficiency did not cause any changes in the stress tolerance (Table 2). And while the stress tolerance of *truA*-deficient strain varied greatly between different species, the effect of *rluA*-deficiency was comparable in all three bacterial species studied. The only exception was the tolerance to low temperatures in *E. coli* where the lack of RluA remarkably decreased the growth at 20 °C (Table 2). Many *E. coli* strains with ribosome assembly defects have a cold-sensitivity phenotype (Kaczanowska and Rydén-Aulin, 2007; Liljeruhm et al., 2022). Since RluA with its dual-specificity also modifies 23S rRNA, the observed cold sensitivity phenotype of *E. coli rluA*-deficient strain could be attributable to the rRNA modification and not to the tRNA modifications.

Although the *P. putida* ΔtruA strain's noticeably changed tolerance to different stressors could contribute to increased spontaneous mutation rate, it is hard to believe that this could be the main reason of the mutator phenotype, since the absence of *rluA* did not change the stress tolerance of studied chemicals at all. Furthermore, there is no obvious external stressor in the experimental conditions of mutant frequency analyses.

3.4.6. The proteome of *P. putida* ΔtruA and ΔrluA strains

To investigate the cellular response to the absence of TruA and RluA, we performed a label-free proteome analyses with exponentially growing *P. putida* PaW85 wild-type, ΔtruA and ΔrluA cells. In the ΔtruA and wild-type strain comparison we were able to quantify 2856 proteins and in the ΔrluA and wild-type strain comparison 2842 proteins. In ΔtruA strain there were 158 proteins which expression levels were changed statistically significantly, but only 18 of those differed at least two-fold (Ref. III, Fig 4B, Table 1, dataset PXD022353 in ProteomeXchange). In addition, there were 5 "on-off" regulated proteins, two of which were only detectable in wild type and three only in ΔtruA strain (Ref. III, Table 1). In ΔrluA strain only the downregulation of two proteins transcribed from the same operon was statistically significant (Ref. III, Fig. 4A, Table 1). Also, there were 2 proteins only detectable in wild type and one protein only detectable in ΔrluA strain (Ref. III, Table 1). Although the downregulation of proteins from a prophage-origin operon *PP5487-PP5489* and upregulation of an oppositely transcribed regulator PP1935 just before this operon were the only changes detectable in both ΔtruA and ΔrluA mutant, the mutator phenotype was not caused by changes in the expression pattern (Ref. III, Fig. 5).

Overall, most of the proteins with statistically significant expression change in ΔtruA strain were functionally unrelated. There were several proteins which genes belong to the same operon or are located consecutively in chromosome, e. g. *PP5487-PP5489*, *hisC-hisD*, *PP1788-17989*, but the genes of most of the proteins with changed abundance are not co-transcribed (Ref. III, Fig. 4A, Table 1). The reasons behind the changes of some protein's expression have been discussed in the Discussion of Ref. III. The proteome results support many our conclusions presented above. For instance, in both ΔtruA and ΔrluA strain none of the main

repair pathway enzymes (MMR enzymes, NER enzymes) or replicative polymerase subunits had changed protein abundance level nor where there any changes in the cellular amount of SOS response regulators (LexA1, LexA2, RecA), or in the amount of RpoS (dataset PXD022353). If a strain would have slightly higher mistranslation, it would be not detected at the protein expression level, but the most common response to accumulating mistranslated proteins is the upregulation of proteases and chaperons (Hartl et al., 2011; Ruan et al., 2008; Samhita et al., 2020). However, in the ΔtruA and ΔrluA proteome the abundance of detected well-known proteases and chaperons (e.g., LonI, LonII, ClpABPSX, DnaJK, GroLS, CspA-II) was not changed. The abundance of major cold shock protein CspA-I was slightly increased (1.77x) in ΔtruA stain, but this change was not statistically significant and in ΔrluA the CspA-I levels were not changed (dataset PXD022353).

