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Characterization of stringent response inhibitors: Thiostrepton and ppGpp analogues

Master's Thesis

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Contents:

Abbreviations

A-site	Aminoacyl site of the ribosome
aa-tRNA	aminoacyl-tRNA
CC	Conserved cysteine residues
CTD	C-terminal domain
EF-G	Elongation Factor G
EF-Ts	Elongation Factor Ts
EF-Tu	Elongation Factor Tu
E-site	Exit site of the ribosome
GAP	GTPase activating protein
GAR	GTPase center of the ribosome
GDI	Guanine-nucleotide Dissociation Inhibitors
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide Exchange Factor
Gpp	Guanosine pentaphosphate phosphohydrolase
IC	Initiation Complex
IF1	Initiation Factor 1
IF2	Initiation Factor 2
IF3	Initiation Factor 3
LB	Luria-Bertani broth medium
NTD	N-terminal domain of RelA
P-site	Peptidyl site of the ribosome
PIC	Preinitiation Complex
ppGpp	Guanosine 3', 5' – bispyrophosphate
RF1/2	Release Factor 1 or 2
RF3	Release Factor 3
RNAP	RNA polymerase
RRF	Ribosome Recycling Factor

RSHs	RelA/SpoT Homologue Proteins
SAH	Small Alarmone Hydrolase
SAS	Small Alarmone Synthetase
SCV	Small Colony Variant
SRL	Sarcin-Ricin Loop
TC	Ternary Complex
TLC	Thin Layer Chromatography
trGTPases	Translational GTPases
β-Me	β-mercaptoethanol

Abstract

The bacterial stringent response is one of the key mechanisms of bacterial adaptation to stress. It is mediated by ppGpp alarmone nucleotide that exerts its regulatory role by modulating the activity of numerous cellular enzymes, including RNA polymerase, translational GTPases, DnaG primase, and, as it was shown in our laboratory recently, of the very protein producing ppGpp – the ribosome-dependent stringent factor RelA. Since the stringent response is implicated in bacterial virulence, antibiotic tolerance and biofilm formation, identification of specific stringent response inhibitors is of great medicinal interest.

In this thesis I have investigated two types of potential stringent response inhibitors: antibiotic thiostrepton and ppGpp-analogues. The primary target of thiostrepton is translational GTPases. Inhibition of RelA by thiostrepton is well-documented but was never studied in detail, necessitating the current investigation. ppGpp-based RelA inhibitors are developed during the last five years, and in their current form have low efficiency and bioavailability.

By comparing thiostrepton effects on RelA and translational GTPase EF-G in the presence of wild type and thiostrepton-resistant 70S ribosomes, we conclude that inhibition of RelA by thiostrepton is non-specific and is mediated by the precipitation of the antibiotic at high concentrations. This suggests that modification of the thiostrepton scaffold is highly unlikely to yield an efficient and specific stringent response inhibitor. On the other hand, we demonstrated two ppGpp analogues – ppApp and 6-thio-ppGpp – are at least one order of magnitude more potent as any of the ppGpp-based RelA inhibitors characterized to date. These findings indicate that chemical modifications of the nucleotide base as a promising strategy for developing specific and potent stringent response inhibitors.

Introduction

1. The stringent response

Bacterial cells live in continually changing and often hostile environmental conditions. In order to survive, they must be able to quickly sense and adapt to changing surroundings. Complex regulatory mechanisms have evolved to modulate bacterial physiology when faced with nutritional limitation or other stress conditions [1]. To conserve energy under nutrient starvation bacteria employ a mechanism named 'the stringent response' – a pleiotropic physiological process leading to succession of growth and division and simultaneous upregulation survival responses, such as amino acid synthesis [1, 2]. These changes are accomplished by reallocation of cellular resources so that synthesis of DNA, stable RNAs, ribosomal proteins and membrane components is repressed, whereas production of proteins involved in the stress resistance, glycolysis and amino acid synthesis dramatically increases [3]. The stringent response is mediated by an alarmone molecule named guanosine 3', 5' - bispyrophosphate or ppGpp, which is, amongst other things, coordinating this reallocation of gene expression at the transcriptional level by binding to RNA polymerase (RNAP) and regulating certain promoters [4].

1.1 RSH proteins

The intracellular levels of the ppGpp are controlled by the RelA/SpoT Homologue proteins (RSHs), with the name coming from two RSH representatives in *Escherichia coli*: RelA and SpoT. Under the conditions of amino acid deprivation accumulation of deacylated tRNA in the ribosomal A-site is sensed by stringent factor RelA. Activated by the tRNA RelA catalyzes production of ppGpp (or pppGpp) using ATP and GDP (or GTP) as substrates (**Figure 1**). ppGpp acts as a primary regulatory nucleotide, as pppGpp after its synthesis is rapidly degraded to ppGpp by guanosine pentaphosphate phosphohydrolase (gpp) [5].

