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Investigating Ribosomal Catalysis: Optimizing Assays with Thermostable Bacterial Ribosomes for Defining Minimal Components Necessary for Peptidyl Transferase Activity

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Investigating Ribosomal Catalysis: Optimizing Assays with Thermostable Bacterial Ribosomes for defining minimal components necessary for Peptidyl Transferase Activity.

Abstract:

Ribosomes are cellular molecular machinery, facilitating the peptidyl transferase activity – a crucial reaction for the elongation of the peptidyl chain during translation. The process of how exactly ribosome catalyzes peptidyl transferase activity remains a subject of study. Researchers are determined to establish the minimal ribosomal components essential for ribosomal activity. Studies with E. coli 70S ribosome showed that only large ribosomal subunit 50S is essential for performing the peptidyl transferase activity. Later, it was discovered that only one of 50S rRNAs, 23S rRNA, is essential, as it contains the peptidyl transferase center. Later studies with more thermophilic bacteria species paved the way to create a minimal synthetic ribosome reconstructed from the *in vitro* synthesized ribosomal rRNAs. This project aims to create a minimal *Thermus thermophilus* ribosome from *in vitro* transcribed (IVT) rRNAs and naturally extracted 50S total protein components and assay its peptidyl transferase activity with fragment reaction to test the functionality of the reconstructed large ribosomal subunit. For this purpose, a new assay with f[^{35}S]Met-tRNAfMet was assembled. The results of this work show that using [^{35}S] radiolabel leads to more sensitive peptidyl transferase activity detection. On the other hand, the detected activity of the reconstructed *Thermus thermophilus* 50S subunit is still low, which significantly differs from studies with closely related bacteria species. That opens a question of whether post-translational modifications matter for the activity of *Thermus thermophilus* ribosomes.

Keywords:

Ribosome, RNA, 50S ribosomal subunit, 23S rRNA, peptidyl transferase activity, *Thermus thermophilus*

CERCS:

T490 Biotechnology; P320 Nucleic acids, protein synthesis

Ribosoomi katalüüsi uurimine: termostabiilsete bakteriaalsete ribosoomide analüüside optimeerimine, et määratleda minimaalsed komponendid, mis on vajalikud Peptidyl Transferase aktiivsuse jaoks

Lühikokkuvõte:

Ribosoomid on rakusisesed molekulaarmasinad, mis toetavad peptidüültransferaasi aktiivsust – peptiidahela pikendamise reaktsiooni katalüüsimine tranlsatsiooni käigus. Protsess, kuidas ribosoom katalüüsib täpselt peptidüültransferaasi aktiivsust pole veel täielikult arusaadud. Üheks teadlaste eesmärgiks on kindlaks teha ribosoomi aktiivsuse jaoks olulised minimaalsed ribosoomikomponendid. Uuringud *E. coli* 70S ribosoomiga näitasid, et ainult suur ribosoomi alaühik 50S on oluline peptidüüli transferaasi aktiivsuse saavutamiseks. Hiljem avastati, et ainult üks rRNA 50S rRNA kompleksist – 23S rRNA, on oluline, kuna see sisaldab peptidüültransferaasi keskust. Hilisemad uuringud termofiilsemate bakteriliikidega sillutasid teed minimaalse sünteetilise ribosoomi loomiseks, mis on rekonstrueeritud *in vitro* sünteesitud ribosoomalsest rRNA-st. Käesoleva töö eesmärk on luua minimaalne *Thermus thermophilius* ribosoom *in vitro* transkribeeritud (IVT) rRNA-dest ja looduslikult ekstraheeritud 50S ribosoomi valkudest ning analüüsida saadud kuntsliku ribosoomi peptidüül-transferaasi aktiivsust. Selleks, et testida rekonstrueeritud suure ribosoomi alaühiku funktsionaalsust kasutati fragmendireaktsiooni f[^{35}S]Met-tRNAfMet. Selle töö tulemused näitavad, et [^{35}S] radiomärgi kasutamine põhjustab tundlikumat peptidüültransferaasi aktiivsuse tuvastamist. Samuti, rekonstrueeritud *T. thermophilius* ribosoomi 50S alaühiku aktiivsus on madal võrreldes sarnaste uuringutega lähedaste bakteriliikidega. See avab küsimuse, kas translatsioonijärgsed muudatused on olulised *T. thermophilius* ribosoomide aktiivsusele.

Võtmesõnad:

Ribosoom, RNA, 50S ribosoomi alaühik, 23S rRNA, peptidüültransferaasi aktiivsus, *Thermus thermophilus*

CERCS:

T490 Biotehnoloogia; P320 Nukleiinhappesüntees, proteiinisüntees

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TERMS, ABBREVIATIONS AND NOTATIONS

DNA – deoxyribonucleic acid

E. coli* - *Escherichia coli

EDTA - ethylenediaminetetraacetic acid

FA – formic acid

fMet-tRNA - Formyl-methionine charged transfer RNA

HPLC – high performance liquid chromatography

IVT – *in vitro* transcription

PTC – peptidyl transferase center

Pmn – puromycin

RNA - ribonucleic acid

rRNA – ribosomal RNA

r-proteins – ribosomal proteins

SHAPE - Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension

tRNA – transfer RNA

TP – total protein component

T.t.* - *Thermus thermophilus

INTRODUCTION

Ribosomes are macromolecular complexes responsible for protein synthesis in all living cells. One of the most crucial steps in comprehending how cells function is the process of catalyzing translation. The process by which ribosomes catalyze the formation of peptide bonds, particularly during the initiation phase of protein synthesis, is still not fully understood and remains an active area of research. This work focuses on investigating the peptidyl transferase activity of the ribosome, particularly focusing on the possibility to study the possibility of creating a minimal ribosome – the minimal set of ribosomal components needed to achieve a functional ribosome.

Peptidyl transferase activity, which is dependent on the peptidyl transferase center within the large ribosomal subunit, is crucial for the elongation of polypeptide chains during translation. Previously, many studies with *E. coli* ribosomes were carried out to establish the minimal set of ribosomal components needed for achieving peptidyl transferase activity. Thus, studies have demonstrated that functional minimal ribosomes can be assembled using only essential components of the large ribosomal subunit, 50S, such as 23S rRNA, 5S rRNA and a subset of crucial proteins from the total protein component of the 50S subunit. However, the limitations in the stability of the *E. coli* ribosomes and their dependence on the post-transcriptional modifications have posed significant challenges in creating a functional minimal synthetic ribosome using *in vitro* transcribed rRNAs and naturally extracted ribosomal proteins. These limitations have shifted researchers' attention towards more robust systems, such as ribosomes from thermophilic bacteria. Thermostable ribosomes, for example, the ones from the *Thermus aquaticus*, allow the creation of the functional 50S subunit from the *in vitro* reconstructed ribosomal RNAs and total protein component of 50S (TP50).

The aim of this research is to assemble a sensitive fragment reaction assay with f[^{35}S]Met-tRNA^{fMet} substrate to test the peptidyl transferase of the 50S ribosomal subunit from the *Thermus thermophilus* bacteria. This subunit is constructed from the *in vitro* transcribed (IVT) rRNAs and total protein component of the 50S subunit. The goal is to determine if the hypothesis that the minimal ribosome can remain active without any post-transcriptional modifications is true.

1 LITERATURE REVIEW

1.1 WHAT IS A RIBOSOME?

1.1.1 Ribosomal structure and types

A ribosome is a macromolecular complex involved in translating messenger RNAs (mRNAs) into proteins in every living organism. Each ribosome is composed of two subunits – small and large, each of them is made up of ribosomal RNA (rRNA) and protein molecules (r-proteins). Small ribosomal subunits contain mRNA binding sites that bind the mRNA molecule near the start codon by interacting with specific elements of the rRNA (Ramakrishnan, 2002). Moreover, it is also responsible for the recruitment of the charged initiator transfer RNA (tRNA) to the start codon (Ramakrishnan, 2002). Another vital function of the small ribosomal subunit is decoding genetic information during translation. It contains a ribosomal decoding center, a crucial region responsible for the correct protein translation (Schluenzen *et al*., 2000). In translation initiation, the small ribosomal subunit binds mRNA at the 5′-end and scans in the 5′ to 3′ direction to place the initiation codon, forming an initiation complex and synthesizing proteins (Choi *et al*., 2000).

There are three tRNA docking sites on the peptidyl transferase center: the E site, the P site and the A site. These sites allow it to catalyze one of the critical chemical reactions in the cell- peptide bond formation along with binding initiation, termination, and elongation factors (Figure 1.) (Ban *et al*., 2000). Each of these three tRNA docking sites is oriented 5' to 3' E-P-A concerning the mRNA and plays a specific role in the process of peptide bond formation. The A site, which stands for the aminoacyl site, binds to the aminoacyl tRNA, carrying new amino acids that are added to the polypeptide chain. The Peptidyl (P) site is responsible for holding the tRNA with the growing polypeptide chain. E site stands for the exit site, where the empty tRNA, without any amino acids, is kept being later released by a ribosome.

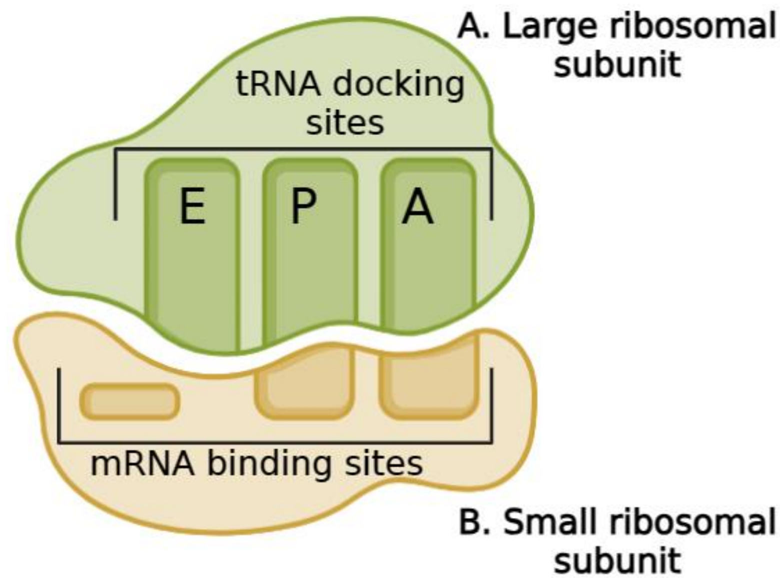

Figure 1. The structure of the ribosome. **A.** The structure of the large ribosomal subunit, showing the locations of three tRNA docking sites – A, P and E, oriented 5’ to 3’. Aminoacyl tRNA binds to the A site, carrying new amino acids. tRNA binding the growing polypeptide chain is held on the P site. Then peptide bond formation is over, and empty tRNA is released from the ribosome via the E site (Ban *et al*., 2000). **B**. The structure of the small ribosomal subunit, where mRNA binding sites are located. This figure was made with “BioRender” software.