Intriguing results with yeast have shown that tRNA modifications can participate in translational reprogramming by changing the expression of specific ribosomal proteins (Chan et al., 2012). With this in mind, we also analyzed the expression of ribosomal proteins. In both ΔtruA and ΔrluA strains, the amounts of ribosomal proteins detected was not changed except for the amount of L34 (gene *rpmH*). The protein L34 was 7.7x upregulated in ΔtruA and 8.3x in ΔrluA strain but in neither strain the upregulation was not statistically significant. It should be mentioned that the ribosomal protein L34 is a small protein (5.1 kDa) and only 4 unique peptides of this protein were detected in the analysis. Overall, there is not much knowledge about the precise function of L34. L34 is the ribosomal large subunit protein, it belongs to the minority of ribosomal proteins which are known to be not essential, and the corresponding genes can be deleted from the chromosome. However, the lack of L34 causes severe growth defects (Akanuma et al., 2014; Shoji et al., 2011). Also, the chromosomal location of *rpmH* is intriguing: it is in the same operon with *rnpA* and is located consecutively with in opposite direction transcribed *dnaA*. The RnpA is the protein subunit of RNase P which is a ribonuclease necessary for the processing of tRNAs (Evans et al., 2006) and DnaA is a protein necessary for the DNA replication initiation of bacterial chromosome. Both proteins take part in central processes of a cells, but the abundance of neither has changed in ΔtruA or ΔrluA strains (dataset PXD022353). Thus, the possible role of TruA and RluA in changed amount of L34 needs further investigation.

Although the changed amount of several proteins in the ΔtruA proteome gives hints about the molecular mechanisms behind different phenotypes observed, it is hard to point out a specific change that could explain the increase in spontaneous mutation frequency.

From the proteome analyses it can be seen that except for the downregulation of proteins in one operon, there are no significant differences in the ΔrluA strain compared to wild-type *P. putida* (Ref. III, Fig. 4A). This correlates with above-described results demonstrating that the lack of *rluA* does not have any effect on translation accuracy nor in stress tolerance. One of the few *rluA*-deficiency phenotypes described previously, is the outcompeting of the *E. coli* cells lacking RluA

by wild-type cells (Raychaudhuri et al., 1999). Based on this we conducted competition experiment in *P. putida*. We marked the wild-type cells and ΔrluA or ΔtruA cells with either streptomycin (Sm) or gentamycin (Gm) resistance genes, mixed the cells together in 1:1 ratio and observed the population dynamics in 30 days. While ΔtruA cells were almost outcompeted by wild-type cells by 30th day (Fig. 5A), the proportion of ΔrluA cells remained in similar range with wild type (Fig. 5B). We conducted three independent competition experiments, and in each experiment, there were four parallels with Sm and four parallels with Gm marked ΔrluA cells. In each experiment the dynamics between wild-type and rluA population varied slightly, but the changes in CFU in ΔrluA and wild-type populations seemed to be random, and by the 30th day the differences between these two populations CFUs was not more than an order of magnitude. From the competition experiment it can be concluded that compared to the wild type the lack of *truA* considerably reduces the fitness of *P. putida*, while the lack of *rluA* does not.

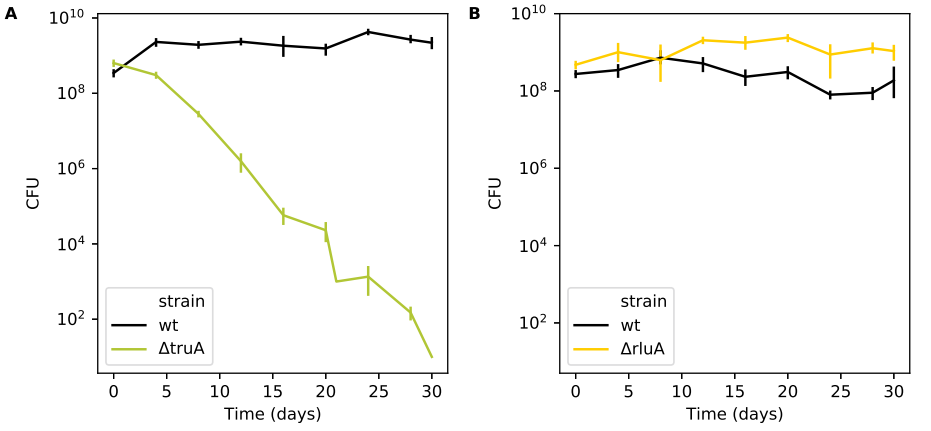

Figure 5. The results of representative competition experiment between *P. putida* PaW85 wild type and ΔtruA strains (A) and wild type and ΔrluA strains (B). The cells were chromosomally marked with antibiotic resistance genes (either Sm or Gm) as a part of mini-Tn*7*, grown overnight at 30 °C and mixed in 1:1 ratio based on OD. 10 µl of mixture was diluted into 5 ml of fresh LB every second day during the 30 days-lasting experiment. The CFUs were determined on every 4th day. The experiment was conducted three times with 8 independent parallels: in half of these experiments wild type was carrying Sm resistance gene and mutant Gm resistance and the other half the strains had reverse marking. The figure represents the average CFUs of four independent cultures in one representative experiment with 95% CI. In the figure the wild type is marked with Gm and the mutant (ΔtruA on figure A and ΔrluA on figure B) is marked with Sm.

In conclusion, the results of the proteome analysis revealed that the protein expression pattern of ΔrluA in exponential phase is comparable to that of the wild-type proteome. Also, in *P. putida* the lack of *rluA* does not affect noteworthily the stress tolerance of measured chemicals nor the translation accuracy,

and the *rluA*-deficient cells are not outcompeted by the wild-type cells. We cannot exclude the possibility that the critical role of Ψ32 becomes evident under specific harsh environmental conditions. Nonetheless, it does not explain the increasing effect of RluA on mutation frequency in exponential growth phase.

3.4.7. Concluding remarks on TruA and RluA

Although we were able to find a new and intriguing link between pseudouridines in the ASL of *P. putida* and *P. aeruginosa* tRNAs and mutant frequency, we were not able to pinpoint the molecular mechanism behind it. To generalize the only comparable effect caused by the lack of TruA and RluA in *P. putida* was the increased mutant frequency, and although unlikely, we cannot eliminate the possibility that TruA and RluA affect mutations via different mechanisms. In all the other investigated phenotypes, the RluA-deficiency had almost no effect, whereas the TruA-deficiency greatly affected the cell's wellbeing – it affected the translation accuracy, stress tolerance, protein expression and general fitness. All this could contribute to the *P. putida* ΔtruA strains mutator phenotype, but then the question remains – what causes the mutator phenotype in ΔrluA background?

The role of tRNA modifications can be versatile. For instance, the MiaA, which also affects mutation frequency, regulates the gene expression through UUN-Leu codons (Aubee et al., 2016; Thompson and Gottesman, 2014). It could also be that both TruA and RluA affect the gene expression through specific codons, most probably through the RluA affected codons, that overlap with TruA ones. But if the expression of specific protein would drastically change, we should detect it in the proteome analyses. Yet, the only proteins with remarkably altered abundance in both mutant strains did not cause any changes in mutant frequency (Ref. III, Fig. 4–5, Table 1). However, it would be interesting to analyze the codon usage of genes with changed protein expression in ΔtruA and ΔrluA strain. Furthermore, the effect of tRNA modifications on translation accuracy varies greatly. For instance, in *P. aeruginosa* the lack of TrmB remarkably diminishes the translation accuracy of repeated Phe and Asp codons, but has no significant effect on other codons which respective tRNAs are also substrates for TrmB (e.g., Ala, Arg, and Val) (Thongdee et al., 2019). This illustrates that although we did not detect any remarkable changes in translation accuracy in the strain lacking RluA, it still could affect the translation accuracy of very specific transcripts, for example transcripts with repeated UUC-Phe or UUG-Leu codons.