Eukaryotic ribosomes are named 80S ribosomes, and prokaryotic ones are named 70S ribosomes (“S” stands for the Svedberg coefficient, which is a measure of the rate of sedimentation under the influence of density gradient sedimentation) (Uzman *et al*., 2000).

An 80S ribosome comprises one 60S large ribosomal subunit and one 40S small ribosomal subunit.The 60S subunit is composed of three rRNA molecules (5S, 28S and 5.8S) and around 50 r-proteins, whereas the 40S subunit is composed of 18S rRNA and about 33 r-proteins (Figure 2). The total number of proteins can be different in various organisms.

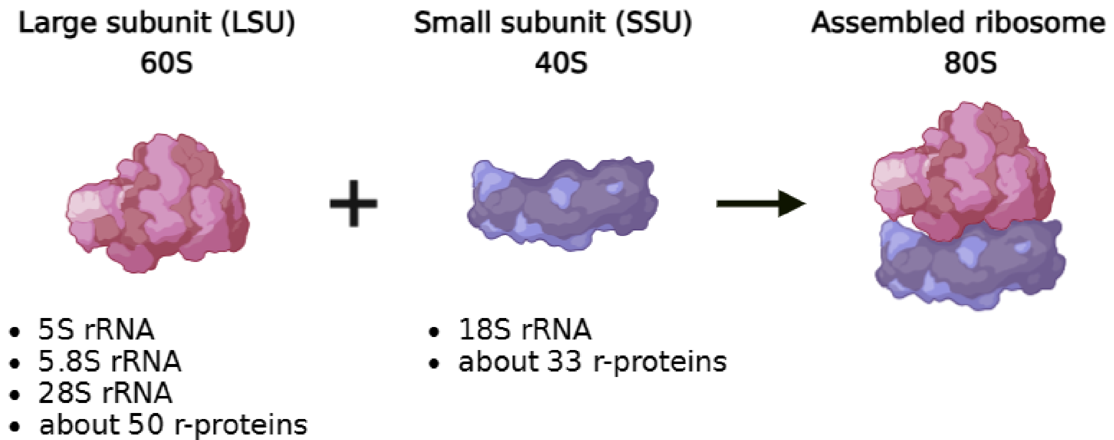

Figure 2. The structure of Eukaryotic ribosome (80S). This figure shows the basic composition of the 80S ribosome, which consists of a large (60S) ribosomal subunit and a small (40S) ribosomal subunit. 60S is composed from three molecules of rRNA (5S, 5.8S and 28S) and approximately 50 ribosomal proteins (r-proteins). A small (40S) ribosomal subunit consists of one rRNA molecule (18S) and about 33 ribosomal proteins (r-proteins). This figure was made with "BioRender" software.

Prokaryotic and eukaryotic ribosomes are different in size (eukaryotic ribosomes are larger than prokaryotic ones) and in the types of proteins and rRNA molecules they are made from. In 70S ribosomes, a large ribosomal subunit (50S) consists of two rRNA molecules (5S and 23S) and 31 ribosomal proteins. Small ribosomal subunit (30S) in prokaryotes contains one rRNA molecule (16S) and about 21 ribosomal proteins (Figure 3). The number of proteins may vary in different organisms.

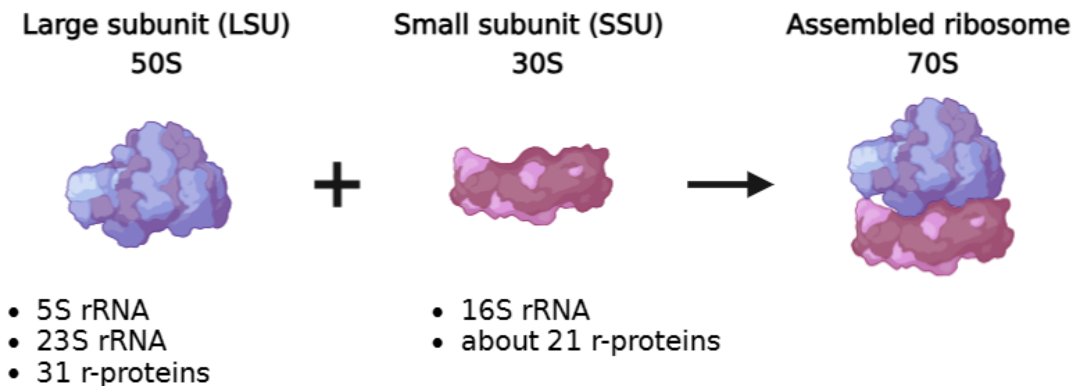

Figure 3. The structure of prokaryotic ribosome (70S). This figure shows the basic structure of the 70S ribosome, which consists of a large (50S) ribosomal subunit and a small (30S) ribosomal subunit. 50S is composed of two rRNA molecules (5S and 23S) and 31 ribosomal proteins (r-proteins). 30S consists of one rRNA molecule (16S) and 21 ribosomal proteins (r-proteins). This figure was made with "BioRender" software.

1.1.2 Functional role and importance of ribosomal RNAs and ribosomal proteins

Both components of ribosomes play essential parts in protein synthesis. Ribosomal RNAs serve two primary functions: forming the structural scaffold and performing catalytic activity. The structural core framework of the ribosome is made from RNAs, which serve as a platform for ribosomal proteins to bind and assemble. These interactions stabilize the ribosome's structure and provide functional surfaces for substrate binding and catalysis during translation (Catalanotto *et al*., 2023). Moreover, rRNA plays a significant role in the function of the peptidyl transferase, which is the catalytic ribosomal center responsible for bond formation. The correct placement of the peptidyl transferase substrates and peptidyl-tRNA and aminoacyl-tRNAs is crucial for transpeptidation catalysis and protein synthesis (Bocchetta *et al*., 1998). Ribosomal RNAs are involved in peptidyl transferase activity by catalyzing the formation of peptide bonds between amino acids during protein synthesis (Moore & Steitz, 2011). Small ribosomal subunits, both in eukaryotes and prokaryotes, contain specific elements of rRNA, which nowadays are suggested to recognize unique sequences – the Shine-Dalgarno sequence in prokaryotes and Kozak sequence in eukaryotes, which are located close to the start codon (AUG) on the mRNA (Martin *et al*., 2016).

Moreover, rRNAs bind the mRNA near the start codon and tRNA during translation (Bastide & David, 2018). Ribosomal proteins ensure proper folding and stable ribosomal structure by

binding to rRNAs (Draper & Reynaldo, 1999). This allows the accurate positioning of the ribosomal functional sites during protein synthesis (Gopanenko *et al*., 2015). More than that, with rRNAs, specific r-proteins contribute to distinct aspects of the ribosomal function and regulation of gene expression (Komili *et al*., 2007).

1.1.3 Peptidyl transferase activity

The primary function of the ribosome is peptidyl transferase activity, which naturally lies within the peptidyl transferase center (PTC) (Alonso & Mondragón, 2021). The peptidyl transferase center is located in the large ribosomal subunit; in prokaryotes, it lies at the domain V at the 23S rRNA. As mentioned in section 1.1.1, PTC contains three tRNA binding sites – A, P and E, each of which has its specific function. The process of translation elongation of the peptidyl transferase center is illustrated in Figure 4 (Aviner, 2020). Protein synthesis starts with binding the charged initiator tRNA (fMet-tRNA) to the P-site of the ribosome, and the mRNA guides the second aminoacyl-tRNA to the A-site. Then, the ribosome catalyzes the crucial reaction - nucleophilic attack by the amino group of the A-site amino acid on the carbonyl carbon of the P-site amino acid linked to fMet-tRNA. This forms a peptide bond between the two amino acids, producing a dipeptide. A-site tRNA then becomes the peptidyl-tRNA by carrying the growing polypeptide chain. P-site is now empty, and tRNAs are translocated in one position. This places the peptidyl-tRNA in the P-site and the deacylated tRNA in the E-site, through which empty tRNAs leave the ribosome. A new aminoacyl-tRNA can now enter the A-site, and the new cycle of the peptide bond formation starts. This process repeats until a stop codon on the mRNA enters the PTC. Peptidyl transferase activity relies not only on the ribosome with its rRNA and ribosomal proteins but also on the complex mix of the various translation factors with mRNAs and GTP.

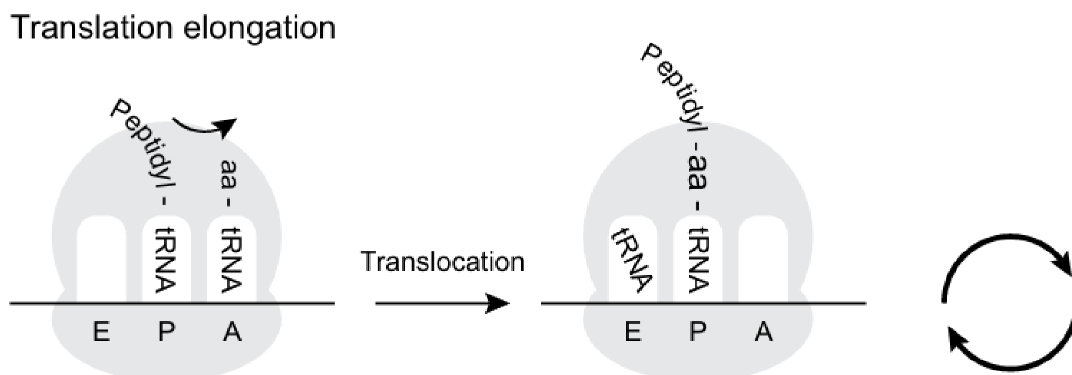

Figure 4. The process of the translation elongation. This is the basic illustration of the process of translation elongation. Firstly, aminoacyl tRNA (aa-tRNA) enters the A-site, while

charged initiator tRNA (fMet-tRNA) binds to the P-site. Aa-tRNA accepts the nascent polypeptide chain from the peptidyl-tRNA. After the translocation, the A-site accommodates the next aa-tRNA, while the P-site now carries the growing polypeptide chain. Empty tRNA leaves the ribosome via the E-site (Aviner, 2020). The figure is taken from the cited article.