We have eliminated the possibilities that the increase in mutant frequency in ΔtruA and ΔrluA strains of *P. putida* is caused by endogenously elevated ROS (Ref. III, Fig. 3C), deficiency in MMR and NER (Ref. III, Fig. 3A), induction of SOS response and upregulation of TLS polymerases (Ref. III, Fig. 3B), and recombination (Fig. 5). In bacterial cells the main source of spontaneous mutations is the replicating polymerase itself. Although the abundance of subunits of DNA polymerase were not changed in the proteome of *P. putida* ΔtruA and ΔrluA strains (dataset PXD022353), this method does not detect the wellbeing of

proteins. For instance, if only a small subset of population would carry a DNA Pol III complex with defective proofreading or replicative subunit, this would already influence the mutation frequency. Furthermore, a bacterial population can carry a great cell-to-cell heterogeneity. Subpopulations of cells may have higher level of endogenic stresses or translation errors, which in turn can lead to more spontaneous mutations in replication (Matic, 2019; Woo et al., 2018). Also, the *in vitro* replication fidelity of purified DNA Pol III complex from *E. coli* mutant mistranslating due to the mutator tRNA, was shown to be lower than the DNA Pol III fidelity from wild-type cells (Al Mamun et al., 2002). However, the effect was mild and the same mutant exhibited greater mutator phenotype *in vivo* (Murphy and Humayun, 1997; Ren et al., 1999). Also, the less accurate DNA polymerase from mutant cells had changed mutation spectrum (Al Mamun et al., 2002), but we did not observe any changes in the Rif^R mutant spectrum (Ref. III, Table S6). Nevertheless, erroneous DNA polymerase is one possibility what could explain our observed phenotype.

Another interesting aspect about TruA and RluA is the dissimilarities in different but related bacterial species. We compared the phenotypes of strains lacking pseudouridines synthases TruA and RluA in *P. putida*, *P. aeruginosa*, and *E. coli*. It was surprising that although all strains have very similar TruA and RluA substrate patterns (Table 1), the observed phenotypes are eminently different (Table 2, Ref. IV, Fig. 2–6). Furthermore, while in our experiments with *P. putida* the *truA*-deficiency had moderate effect on growth, it has been recorded previously that in *E. coli* the *truA*-deficiency significant affects growth (Tsui et al., 1991) and in *P. aeruginosa* there is no growth retardation (Ahn et al., 2004). This is in good accordance with our observations. The dissimilar importance of TruA and RluA in strains studied can be partially due to differences in codon usage or in the abundance of tRNAs. Nevertheless, this clearly illustrates the need to study the mutational as well as other processes in a broader selection of species when even a conserved protein with seemingly the same function can cause different phenotypes in closely related bacteria.

CONCLUSIONS

It is widely acknowledged that the spontaneous mutation frequency of a bacterial cell is shaped by several independent but simultaneous processes – the DNA replication, the efficiency of repair enzymes, transcription, the presence of endogenic and exogenic stressors, the ability to adapt new conditions and many more. Furthermore, in cell, there are also other processes and reactions that do not so obviously affect the outcome of DNA mutations but still play a part in the overall wellbeing of a cell and consequently affect the mutation frequency, for instance, the faithful translation. The current study presents insight into some of the factors affecting mutation frequency of *P. putida* PaW85. The main findings can be summarized as follows:

The nucleoid-associated protein integration host factor IHF

- IHF affects the mutation frequency in *P. putida* PaW85. The effect appeared to be dependent of the growth phase of bacteria, the chromosome location of the mutational target, and the amount of IHF. To get insight how exactly the nucleoid structure and nucleoid-associated proteins (NAPs) affect the mutagenesis, it would be useful to investigate the role of other NAPs as well.

The test system created

- The created levan papillae formation-based test system lac-lsc enables monitoring both exogenously and endogenously increased mutation frequency in a single bacterial colony.
- The lac-lsc test system is applicable in a wide variety of non-levan producing environmental and laboratory strains from genus *Pseudomonas*. However, the strain-specific optimization would be necessary before conducting experiments with other species.
- The transposon mutagenesis screen with *P. putida* tester strain enabled to identify among the already known genes affecting mutation frequency also several novel genes whose inactivation increased mutation frequency, e.g., *truA* that encodes for tRNA pseudouridine (Ψ) synthase.