1.2 PEPTIDYL TRANSFERASE ACTIVITY ASSAY

1.2.1 The mechanism of the fragment reaction

Peptidyl transferase activity was first separated from the other ribosomal functions in 1967 when Monro established his first *in vitro* assay, also named "fragment reaction". The initial idea of this research was to investigate the role of the *E. coli* 50S ribosomal subunit in protein synthesis, testing its ability to catalyze peptide bond formation (Monro, 1967). In this reaction, an antibiotic puromycin (Pmn) binds to the A-site of the ribosome, while fMet-$tRNA^{fMet}$ binds to the P-site. The binding of the puromycin to the fMet leads to the stopping of the elongation of the peptide bond by having an amide bond instead of an ester one. Two products of the reaction, $tRNA^{fMet}$ on the P-site and fMet-Pmn on the A-site, formed, and the reaction's efficiency is then measured by the amount of the fMet-Pmn product (Figure 5). The radioactive label [^{35}S] addition to the fMet enables the reaction readout, which is done in the Forster lab using the high-performance liquid chromatography (HPLC) assay technology. Monro established that isolated *E. coli* 50S subunits maintain the capability of catalyzing peptide bond formation in an *in vitro* system. It proved that the peptidyl transferase activity depends on the 50S subunit. It also investigates that magnesium ions (Mg^{2+}) are necessary to bind phosphate groups to tRNA molecules and stabilize reaction intermediates (Monro, 1967).

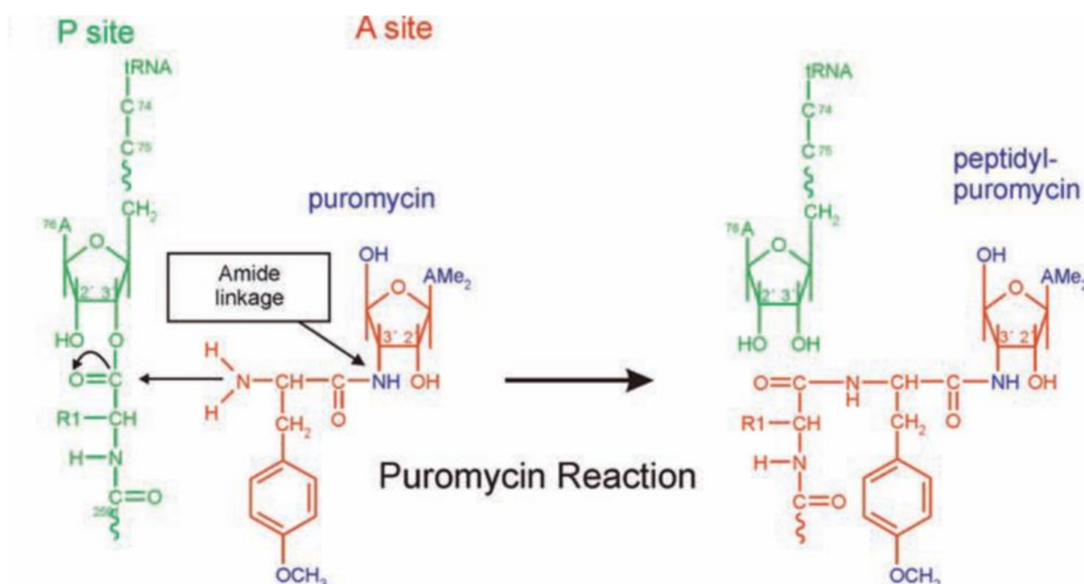

Figure 5. Mechanism of the puromycin action. Puromycin has an amide group in its structure, which amino groups cannot attack. The amino group on Pmn launches a nucleophilic attack on the ester bond on the fMet-tRNA, which leads to the formation of the peptide bond and a product fMet-Pmn. The tRNA is now empty (Wilson & Nierhaus, 2006). The scheme is taken from the cited article.

1.2.2 The search for the essential components for the peptidyl transferase activity of *E. coli*

When Monro discovered that isolated E. coli 50S subunit was enough to enhance the peptidyl transferase activity, it reduced the components needed for the peptidyl transferase activity. Further research shows that following the two-step reconstitution protocol, the 50S *E. coli* subunit can be successfully *in vitro* reconstructed with isolated *E. coli* total protein component (TP50) and ribosomal RNAs (Dohme & Nierhaus, 1976). Later, studies showed that not all the components of 50S are needed to perform a successful and functional *in vitro* reconstruction of the 50S subunit (Schulze & Nierhaus, 1982). Hampl's research shows that the deletion of single proteins L1, L5, L7, L12, L9, L19, L23, L29, L31, L32 and L33 did not affect the activity of the peptidyl transferase in the 50S subunit, as well as its *in vitro* reconstruction (Hampl *et al.*, 1981). Later, *in vitro* reconstruction of the active 50S subunit was performed with only 23S rRNA and the total proteins from the large ribosomal subunit (Giuliano & Engl, 2021). Further investigations show that the 50S subunit still can be actively reconstructed only with the 23S rRNA and 16 r-proteins, such as L2, L3, L14, L15, L16 and others (Schulze & Nierhaus, 1982). Removing the 5S rRNA and other r-proteins did not significantly influence the peptidyl transferase activity and successful *in vitro* reconstruction of the 50S subunit. Proteins such as L2, L3, L14, L15, and L16 were found to be essential for the peptidyl transferase activity as

their absence significantly impaired the assembly of the 50S ribosomal subunit (Schulze & Nierhaus, 1982).

Two-step reconstitution of the *E. coli's* 50S ribosomal subunit showed that the peptidyl transferase activity is dependent on specific post-transcriptional modifications of the 23S rRNA around the peptidyl transferase center at the domain V (Green & Noller, 1996). The reconstructed 50S lacked stability and activity compared to the native subunits, which proposed switching to more stable organisms, such as thermostable bacteria.

1.3 STUDIES WITH THERMOSTABLE BACTERIAL RIBOSOMES

1.3.1 Thermophilic bacteria

Thermophilic bacteria (also referred to as thermostable) are microorganisms whose optimum growth temperature is between 60°C and 80°C. Those whose optimal growing condition temperature is above 80°C are referred to as hyperthermophiles (Santos & Da Costa, 2002). Due to their ability to grow and live under high temperatures, thermophiles usually possess high metabolism and more stable, chemically and physically, cellular components, such as enzymes, proteins, and nucleic acids (Haki & Rakshit, 2003). Thus, thermostable organisms are good candidates for research studies on ribosomal catalysis and *in vitro* reconstruction. The stability of the thermophilic bacteria ribosomes and their rRNA at high temperatures allows them to maintain catalytic activity and intricate structure in harsh conditions.

1.3.2 Ribosomal catalysis studies with thermostable bacterial ribosomes

The instability of reconstructed *E. coli* ribosomes made researchers look for a more stable organism that can be used to further investigate ribosomal catalysis. Thus, studies shifted towards thermostable bacteria ribosomes. In 1992, the 50S subunit, isolated from the thermophilic bacterium *Thermus aquaticus,* remained 80% active after treatment with proteinase K, SDS and phenol extraction, knowing that almost all the proteins were removed (95% of the TP) (Noller *et al*., 1992). At the same time, the same treatment of the *E. coli* 50S subunit, which has also removed all proteins, leads to the wholly abolished peptidyl transferase activity (Noller *et al*., 1992). This study supported the idea that peptidyl transferase activity of the ribosome primarily lies on the 23S ribosomal RNA.

Here again comes the question of the essential components needed for a successful ribosomal reconstruction. The *E. coli* 50S cannot be reconstructed to be functional only from its *in vitro*

transcribed (IVT) 23S rRNA and TP50, which were thought to be a problem of lacking some crucial 23S rRNA modifications present in native 23S rRNA and introduced post-transcriptionally (Green & Noller, 1996). In comparison, studies with thermophilic bacteria such as *Thermus aquaticus* and *Bacillus stearothermophilus* show that functional 50S subunit can be reconstructed from IVT 23S rRNA and TP50 without any additional modifications of the components (Khaitovich *et al*., 1999) (Green & Noller, 1999). These studies also noted that 50S subunits, reconstructed from the *in vitro* transcribed 23S rRNA and TP50, require the presence of 5S rRNA for activity.

1.3.3 *Thermus thermophilus* as a model organism for peptidyl transferase activity studies

Thermus thermophilus (*T.t.*) is a gram-negative, aerobic bacteria belonging to the phylum *Deinococcus-Thermus* (Ohtani *et al*., 2010). It is a highly thermophilic organism with an optimal growth temperature of around 65°C – 75°C, and it was first isolated from a hot spring in Izu, Japan. *Thermus thermophilus* makes a good candidate for studying the peptidyl transferase activity not only because its ribosomes are thermostable but also because it is a closely related speech to *Thermus aquaticus*, whose ribosomes were proven to catalyze peptidyl transferase activity and perform fragment reaction with only 5% of TP50 and without any post-transcriptional modifications, as it was previously mentioned in 1.3.2 section. In 2006, the first crystal structure of *T.t.* 70S ribosome in a functional state was published, and later, in 2017, SHAPE (Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension) analysis of the protein-free *Thermus thermophilus* 23S rRNA was made (Korostelev *et al*., 2006) (Lenz *et al*., 2017). SHAPE analysis showed 23S rRNA could adopt its near-native state in the presence of $Mg2^{+,}$ and crystal structure shows us to see which proteins are the closest to the PTC, giving the idea that they might be a primary candidate needed for effective catalysis of the peptidyl transferase.

1.4 MINIMAL COMPONENTS ESSENTIAL FOR THE PEPTIDYL TRANSFERASE ACTIVITY

1.4.1 Defining minimal components for the peptidyl transferase activity

Defining which components are essential for the peptidyl transferase activity is still challenging. Despite many earlier published studies, there is still no exact evidence that 23S ribosomal RNA alone can catalyze an active protein synthesis reaction. Thus, as has been already mentioned, reconstruction of a functional 50S ribosomal subunit from IVT 23S rRNA has shown to require at least some set of ribosomal proteins. In the case of *E. coli,* some post-transcriptional modifications are needed. On the other hand, there have been no previously published studies with *Thermus thermophilus*, which raises the question of whether the active reconstituted 50S subunit can be achieved only with IVT rRNAs and TP50, as it has been shown for other thermostable bacteria such as *Thermus aquaticus* and *Bacillus stearothermophilus* (Khaitovich *et al*., 1999). In the case of *T.t*., it is a subject of further investigation whether its peptidyl transferase activity can be achieved via 50S reconstitution with IVT rRNAs and TP50 without any post-transcriptional modifications.

Identifying minimal components needed for peptidyl transferase activity is also relevant in the search for the minimal ribosome. This concept is closely related to the protoribosome, which evolved from the idea of how the first ribosome was structured. The protoribosome, a small RNA-like pocket segment, is an evolutionary precursor for the modern ribosome. In 2022, researchers designed and synthesized some RNA constructs, including PTC, which mimicked the possible structure of the protoribosome (Bose *et al*., 2022). The ability of this construct to catalyze peptide bond formation supported the idea that protoribosome is believed to be a hypothesized ancient RNA-based machinery.

Another similar concept is the minimal synthetic ribosome. This idea is based on the proposal that the smallest number of synthesized components is needed to catalyze peptidyl transferase activity of the ribosome effectively. These components are usually isolated from the ribosome and *in vitro* transcribed for the further *in vitro* reconstitution of the 50S ribosomal subunit, which includes PTC.

1.4.2 Previous work with *Thermus thermophilus* in the Forster lab

Previously, experiments in the Forster lab showed that 50S reconstructed with *in vitro* transcribed ribosomal RNAs, 23S and 5S, and extracted TP50 from *T.t.* had a minimal detectible peptidyl transferase activity. The question is raised whether post-transcriptional modifications matter, in the case of this bacteria species, or a more sensitive assay should be established to detect any peptidyl transferase activity of the *in vitro* reconstituted *T.t.* 50S ribosomal subunit.

2 THE AIMS OF THE THESIS

The aims of this work are:

- Create a minimal *Thermus thermophilus* ribosome from its *in vitro* transcribed 23S rRNA, 5S rRNA and total protein content of the 50S ribosomal subunit.
- Assaying peptidyl transferase activity with the constructed minimal ribosome, using f[^{35}S]Met-tRNA^{fMet}, as a substrate for the fragment reaction.
- Test if minimal *T.t.* ribosome can be constructed without any post-transcriptional modifications.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Ribosomal components

Thermus thermophilus 23S rRNA was cloned into pUC19 (the pUC19_23S plasmid) and supplied by Prof. Loren Williams. Another student, Sadat Chowdhury, extracted the total protein content of the *T.t.* 50S and the 50S subunit itself. *E. coli's* rRNAs and TP50 were prepared by Letian Bao, as well as [^{3}H]fMet-tRNA substrate. T7 RNA polymerase, used for the *in vitro* transcriptions, was made by Raymond Fowler. *Thermus thermophilus* IVT 5S rRNA was made by a previous student who worked on this project – Julia Langer. *E. coli's* IVT 5S rRNA and 23S rRNA were prepared by Letian Bao.

3.1.2 Restriction digestion of 23S-encoding plasmid

Plasmids encoding for *Thermus thermophilus* 23S rRNA need to be linearised via enzyme digestion with the corresponding enzyme FastDigest HindIII before further *in vitro* transcription. The digestion mixture also contained 10X FD buffer and H_2O. The digestion mix was incubated at 37°C for 1 hour, followed by a phenol-chloroform extraction and overnight ethanol precipitation. Thermo Fisher Scientific supplied both FastDigest buffer and HindIII enzyme.

3.1.3 Phenol-chloroform extraction

To purify the desired product phenol-chloroform extraction method was used. DNA or RNA is extracted from the previous reaction mix by adding the same volume of (1:1 ratio) phenol (pH 8.0) / chloroform mixture (1:1 ratio) to the same reaction tube. The sample is later vortexed and then centrifuged at 14 000 rpm for 30 seconds. Later, the aqueous (top) phase is collected in the new tube, where one volume of chloroform is added. The sample again vortexed and spined at 14 000 rpm for 30 seconds. This step is repeated twice to remove all traces of phenol. The aqueous phase obtained from the second chloroform extraction round now contains the purified product of interest. It can be stored at -20°C or -80°C, depending on the product's origin, until further use.

3.1.4 Ethanol precipitation

Ethanol precipitation is performed right after the phenol-chloroform extraction to remove traces of chloroform and concentrate purified nucleic acids. $1/10^{th}$ of 3 M sodium acetate (pH 5.4) was measured from the exact volume of previously purified nucleic acids mixed with them. Later, 2.5X volumes of ice-cold 95% ethanol measured from the previously gained mixture were added. The sample was incubated at -20°C overnight and centrifuged at 14 000 rpm for 30 minutes at 4°C the following day to pellet the precipitate. The supernatant was removed by pipetting, and the pellet was rinsed with 500 µl ice-cold 70% ethanol. The sample was then centrifuged for 5 minutes at 14 000 rpm at 4°C. Ethanol was removed, and the pellet was air dried at 37°C. After drying, the pellet was resuspended in 50 µl ddH2O and stored at -20°C or -80°C.

3.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to ensure the desired product was achieved. A small 0.8% agarose gel was cast using 0.4 grams of agarose, 50 ml of 0.5X TAE buffer and 5 µl SybrSafe. First, agarose was mixed with TBE buffer and heated in a microwave. Once the solution cooled down and became touchable, SybrSafe was added, and the mixture was poured into a casting stand to solidify. TBE buffer was prepared from 1M Boric acid, 1M Tris base, 0.02M EDTA solution and H_20 and filled into the desired volume.

DNA samples were mixed with 6X DNA loading dye (Thermo Fisher Scientific) and run alongside 6X DNA loading buffer (Thermo Fisher Scientific). RNA samples were mixed with 2X RNA loading dye and ran with the same 6X DNA loading buffer. The gel electrophoresis takes place in 0.5 TAE buffer. Agarose gels were run at 100 V for 30-45 minutes and later visualized under UV light.

3.1.6 *In vitro* transcription and purification of *T.t.* 23S rRNA

In vitro transcription (IVT) allows the direct synthesis of RNA molecules from the template DNA of any sequence and size range (Beckert & Masquida, 2011).

23S rRNA was transcribed from the corresponding plasmid. The reaction mixture contained 1M of 5 TMSD buffer, 4.4 mM of each ATP, GTP, UTP and CTP mixes, 1 µM of 50 DTE reagent, 2% of Triton-x 100 solution, 1.25 µM of the Promega T7 RNA polymerase and

linearized DNA template. The reaction mix was incubated at 37°C for 3 hours and later purified via phenol-chloroform extraction, followed by the ethanol precipitation.

The desired amount of purified mixture was later mixed with 2X RNA loading dye (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanole FF 0.5 mM EDTA) and loaded on the agarose gel as it was described above to ensure that *in vitro* transcription was successful.

3.1.7 *In vitro* reconstitution of *T.t.* 50S RNA

Reconstitution of *T.t.* 50S RNA was made using IVT 23S rRNA and IVT 5S rRNA alongside naturally extracted *T.t.* TP50. The reconstruction mix contained 20 mM of Tris-HCl buffer, 400 mM of NH_4Cl, 10 mM $MgCl_2$, 6 mM of spermidine, 5 mM BME, and 0,2 mM of EDTA (Ethylenediaminetetraacetic acid). Ribosomal RNA was added in a concentration of 1 A_{260} unit each and combined with 1.2 equivalent units (e.u.) of TP50. H_2O was added up to the final volume, 40 µl. The reaction was mixed in the PCR tube and then loaded into the Thermocycler for the 3-step reaction. The first step included incubating the reaction mixture at 44°C for 20 minutes. After that, the concentration of Mg^{2+} was adjusted to 20 mM to allow it to fold correctly (Kognole & Mackerell, 2020). After that, the program proceeded with the second step: incubating the reaction at 50°C for 90 minutes. The program cycle ends with cooling down the sample at 4°C.

3.1.8 *In vitro* reconstitution of *E. coli* 50S

Reconstitution of *E. coli* 50S is aimed to be done with IVT 23S rRNA, IVT 5S and the total protein content of the 50S subunit. Each reconstruction mix contained 20 mM of Tris-HCl buffer, 400 mM of NH_4Cl, 10 mM $MgCl_2$, 6 mM of spermidine, 5 mM BME, and 0,2 mM of EDTA (Ethylenediaminetetraacetic acid). Ribosomal RNA was added in a concentration of 1 A_{260} unit each and combined with 1.2 equivalent units (e.u.) of TP50. H_2O was added up to the final volume, 40 µl. The reaction was mixed in the PCR tube and then loaded into the Thermocycler for the 3-step reaction. The first step included incubating the reaction mixture at 44°C for 20 minutes. After that, the concentration of Mg^{2+} was adjusted to 20 mM to allow it to fold correctly (Kognole & Mackerell, 2020). After that, the program proceeded with the second step: incubating the reaction at 50°C for 90 minutes. The program cycle ends with cooling down the sample at 4°C.

3.1.9 Preparation of the f[^{35}S]Met-tRNA^{fMet} substrate

Substrate f[^{35}S]fMet-tRNA^{fMet} was prepared by Prof. Forster. Reaction mix contained 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM PEP, 3 mM BME, 0.005 g/l Ppiase, 0.05 g/l PK, 0.002 unit MK, hot Met and cold Met at 160 µM, 150 µM tRNA-fMet, 1.1 u MetRS, 2.84 µM FMT, 1mM CHO-THF and H_2O up to the final volume, 1000 µl.

3.1.10 Fragment reaction with *in vitro* reconstituted *Thermus thermophilus* 50S

Three samples were prepared for each fragment reaction experiment. First, the main reaction mix contains previously made *in vitro* reconstituted 23S rRNA at a whole volume of the reconstruction reaction together with IVT 5S rRNA and naturally extracted *T.t.* TP50. The second sample contained naturally extracted *Thermus thermophilus* 50S ribosomal subunit, used as a positive control of the fragment reaction. The last reaction mix contained no structural ribosomal parts and was used as the negative control. Each sample included 50 mM of the Tris-HCl (pH 7.9), 400 mM of KCl, 60 mM $MgCl_2$, 1 mM of puromycin, 0,5 µM of f[^{35}S]Met-tRNA^{fMet} and H_2O up to the 60 µl. Then, to start the reaction, methanol was added to the partial volume of each reaction at a percentage of 33,3% of the total volume, which was 90 µl for each mix. Later, all samples were placed at 37°C in the heat block for 1-hour incubation. Then, 45 µl of 2M KOH was added into each reaction tube, and the 37°C incubation proceeded for another 5 minutes. The next step was the addition of 13 µl of the formic acid (FA) to each of the tubes. After quick vortexing, all the reaction mixes were placed on ice for 5 minutes, and then 15 minutes of spin was performed at 4°C at a maximum speed (14 000 RPM). After that, all reactions were transferred into separate HPLC tubes.

3.1.11 Fragment reaction with *in vitro* reconstituted *E. coli* 50S

Three various samples were prepared for each fragment reaction experiment. First is the main reaction mix, containing previously made *in vitro* reconstituted rRNA at a whole volume of the reconstruction reaction and naturally extracted TP50. The second sample contained a naturally extracted *E. coli* 50S ribosomal subunit, used as a positive control of the fragment reaction. The last reaction mix contained no structural ribosomal parts and was used as the negative control. Each sample included 50 mM of the Tris-HCl (pH 7.9), 400 mM of KCl, 60 mM $MgCl_2$, 1 mM of puromycin, 0,5 µM of [^{3}H]fMet-tRNA and H_2O up to the 60 µl. Then, to start the reaction,

methanol was added to the partial volume of each reaction at a percentage of 33,3% of the total volume, which was 90 µl for each mix. Later, all samples were placed at 37°C in the heat block for 1-hour incubation. Then, 45 µl of 2M KOH was added into each reaction tube, and the 37°C incubation proceeded for another 5 minutes. The next step was the addition of 13 µl of the formic acid (FA) to each of the tubes. After quick vortexing, all the reaction mixes were placed on ice for 5 minutes, and then 15 minutes of spin was performed at 4°C at a maximum speed (14 000 RPM). After that, all reactions were transferred into separate HPLC tubes.