Phenotypes of TruA and RluA in *P. putida*

- We were able to find an intriguing link between the lack of pseudouridylation activity of Ψ synthases TruA and RluA and the increased mutation frequency in exponentially growing *P. putida* PaW85. The observed mutator phenotype was not caused by the upregulation of TLS polymerases, malfunction of MMR or NER, recombination, or increased intracellular ROS.
- The lack of TruA reduced remarkably the cells tolerance to chemicals affecting the translation, producing ROS or inhibiting peptidoglycan synthesis. These results indicated that TruA has a role in cells stress tolerance. At the same time, the absence of RluA did not change the tolerance to the same chemicals. Also, the TruA-deficiency caused remarkable reduction in general fitness, whereas the fitness of RluA-deficient mutant was comparable to the wild type.

- The proteome of exponentially growing *P. putida* remarkably differed in the absence of TruA but the lack of RluA evoked only minor changes. These results indicate that the Ψs at the positions 38–40 in tRNAs (done by TruA) have greater importance in the *P. putida* cells than Ψ at the position 32 (done by RluA).
- In translation accuracy measurements we saw that the Ψs at the positions 38–40 in the tRNA ASL increase the translation accuracy, although the effect greatly depends on the measured context. This demonstrated that at least one of the roles of tRNA modification Ψ in prokaryotic tRNAs is improving the translation fidelity.

The comparison of *P. putida*, *P. aeruginosa*, and *E. coli* TruA and RluA

- We demonstrated that the target positions of *P. putida* TruA and RluA in the ASL of tRNAs are the same as in *E. coli*, and the overall substrate pattern of *P. putida*, *P. aeruginosa*, and *E. coli* TruA and RluA is comparable. Based on this, it was surprising that mutants without TruA had noticeably different phenotypes in all three bacteria studied. Both the strains tolerance to different stressors and the translation accuracy varied greatly between the species.

Altogether, it can be concluded that the mutational processes are affected by a complex network of obvious and not so obvious factors acting together and also depend upon exogenic and endogenic stressors. With the aid of this study, both the nucleoid-associated proteins and the tRNA modification enzymes can be added to the list of factors affecting the mutational processes in *P. putida* PaW85.

SUMMARY IN ESTONIAN

Mutatsioonisagedust mõjutavate tegurite otsinguil: tRNA modifikatsiooniensüümid TruA ja RluA mutatsiooniprotsessides

Bakterid suudavad elada väga erinevates keskkonnatingimustes, ka paikades kus elu tundub esmapilgul võimatu. Muutlikes keskkonnatingimustes kohanemise ja ellujäämise tagab geneetiline varieeruvus. Bakterites on geneetilise varieeruvuse allikateks mutatsioonid ja horisontaalne geeniülekanne. Potentsiaalselt võivad mutatsioonid olla rakkudele ohtlikud või isegi surmavad ja seetõttu on mutatsioonide tekkesagedus rakkudes nii madal kui võimalik. DNA terviklikkuse säilitamiseks on rakkudes hulgaliselt ensüüme, mis aitavad ära hoida mutatsioonide teket või vastutavad DNAsse tekkinud kahjustuste parandamise eest. Ent peaaegu alati leidub keskkonnas midagi, mis on elutegevusele kahjulik või piirav, ja sellest tulenevalt võib kahjustuda DNA. Samuti võivad DNAd kahjustada loomuliku elutegevuse kõrvalproduktid, näiteks reaktiivsed hapnikuühendid. Seega, mutatsioonid siiski tekivad ja on aluseks evolutsioonile. Mõistmaks evolutsiooni, on tarvis esmalt mõista mutatsioonide tekke molekulaarseid tagamaid.