3.1.12 HPLC analysis

A reverse-phase HPLC (C18 column) assay was carried out at 0°C overnight. For the HPLC, two different buffers were used – 60% of the buffer A and 40% of the buffer C. Buffer A consists of 90% H_2O and 10% MeOH. Buffer C was made from 10% H_2O and 90% MeOH.

3.2 RESULTS AND DISCUSSION

3.2.1 *In vitro* transcription of *T.t.* 23S rRNA

There were several attempts before the first successful *in vitro Thermus thermophilus* 23S rRNA transcription was achieved. The first attempts of the IVT reactions with a previously digested 23S RNA plasmid template showed no defined product on the agarose gel (Figure 6B). To find out the reason for these failures, it was decided to carry out the IVT with another DNA template, tProB, so as not to waste much of the template. Two reaction mixes were prepared, one with the new DNA template and several components previously used in unsuccessful *T.t.* 23S RNA *in vitro* transcription experiments. The second reaction mix contained a new bunch of the components (NTPs – ATP, CTP, UTP and GTP, mixed, and T7 RNA polymerase, described in the 3.1.6 section) and the same tProB plasmid. This test showed that only the reaction, containing a new set of the initial IVT components, was successful. The main hypotheses were that previously used T7 RNA polymerase was not active due to some contamination, and old NTPs activity could be inhibited entirely due to their degradation over time.

After the control IVT reaction was done successfully, it was decided to proceed with the new set of the reaction components and try them out with the 23S RNA template on a small scale to test the hypothesis that there might be some impurities in the template, leading to inability to perform the successful *in vitro* transcription. Small scale IVT reaction had a total volume of 200.5 µL. This attempt was successful, and there was a detectable band of around 3000 nucleotides, which corresponded to the size of the desired product – 23S rRNA of *Thermus thermophilus* (Figure 6A). Several large-scale *in vitro* transcriptions of the *T.t.* 23s rRNA were done to obtain enough material for further *in vitro* reconstitution. Phenol-chloroform extraction and ethanol precipitation were performed after each successful IVT reaction to purify and concentrate the formed 23S rRNA product for further use. Final products were again tested on the agarose gel. Purified *T.t.* 23S rRNA was snap-freezed with the liquid nitrogen and stored at -80°C until further use.

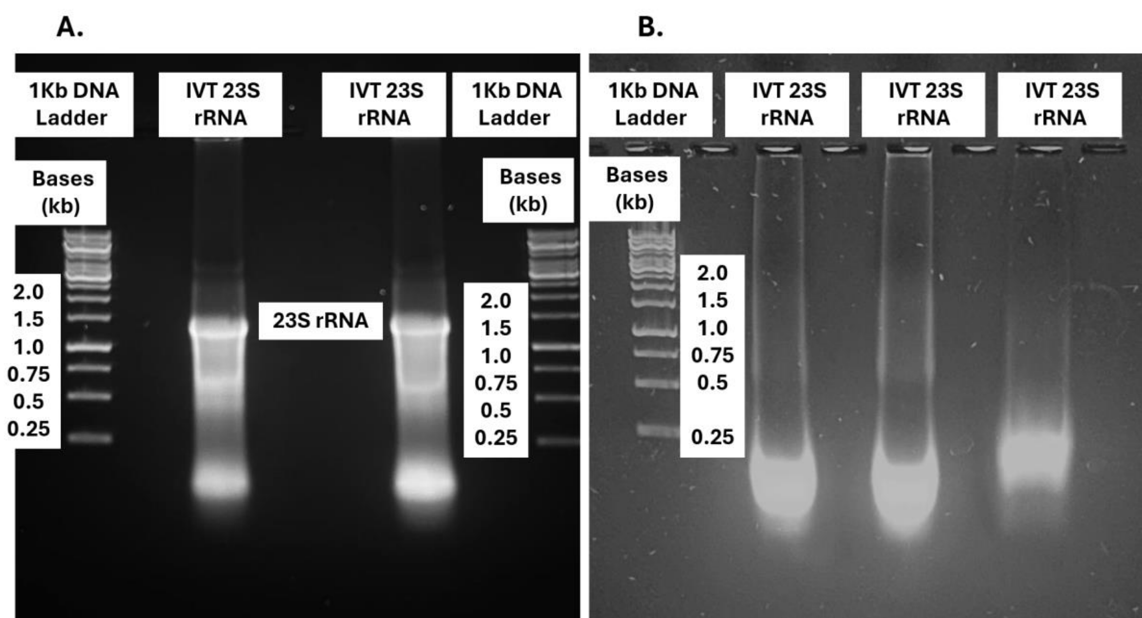

Figure 6. Agarose gels showing results from both successful and unsuccessful IVT 23S rRNA attempts. A. Agarose gel showing successful *in vitro* transcription of T.t. 23S rRNA. Desired product has a size of 3000 nucleotides, which corresponds to the appearance of the band, which is in size close to the 1500 bp band of the DNA ladder. **B.** Agarose gel showing results of an unsuccessful attempt of *in vitro* transcription of T.t. 23S rRNA. There is no formation of a band, corresponding to the formation of a desired product.

3.2.2 Assaying peptidyl transferase activity of the reconstructed *Thermus thermophilus* 50S ribosomal subunit

Previous studies with reconstructed *Thermus thermophilus* 50S subunits in the Forster lab were carried out with the different fragment reaction substrate, f[^{3}H] Met-tRNA^{fMet}. Results of these analyses depict almost no detectable amount of the f[^{3}H]Met-puromycin product due to the 50S *T.t.* reconstitution with the IVT rRNAs and total protein component of the 50S subunit. These results were unexpected because of the published similar *in vitro* reconstitution of the 50S thermostable bacteria subunit from the very closely related species also made with the IVT rRNAs, and TP50 shows peptidyl transferase activity around 25% of the native subunit (Khaitovich *et al.*, 1999). This raises the question of whether the reconstructed 50S subunits were inactive due to the lack of post-transcriptional modifications or a minimal amount of the product formed during this fragment reaction. However, the current HPLC assay is not sensitive enough to measure it. Another hypothesis proposed is to try to add a different, higher concentration of Mg^{2+}, as it might help to make the reconstructed 50S more stable and influence better protein folding. A more sensitive HPLC assay for the fragment reaction was established to test these hypotheses. This time, a new substrate for the fragment reaction, f[^{35}S]Met-tRNA^{fMet}, was prepared with a new radiolabel [^{35}S]. It was thought that

changing the [^{3}H] to the [^{35}S] might allow us to detect very small amounts of the fragment reaction product, as [^{35}S] can emit high-energy beta particles, which lead to more sensitive detection. With that, a higher concentration of $MgCl_2$ was used – 10 mM, as in previous experiments, its concentration was 4 mM.

Reconstitution of the *T.t.* 50S subunit was made with both *in vitro* transcribed 23S and 5S rRNA and the total protein component of the 50S subunit, following previous studies with thermostable bacteria species (Khaitovich *et al*., 1999). Reconstitution was done in the presence of $MgCl_2$, as Mg^{2+} has been proven to positively affect the stability of the ribosomal subunit by proper folding (Fischer *et al*., 2018). For this assay, 4 fragment reactions were prepared: the negative control sample, which did not contain any of the functional ribosomal components; the positive control sample with functional 50S ribosomal subunit extracted from *E.coli*, as it has been previously proven to be able of catalyzing the fragment reaction; and two samples testing out the ability to catalyze the peptidyl transferase activity of *in vitro* reconstituted *Thermus thermophilus* 50S ribosomal subunit. Two *in vitro* reconstitution reactions were prepared with previously made IVT 23S rRNA, 5S rRNA and total protein component of 50S subunit. These two samples varied in IVT 23S rRNA concentration – 202 A_{260}/ml in the second reaction and 279 A_{260}/ml in the first. All reactions were carried out with the f[^{35}S]Met-tRNA^{fMet} substrate.

On the HPLC chromatogram, corresponding to the negative control sample, only two peaks are visible in Region 1 (marked in red) (Figure 7). The red region and its peaks correspond to the substrate f[^{35}S]Met-tRNA^{fMet}. The absence of the other visible peaks in Region 2 indicates that there is nothing to detect, corresponding to the fact that no product was formed during the fragment reaction.

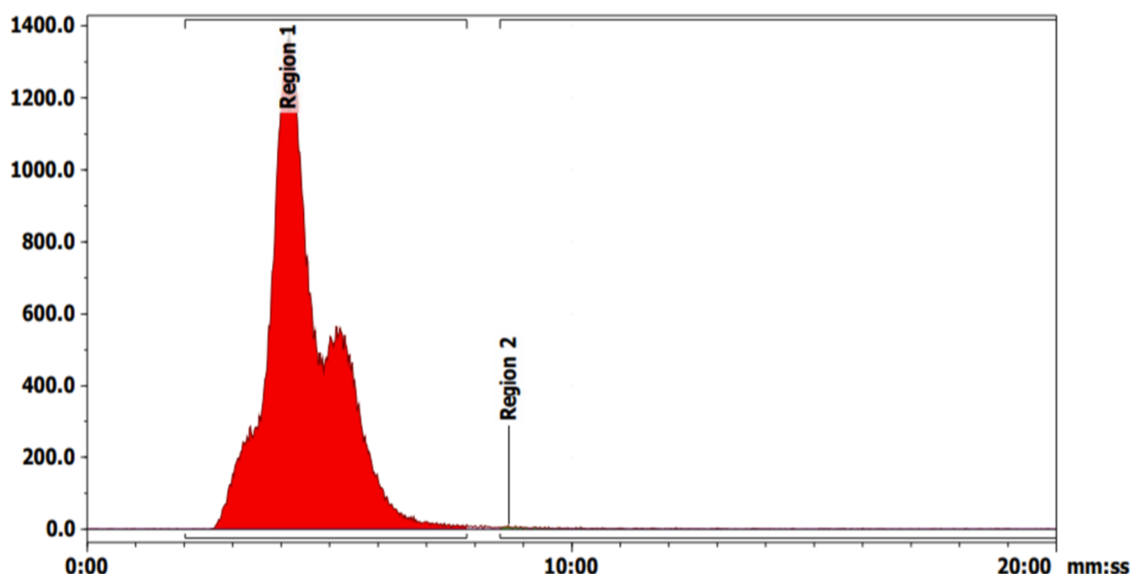

Figure 7. Negative control sample for the fragment reaction with f[^{35}S]Met-tRNA^{fMet} substrate. The red-coloured region corresponds to the presence of the f[^{35}S]Met. This sample contains all the components for the fragment reaction except for any functional 50S particles. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

In comparison, two regions are visible on the HPLC chromatogram corresponding to the positive control sample with extracted *E. coli* 50S ribosomal subunit (Figure 8). The first peak in Region 1 corresponds to the substrate f[^{35}S]fMet-tRNA^{fMfet}, and the peak in Region 2 corresponds to the formed product f[^{35}S]Met-puromycin. The reaction yield can be calculated by comparing the peaks corresponding to the formed product and initial substrate. The positive control chromatogram shows that the reaction with *E. coli* 50S yielded 77% of the total area.

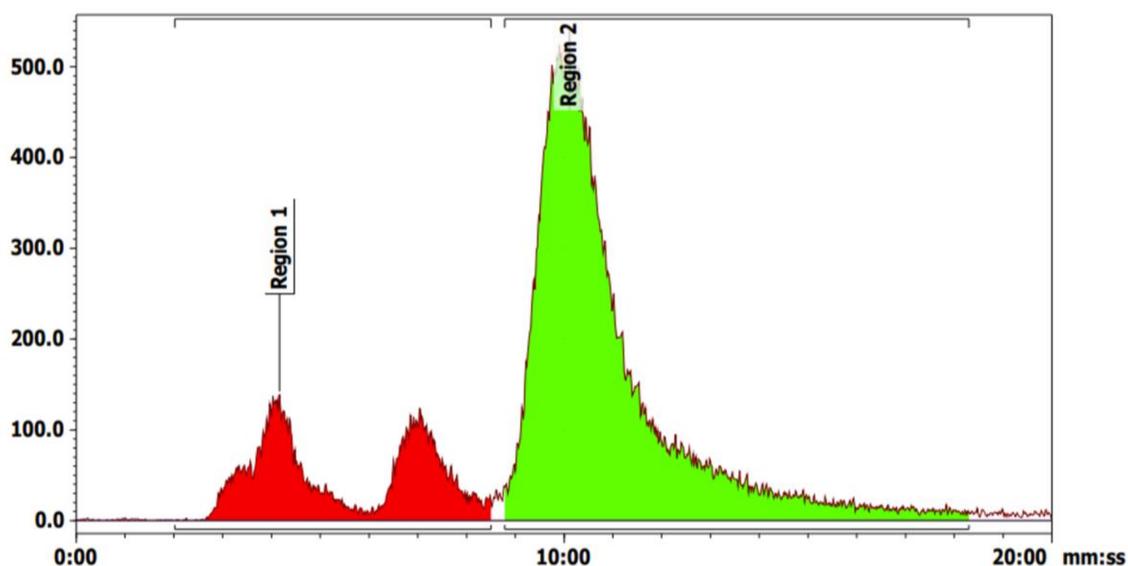

Figure 8. Positive control sample with *E. coli* 50S for the fragment reaction with f[^{35}S]Met-tRNA^{fMet} substrate. Region 1, coloured in red, corresponds to the presence of the f[^{35}S]Met. Region 2, coloured in green, shows the presence of the f[^{35}S]Met-puromycin product formed as a result of a successful fragment reaction. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

Both fragment reactions with reconstituted 50S ribosomal subunit of *Thermus thermophilus* show the appearance of a small amount of the formed f[^{35}S]Met-puromycin product. Figure 9, describing the first fragment reaction with *T.t.* IVT 23S rRNA at the concentration of 279 A_{260}/ml, shows that Region 2 accounts only for 5% of the total area. In contrast, the other 94% corresponds to the initial substrate. The second reaction resulted in an expected smaller amount of the f[^{35}S]Met-puromycin product, as the concentration of the 23S rRNA used in the reconstitution of the 50S ribosomal subunit, which peptidyl transferase activity was tested with the fragment reaction, was smaller than in the concentration of the IVT 23S rRNA used for the first 50S reconstitution reaction - 202 A_{260}/ml (Figure 10). Region 2 accounts for an even smaller percentage of the total area – 3%.

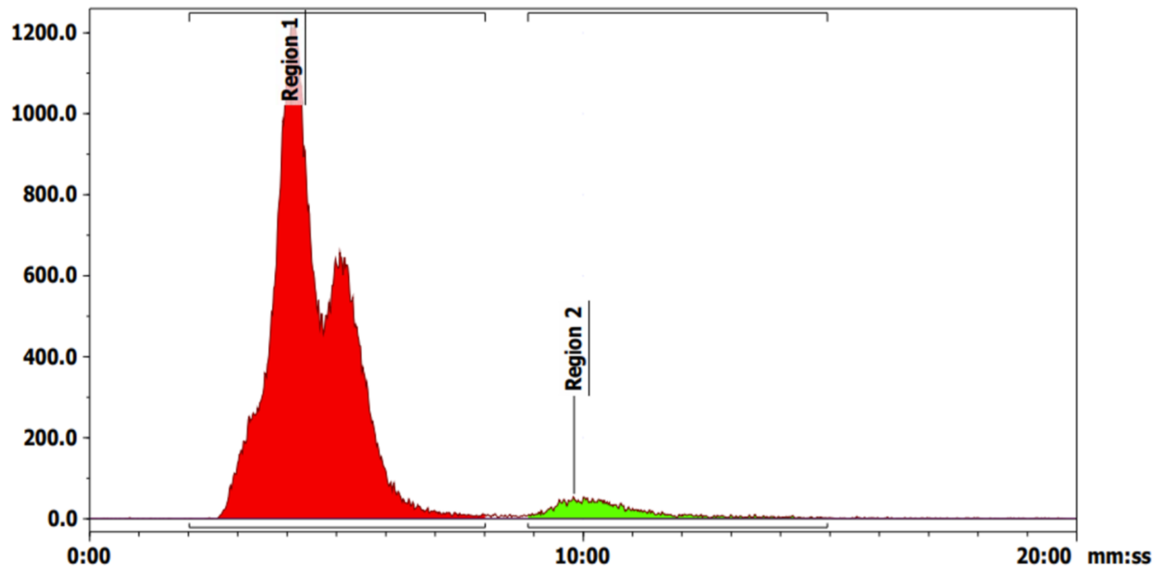

Figure 9. Reconstructed *T.t.* 50S with IVT rRNAs and TP50 (First reaction). Red-coloured Region 1 corresponds to the presence of the f[^{35}S]Met. Region 2, highlighted in green, corresponds to the formed during the fragment reaction f[^{35}S]Met-puromycin product. This reaction uses a 50S *T.t.* subunit, reconstructed with the IVT 23S rRNA with a 279 A_{260}/ml concentration. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

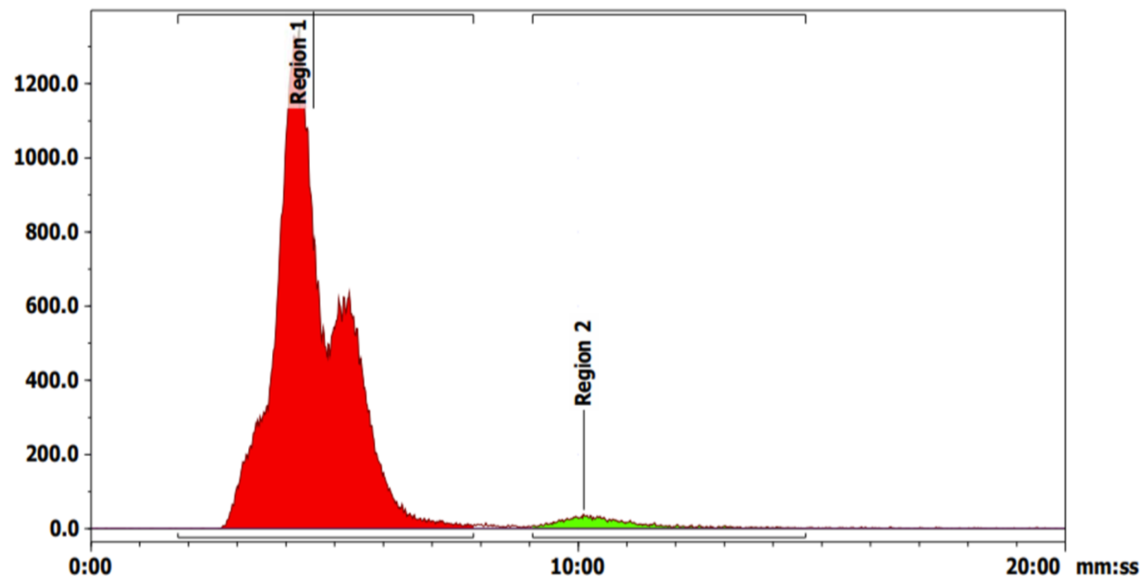

Figure 10. Reconstructed *T.t.* 50S with IVT rRNAs and TP50 (Second reaction). Red-coloured Region 1 shows the presence of the f[^{35}S]Met, while Region 2, highlighted in green, corresponds to the amount of the f[^{35}S]Met-puromycin product formed due to the fragment reaction. *T.t.* 50S subunit, in which peptidyl transferase activity was tested in this experiment, was reconstructed with IVT 23S rRNA, whose working concentration was 202 A_{260}/ml. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

The result of this experiment shows that the presence of the new substrate with the new radiolabel f[^{35}S]fMet-tRNA^{fMet} allows a more sensitive assay, leading to the detection of a smaller amount of the formed fragment reaction product – f[^{35}S]Met-puromycin. Despite the

usage of more $MgCl_2$ during the reconstitution and a new, more sensitive radiolabel [^{35}S], given results show that reconstructed from IVT rRNAs and TP50 *T.t.* 50S subunit does not exhibit the proposed functionality of the similarly reconstructed *T. aquaticus* 50S ribosomal subunit. Taking into account that both of these species are closely related, the obtained result of fragment reactions, showing that *Thermus thermophilus* reconstructed 50S is not as active as it was thought it should be, suggests that there might be a need for post-transcriptional modifications in order to achieve a higher peptidyl transferase activity. Another hypothesis that can be tried in the future is carrying out the same fragment reaction with various higher concentrations of Mg^{2+} and more concentrated IVTs. For that, the purification of IVT might be done slightly differently so as not to lose any of the samples in the purification process, or there might be an attempt to carry it out at a larger scale to achieve a higher yield of the *in vitro* transcription.

3.2.3 Assaying peptidyl transferase activity of the reconstructed *E. coli* 50S ribosomal subunit

The efficiency of the fragment reaction assay with f[^{35}S]Met-tRNA^{fMet} substrate and *in vitro* reconstituted 50S of the *Thermus thermophilus* was compared to the peptidyl transferase activity of the *in vitro* reconstituted from the naturally extracted ribosomal components *E. coli* 50S reaction with f[^{3}H]Met-tRNA^{fMet}. Three fragment reactions were carried out and tested with HPLC assay.