Ajaloolistel põhjustel on kujunenud bakterimaailma mudelorganismiks soolebakter *Escherichia coli* ehk soolekepike ning enamus mutatsiooniuuringuid on läbi viidud *E. coli*ga. Samas, nii mõneski aspektis on *E. coli* erandlik ja üldistuste tegemiseks on kindlasti tarvis uurida laiemalt ka teisi liike. Meie uurimisgrupis uuritakse mutatsiooniprotsesse bakteriperekonnas *Pseudomonas*. *Pseudomonas*e perekonda kuulub hulgaliselt erinevates keskkondades elavaid metaboolselt mitmekesiseid baktereid, näiteks taimede ja inimese patogeenid, aga ka vee- ja mullakeskkonna bakterid. *Pseudomonas aeruginosa* on kindlasti üks tuntumaid selle perekonna esindajaid, kes on oportunistlik inimese patogeen ja on silmapaistev oma võime poolest äärmiselt kiiresti omandada resistentsus antimikroobsete ühendite suhtes. *Pseudomonas putida* on mullabakter, kes talub suurtes kogustes toksilisi ühendeid ja saasteaineid ning on võimeline lagundama ka sünteetilisi ühendeid. Seetõttu kasutatakse *P. putida*t palju biotehnoloogias.

On ilmselge, et vigadevaba DNA replikatsioon ja korrektselt töötavad DNA vigade paranduse ensüümid on aluseks rakkude terviklikkusele ja madala mutatsioonisageduse hoidmisele. Ent palju muudki võib mõjutada mutatsioonisagedust, näiteks geeni asukoht genoomis, ligipääs DNAle, transkriptsioon ja stressitingimused. Samuti ei tohi unustada, et üherakulises bakteris toimuvad kõik kesksed protsessid samaaegselt ja ruumiliselt eraldamata, ning seeläbi võivad need üksteist mõjutada. Paljude faktorite täpne roll mutatsiooniprotsessides pole selge ja samuti pole nende rolli vaadatud laiemalt erinevates bakterites. Sellest tulenevalt sai minu töö eesmärgiks leida ja kirjeldada uusi mutatsioonisagedust mõjutavaid geene bakteriperekonnas *Pseudomonas*.

Esmalt oli selleks tarvis luua tööriist – testsüsteem, mille abil oleks võimalik tuvastada mutatsioonisagedust mõjutavaid geene *Pseudomonas*e perekonna

bakterites. Loodud testsüsteem lac-lsc põhineb limase polümeeri levaani tootmisel bakterikoloonia pinnale. Tulemustest võib järeldada, et see testsüsteem on kasutatav paljudes *Pseudomonas*e perekonna bakterites ja see võimaldab jälgida nii eksogeenselt kui endogeenselt suurenenud mutatsioonisagedust. Bakteris *P. putida* õnnestus testsüsteemi kasutades tuvastada hulgaliselt varsemalt teadaolevaid mutatsioonisagedust mõjutavaid geene, aga ka mitmeid uusi, varasemalt mutatsioonidega mitte seostatud geene. Uute leidude hulgas põhjustas suurima mutatsioonisageduse tõusu geeni *truA* katkestamine.

TruA on tRNA modifikatsiooniensüüm, mis modifitseerib U nukleotiidi pseudouridiiniks (Ψks) tRNA antikoodoni vahetus läheduses. tRNAd on adaptermolekulid, mis valgusünteesi käigus kannavad valkude ehitusplokke, aminohappeid, ribosoomi ja dekodeerivad mRNA järjestuse. Oma keskse rolli täitmisel interakteeruvad tRNA molekulid paljude teiste molekulidega ja arvatavasti seetõttu on nukleotiidid tRNAs nii ulatuslikult keemiliselt modifitseeritud. Ψ on kõige levinum nukleotiidi modifikatsioon, mida leidub nii tRNAdes, rRNAdes kui ka mRNAdes. Lisaks TruAle teeb antikoodonist teisele poole sama modifikatsiooni ensüüm RluA.

Töö teises osas keskendusin TruA ja RluA rolli väljaselgitamisele *P. putida* rakkudes. Leidsime, et nii TruA kui RluA tehtavate Ψde puudumisel suureneb *P. putida* mutatsioonisagedus märkimisväärselt. Esmapilgul tundub üllatav, et ensüümid, mis on seotud ennekõike valgusünteesiga, mõjutavad DNA mutatsioonide teket, kuid see ilmestab väga selgelt, kuidas kõik protsessid võivad ühes terviksüsteemis üksteist mõjutada. Lisaks on see esimene kord, kus on näidatud, et Ψde puudumisel tRNA antikoodon-lingus tõuseb DNA mutatsioonisagedus.