The first fragment reaction was carried out without functional ribosomal components and was used as a negative control. On the HPLC chromatogram, as with the negative control for the *T.t.* experiment, there is only one visible region – red-colored Region 1, corresponding to the presence of the f[^{3}H]Met (Figure 11).

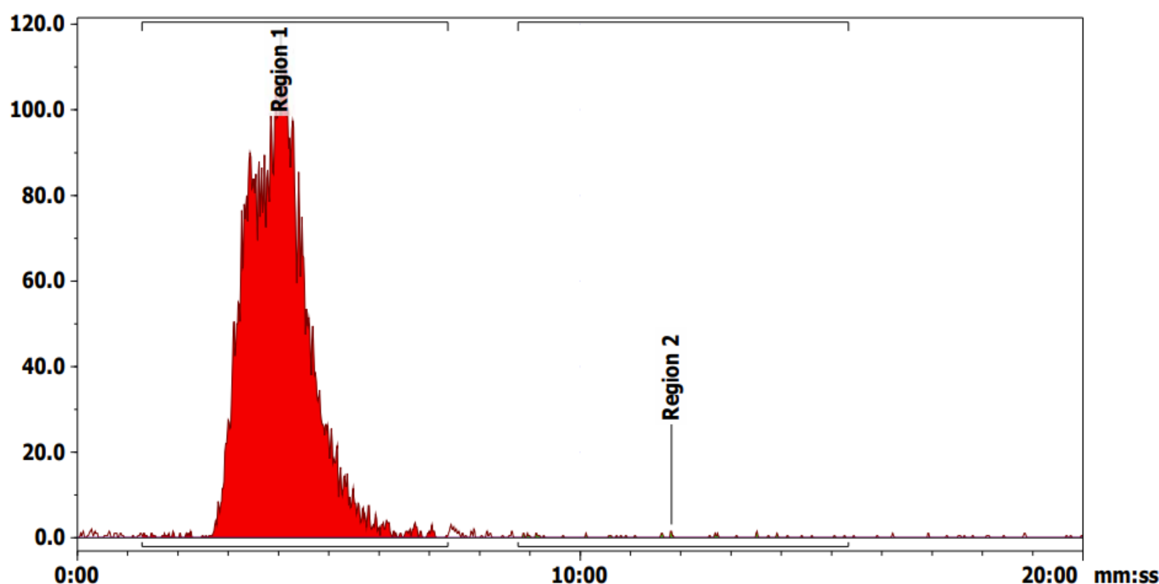

Figure 11. Negative control sample for the *E. coli* fragment reaction with f[^{3}H]Met-tRNA^{fMet}. Red-coloured Region 1 corresponds to the presence of the f[^{3}H]Met. This sample contains all the necessary components for the fragment reaction except for the functional ribosomal components. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

A positive control sample for this test was prepared with a naturally extracted *E. coli* 50S subunit. Again, as for the previous peptidyl transferase activity tests, the positive control sample represents that all components of the fragment reaction are active. In Figure 12, which demonstrates the chromatogram of the positive control sample, both Region 1 and Region 2 are visible. Unlike the test with f[^{35}S]Met-tRNA^{fMet}, substrate with [^{3}H] radiolabel allows the detection of a smaller amount of the formed f[^{3}H]Met-puromycin product formed as a result of the fragment reaction, indicating that the sensitivity of this assay is worse than the one with [^{35}S] radiolabel.

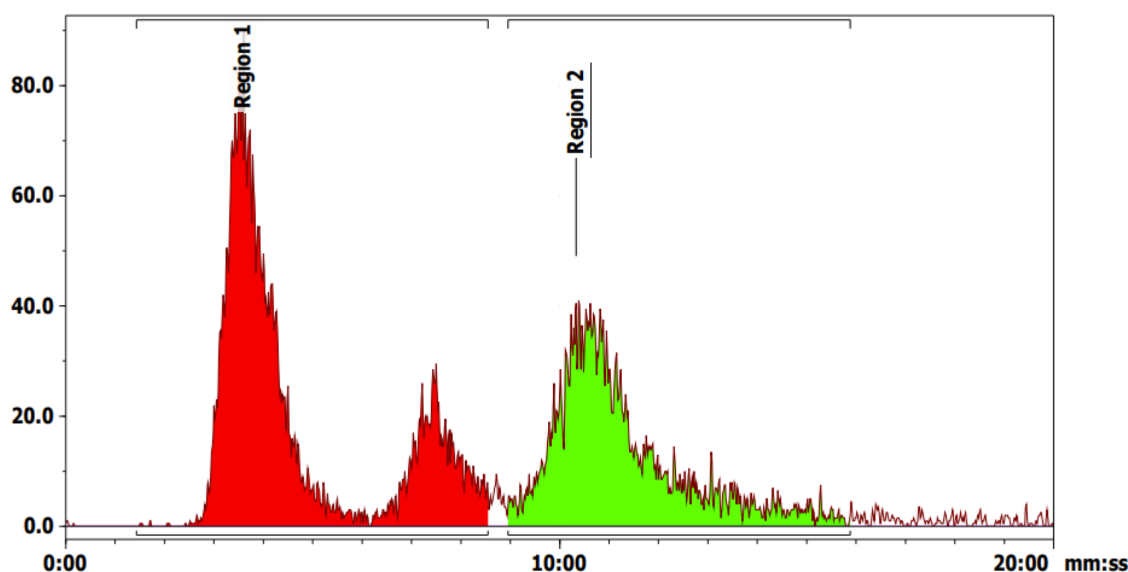

Figure 12. Positive control sample for the *E. coli* fragment reaction with f[^{3}H]Met-tRNA^{fMet}. Red-coloured region 1 corresponds to the presence of the f[^{3}H]Met. Green Region 2 corresponds to the amount of the f[^{3}H]-puromycin product formed because of the successful fragment reaction. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

E. coli 50S subunit was reconstructed from the previously naturally extracted rRNAs and total protein component of the 50S ribosomal subunit. As previously proven, functionally active 50S of *E. coli* can be achieved via *in vitro* reconstitution with naturally extracted 5S rRNA, 23S rRNA and TP50. There have not been any successful reconstructions with *in vitro* transcribed rRNAs stating the importance of the post-translational modifications for this organism's ability to catalyze peptidyl transferase activity. We decided to test how sensitive the fragment reaction assay with f[^{3}H]Met-tRNA^{fMet} would be for this species. The achieved result shows that a minimal amount of the f[^{3}H]Met-puromycin product formed during the fragment reaction, suggesting that there might be a couple of reasons why this happened (Figure 13). First, this result is evidence that a more sensitive assay should be used for these reactions, as even positive control samples, previously tested with the [^{35}S] radioactive label, show the worst fMet-puromycin product detection. Secondly, knowing that both fragment reaction components, fMet-tRNA and puromycin, are active, we can suggest that the reconstructed 50S ribosomal subunit had a deficient activity. Further plans can be carrying out new *in vitro* reconstitution reactions with higher concentrations of Mg^{2+} or even trying a new set of experiments with fresh naturally extracted 50S ribosomal components, and there might be an issue with protein degradation in this experiment. Last but not least, this fragment reaction should also be tested

with f[^{35}S]Met-tRNA^{fMet} substrate as it has been proven to be more sensitive to the smaller amount of the final product formed.

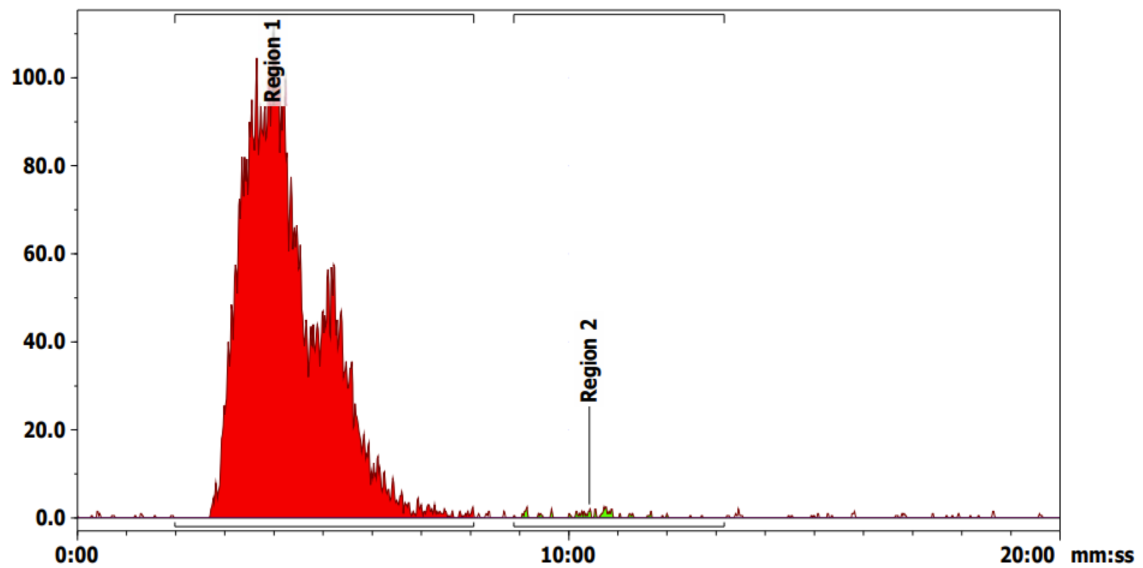

Figure 13. Fragment reaction with *E. coli* 50S reconstructed from naturally extracted ribosomal components and substrate f[^{3}H]Met-tRNA^{fMet}. Region 1, coloured in red, corresponds to the amount of the f[^{3}H]Met and Region 2, green, corresponds to the amount of the f[^{3}H]Met-puromycin product formed due to the fragment reaction. On the x-axis of the graph, time in minutes is depicted. On the y-axis of the graph, counts per second are shown.

SUMMARY

In this work, the *Thermus thermophilus* minimal ribosome was attempted to be constructed. For this, *in vitro* transcription of 23S ribosomal RNA was done to use it along with IVT 5S rRNA and TP50. To test the functional peptidyl transferase activity of the *in vitro* reconstituted *T.t.* 50S, a fragment reaction with radioactive fMet-tRNA was assembled. Knowing that the previous test in the Forster lab with reconstructed *Thermus thermophilus* 50S ribosomal subunit showed almost no detectable activity and was carried out in the presence of f[^{3}H]Met-tRNA^{fMet} substrate with radioactive label [^{3}H], the new substrate f[^{35}S]Met-tRNA^{fMet} with the new radioactive label [^{35}S] was prepared. This radiolabel is believed to give a more sensitive detection even of a tiny amount of the f[^{35}S]Met-puromycin product formed during the fragment reaction due to its higher energy particle emission.