Järgnevatest katsetest *P. putida* tüvedega selgus, et mutatsioonisageduse tõusu TruA- või RluA-defektsetes tüvedes ei põhjusta vead põhilistes reparatsioonisüsteemides, vigu tegevad polümeraasid, rekombinatsioon ega ka rakusiseselt tõusnud reaktiivsete hapnikuühendite hulk. Samuti ei tundu mutatsioonisageduse tõusu põhjuseks olevat muutused translatsiooni täpsuses, sest kuigi TruA puudumisel muutub translatsioon ebatäpsemaks, siis nähtud efekt on väike ja RluA puudumine translatsiooni ei mõjuta. Samuti ei näinud me RluA puudumisel muutuseid teistes fenotüüpides nagu näiteks üldises elulemuses või stressitaluvuses. Seevastu TruA puudumine mõjutas märgatavalt rakkude elulemust, stressitaluvust ja ka teisi fenotüüpe.

Seejärel vaatlesime TruA ja RluA puudumise fenotüüpe võrdlevalt ka *P. aeruginosa* ja *E. coli* rakkudes. Nägime, et TruA ja RluA substraat-tRNAd on kõigis kolmes bakteris väga sarnased, kuid sellest hoolimata varieeruvad fenotüübid liigiti suuresti. Lisaks eelpool kirjeldatud fenotüüpidele *P. putida*s suurenes TruA puudumisel mutatsioonisagedus ka *P. aeruginosa*s, kuid RluA puudmine *P. aeruginosa*s mutatsioonisgedust ei mõjutanud. Ensüümi TruA mõju nii translatsiooni täpsusele kui stressitaluvusele oli eri liikides väga erinev, seevastu RluA ei mõjutanud translatsiooni täpsust ega stressitaluvust üheski uuritud bakteris. Need tulemused ilmestavad hästi seda, miks on vajalik teostada mutatsiooniuuringuid erinevate bakteritega, kuna sama funktsiooniga valgu puudumine võib isegi sama perekonna bakterites põhjustada väga erinevaid fenotüüpe.

Kokkuvõtteks võib öelda, et lõime testsüsteemi, mille abil on võimalik tuvastada mutatsioonisagedust mõjutavad geene ja teostada alusuuringuid paljudes ka vähemuuritud *Pseudomonas*e perekonna bakteriliikides. Lisaks õnnestus tuvastada mitmeid varem teadmata mutatsioonisagedust mõjutavaid geene mullabakteris *P. putida*, näiteks nukleoidiga seostuva valgu IHF geen. Neist üllatavaimad ja suurima efektiga olid aga geenid *truA* ja *rluA*. Käesolev doktoritöö näitab vajalikkust uurida erinevaid baktereid, mõistmaks samade ensüümide rolle erinevas taustsüsteemis.

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PUBLICATIONS

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Tamman, H; Ainelo, A; **Tagel, M**, Hõrak, R (2016). Stability of the GraA antitoxin depends on growth phase, ATP level. and global regulator MexT. Journal of Bacteriology, 18(5), 787–796.

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Member of the Estonian Society for Microbiology, since 2013

Student member of the council of the Institute of Molecular and Cell Biology, 2016–2020

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Co-supervisor of several BSc students (Johanna Elmik 2014, Karl Jürgenstein 2016, Gabriel Agur 2016, Aneth Lvovs 2017)

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Other teaching and supervision:

Member of the Organizing Committee of the Estonian Biology Olympiad, since 2018

Lectures and practical courses for high school students (e.g., lecture in Vanalinna Hariduskolleegium 2018, practical course in genetics for Hugo Treffner Gymnasium 2019, practical lecture for Võnnu Highschool 2020)

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1. **Toivo Maimets**. Studies of human oncoprotein p53. Tartu, 1991, 96 p.
2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
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5. **Maia Kivisaar**. Studies on phenol degradation genes of *Pseudomonas* sp. strain EST 1001. Tartu, 1992, 61 p.
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