The HPLC assay results show that in the presence of [^{35}S] substrate and a slightly higher concentration of Mg^{2+}, a small amount of the f[^{35}S]Met-puromycin product is detectable. There are two fragment reactions with reconstructed 50S *T.t.*, varying in only the concentration of the IVT 23S rRNA used. The activity of the fragment reaction, measured in the reaction sample prepared with the higher concentration of IVT 23S rRNA, is slightly higher than the 50S subunit reconstructed with a less concentrated IVT 23S rRNA. Then, comparing these results to the previously investigated peptidyl transferase activity of the 50S ribosomal subunit of the *Thermus aquaticus*, reconstructed from the IVT rRNAs and TP50, our results appeared to be surprisingly different, as these two species are closely related, and we thought to detect at least similar activity of the reconstructed 50S. Peptidyl transferase activity of the reconstructed *T.t.* 50S ribosomal subunit appears to be very low, raising the question of whether the post-transcriptional modification actually matters for these bacterial speeches.

The sensitivity of the f[^{35}S]Met-tRNA^{fMet} substrate was compared to the sensitivity of the fragment reaction assay with f[^{3}H]Met-tRNA^{fMet} with 50S ribosomal subunit, reconstructed from the naturally extracted components of the *E. coli*. These reconstructed *E. coli* 50S were proven to be functionally active during fragment reactions in previously published research. Interestingly, our fragment reactions show almost no detectable activity of the reconstructed with naturally extracted rRNAs and TP50 *E. coli* 50S assayed with f[^{3}H]Met-tRNA^{fMet}, suggesting a more sensitive assay should be carried out. At the same time, a comparison of both reactions' positive control, fragment reactions carried out with the same extracted *E. coli* 50S subunit and different fMet-tRNA shows that [^{35}S] radiolabel in the fMet substrate allows one to perform a more sensitive assay and detect more fMet-puromycin product than when using the fMet substrate with [^{3}H] radiolabel.

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REFERENCES

Alonso, D., & Mondragón, A. (2021). Mechanisms of catalytic RNA molecules. *Biochemical Society Transactions*, *49*(4), 1529–1535. https://doi.org/10.1042/BST20200465

Aviner, R. (2020). The science of puromycin: From studies of ribosome function to applications in biotechnology. *Computational and Structural Biotechnology Journal*, *18*, 1074–1083. https://doi.org/10.1016/J.CSBJ.2020.04.014

Ban, N., Nissen, P., Hansen, J., Moore, P. B., & Steitz, T. A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. *Science (New York, N.Y.)*, *289*(5481), 905–920. https://doi.org/10.1126/SCIENCE.289.5481.905

Bastide, A., & David, A. (2018). Interaction of rRNA with mRNA and tRNA in Translating Mammalian Ribosome: Functional Implications in Health and Disease. *Biomolecules*, *8*(4). https://doi.org/10.3390/BIOM8040100

Beckert, B., & Masquida, B. (2011). Synthesis of RNA by *in vitro* transcription. *Methods in Molecular Biology (Clifton, N.J.)*, *703*, 29–41. https://doi.org/10.1007/978-1-59745-248-9_3

Bocchetta, M., Xiong, L., & Mankin, A. S. (1998). 23S rRNA positions essential for tRNA binding in ribosomal functional sites. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(7), 3525–3530. https://doi.org/10.1073/PNAS.95.7.3525

Bose, T., Fridkin, G., Davidovich, C., Krupkin, M., Dinger, N., Falkovich, A. H., Peleg, Y., Agmon, I., Bashan, A., & Yonath, A. (2022). Origin of life: protoribosome forms peptide bonds and links RNA and protein dominated worlds. *Nucleic Acids Research*, *50*(4), 1815–1828. https://doi.org/10.1093/NAR/GKAC052

Catalanotto, C., Barbato, C., Cogoni, C., & Benelli, D. (2023). The RNA-Binding Function of Ribosomal Proteins and Ribosome Biogenesis Factors in Human Health and Disease. *Biomedicines*, *11*(11). https://doi.org/10.3390/BIOMEDICINES11112969

Choi, S. K., Olsen, D. S., Roll-Mecak, A., Martung, A., Remo, K. L., Burley, S. K., Hinnebusch, A. G., & Dever, T. E. (2000). Physical and Functional Interaction between the Eukaryotic Orthologs of Prokaryotic Translation Initiation Factors IF1 and IF2. *Molecular and Cellular Biology*, *20*(19), 7183. https://doi.org/10.1128/MCB.20.19.7183-7191.2000

Dohme, F., & Nierhaus, K. H. (1976). Total reconstitution and assembly of 50 S subunits from Escherichia coli Ribosomes *in vitro*. *Journal of Molecular Biology*, *107*(4). https://doi.org/10.1016/S0022-2836(76)80085-X

Draper, D. E., & Reynaldo, L. P. (1999). RNA binding strategies of ribosomal proteins. *Nucleic Acids Research*, *27*(2), 381–388. https://doi.org/10.1093/NAR/27.2.381

Fischer, N. M., Polěto, M. D., Steuer, J., & Van Der Spoel, D. (2018). Influence of Na+ and Mg2+ ions on RNA structures studied with molecular dynamics simulations. *Nucleic Acids Research*, *46*(10), 4872. https://doi.org/10.1093/NAR/GKY221

Giuliano, M. G., & Engl, C. (2021). The Lifecycle of Ribosomal RNA in Bacteria. *RNA Damage and Repair*. https://www.ncbi.nlm.nih.gov/books/NBK594409/

Gopanenko, A. V., Malygin, A. A., & Karpova, G. G. (2015). Exploring human 40S ribosomal proteins binding to the 18S rRNA fragment containing major 3'-terminal domain. *Biochimica et Biophysica Acta*, *1854*(2), 101–109. https://doi.org/10.1016/J.BBAPAP.2014.11.001

Green, R., & Noller, H. F. (1996). *In vitro* complementation analysis localises 23S rRNA post-transcriptional modifications that are required for Escherichia coli 50S ribosomal subunit assembly and function. *RNA*, *2*(10), 1011. /pmc/articles/PMC1369433/?report=abstract

Green, R., & Noller, H. F. (1999). Reconstitution of functional 50S ribosomes from *in vitro* transcripts of Bacillus stearothermophilus 23S rRNA. *Biochemistry*, *38*(6), 1772–1779. https://doi.org/10.1021/bi982246a

Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, *89*(1), 17–34. https://doi.org/10.1016/S0960-8524(03)00033-6

Hampl, H., Schulze, H., & Nierhaus, K. H. (1981). Ribosomal components from Escherichia coli 50 S subunits involved in the reconstitution of peptidyltransferase activity. *Journal of Biological Chemistry*, *256*(5), 2284–2288. https://doi.org/10.1016/S0021-9258(19)69775-9

Khaitovich, P., Tenson, T., Kloss, P., & Mankin, A. S. (1999). Reconstitution of functionally active Thermus aquaticus large ribosomal subunits with *in vitro*-transcribed rRNA. *Biochemistry*, *38*(6), 1780–1788. https://doi.org/10.1021/BI9822473

KOGNOLE, A. A., & MACKERELL, A. D. (2020). Contributions and competition of Mg2+and K+in folding and stabilisation of the Twister ribozyme. *RNA*, *26*(11), 1704–1715. https://doi.org/10.1261/RNA.076851.120/-/DC1

Komili, S., Farny, N. G., Roth, F. P., & Silver, P. A. (2007). Functional specificity among ribosomal proteins regulates gene expression. *Cell*, *131*(3), 557–571. https://doi.org/10.1016/J.CELL.2007.08.037

Korostelev, A., Trakhanov, S., Laurberg, M., & Noller, H. F. (2006). Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. *Cell*, *126*(6), 1065–1077. https://doi.org/10.1016/J.CELL.2006.08.032

Lenz, T. K., Norris, A. M., Hud, N. V, & Dean Williams, L. (2017). *Protein-free ribosomal RNA folds to a near-native state in the presence of Mg 2+ †*. https://doi.org/10.1039/c7ra08696b

Martin, F., Ménétret, J. F., Simonetti, A., Myasnikov, A. G., Vicens, Q., Prongidi-Fix, L., Natchiar, S. K., Klaholz, B. P., & Eriani, G. (2016). Ribosomal 18S rRNA base pairs with mRNA during eukaryotic translation initiation. *Nature Communications*, *7*. https://doi.org/10.1038/NCOMMS12622

Monro, R. E. (1967). Catalysis of peptide bond formation by 50 s ribosomal subunits from Escherichia coli. *Journal of Molecular Biology*, *26*(1), 147–151. https://doi.org/10.1016/0022-2836(67)90271-9

Moore, P. B., & Steitz, T. A. (2011). The Roles of RNA in the Synthesis of Protein. *Cold Spring Harbor Perspectives in Biology*, *3*(11). https://doi.org/10.1101/CSHPERSPECT.A003780

Noller, H. F., Hoffarth, V., & Zimniak, L. (1992). Unusual resistance of peptidyl transferase to protein extraction procedures. *Science (New York, N.Y.)*, *256*(5062), 1416–1419. https://doi.org/10.1126/SCIENCE.1604315

Ohtani, N., Tomita, M., & Itaya, M. (2010). An Extreme Thermophile, Thermus thermophilus, Is a Polyploid Bacterium. *Journal of Bacteriology*, *192*(20), 5499. https://doi.org/10.1128/JB.00662-10

Ramakrishnan, V. (2002). Ribosome structure and the mechanism of translation. *Cell*, *108*(4), 557–572. https://doi.org/10.1016/S0092-8674(02)00619-0

Santos, H., & Da Costa, M. S. (2002). Compatible solutes of organisms that live in hot saline environments. *Environmental Microbiology*, *4*(9), 501–509. https://doi.org/10.1046/J.1462-2920.2002.00335.X

Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F., & Yonath, A. (2000). Structure of functionally activated small ribosomal subunit at 3.3 Å resolution. *Cell*, *102*(5), 615–623. https://doi.org/10.1016/S0092-8674(00)00084-2

Schulze, H., & Nierhaus, K. H. (1982). Minimal set of ribosomal components for reconstitution of the peptidyltransferase activity. *The EMBO Journal*, *1*(5), 609–613. https://doi.org/10.1002/J.1460-2075.1982.TB01216.X

Uzman, A., Lodish, H., Berk, A., Zipursky, L., & Baltimore, D. (2000). Molecular Cell Biology (4th edition) New York, NY, 2000, ISBN 0-7167-3136-3. *Biochemistry and Molecular Biology Education*, *29*(3), Section 1.2The Molecules of Life. https://doi.org/10.1016/S1470-8175(01)00023-6

Wilson, D. N., & Nierhaus, K. H. (2006). The E-site story: The importance of maintaining two tRNAs on the ribosome during protein synthesis. *Cellular and Molecular Life Sciences*, *63*(23), 2725–2737. https://doi.org/10.1007/S00018-006-6125-4

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