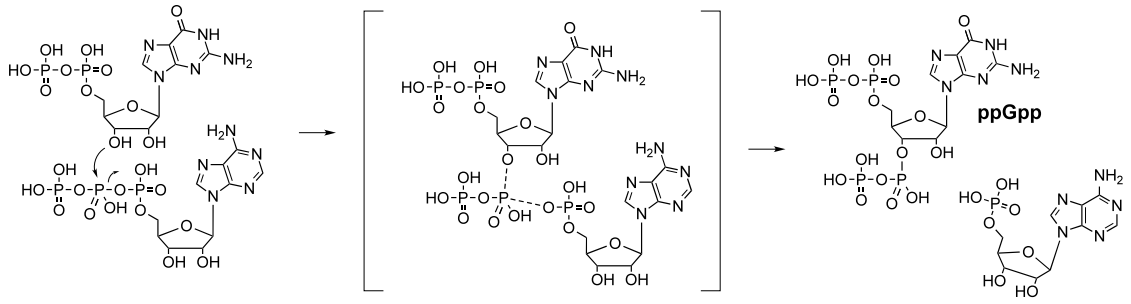

Figure 1. Synthesis of ppGpp from GDP and ATP

RelA's catalytic activity resides in the SYNTH domain (**Figure 2**). The other *E. coli* RSH is SpoT is a bi-functional protein with strong ppGpp hydrolysis activity in HD domain and weak synthetic activity in the SYNTH domain [6]. Synthetic activity of the SpoT protein is induced by many types of nutrient stress other than amino acid starvation, e.g. limitation of phosphate, carbon, fatty acid and iron [4]. SpoT hydrolase activity is essential to bacterial cells, as high concentrations of ppGpp result in cell cycle arrest by inhibiting the DNA replication [7]. HD domain is also present in RelA, but in an inactive form [8]. CTD region of RSH proteins has a regulatory function upon the NTD region as it contacts the HD and SYNTH domains [9].

In the case of many gram-positive model organisms (like *Mycobacteriym tuberculosis* and *Bacillus subtilis*) a single enzyme named Rel is responsible for both synthesis and degradation of the alarmone molecule [10]. Like SpoT, Rel is bifunctional protein with active SYNTH and HD domains. In general, these three enzymes - Rel, RelA and SpoT - are referred to as "long" RSH-s and have a six-domain structure, comprising of HD, SYNTH, TGS, helical, conserved cysteine residues (CC) and ACT domains [8]. However, in addition to "long" RSH-s there are also shorter and specialized RSH-s that contain either the SYNTH or the HD domain. These are Small Armone Synthetases (SASs) and Small Alarmone Hydrolyses (SAHs) (**Figure 2**).

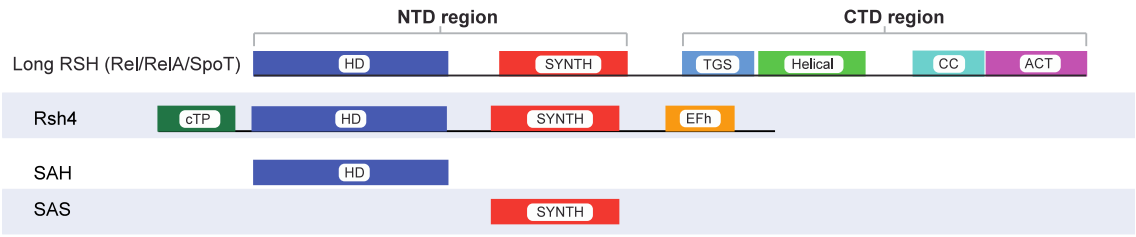

Figure 2. Structure of RSH proteins as per [8]. First line is the structure of "long" RSHs, which comprise of six domains: HD, SYNTH, TGS, helical, conserved cysteine residues (CC) and ACT domains. Rsh4 is a "long" RSH representative in plants – cTP stands for chloroplast transit peptide and Efh for EF hand domain. SAH and SAS are "short" RSHs comprising of only HD or SYNTH domains respectively.

1.2 Molecular mechanisms of ppGpp-mediated regulation

Nucleotides ppGpp and pppGpp were first discovered by Cashel and Gallant [11] as "magic spots" on a thin layer chromatogram while analyzing changes in intracellular nucleotide pools in response to the nutritional stress. They have postulated that accumulation of these compounds led to suppression of RNA synthesis during the stringent response [12]. Since then, extensive genetic and molecular investigations of *E. coli* have established that in this bacteria ppGpp exerts its global regulatory activity mainly by acting on RNA polymerase (RNAP) in two ways: directly, by regulating the RNA polymerase and indirectly, by regulating concentrations of the RNAP sigma factors [4].

Direct effects of ppGpp on RNAP are further potentiated by its cooperation with a protein that binds to the polymerase – DnaK supressor DksA. DnaK is a negative regulator of the heat shock response and *E. coli dksA* gene was first discovered as a dosage dependent suppressor of growth and filamentation phenotypes of *dnaK* deletion mutant strains [13]. Since then DksA have been implicated in a variety of cellular processes including cell division, the stringent response, expression of virulence factors etc. Its role in manifestation of the stringent response has been connected to its ability to bind to the RNAP secondary channel thereby stabilizing ppGpp binding to the RNAP [14]. DksA augments ppGpp effects on initiation and e.g DksA and ppGpp together directly and specifically inhibit the transcription from rRNA promoters, ribosomal protein and tRNA promoters. A strong correlation between the short lifetime of the competitor-resistant promoter complex and negative regulation by

ppGpp and DksA has been found. This leads to suggestion that they inhibit transcription initiation only from those promoters that make short-lived complexes with the RNAP and fail to inhibit from promoters with long-lived complexes [15, 16].

Indirect effects of ppGpp on the RNA polymerase are mediated by so-called called sigma-factor competition [17]. Sigma factors (σ) are dissociable subunits of RNA polymerase, that are responsible for recognition of promoter sequences during initiation of transcription [18]. During the logarithmic growth vegetative sigma factor σ^{70} directs transcription initiation from operons that are essential for synthesis of proteins, lipids and DNA. During the stringent response high concentrations of ppGpp interfere with RNAP binding to the strong σ^{70}-dependent promoters, such as the promoters of ribosomal RNA, ribosomal protein and tRNA genes [19]. This leads to inhibition of the rRNA and tRNA synthesis, and at the same time more core RNAP is available to bind to the alternative σ factors which direct RNAP to transcribe genes involved in adaptive responses [4, 18].

In addition to directing RNAP promoter selection ppGpp exerts broad influence on bacterial physiology [4]. For example, ppGpp controls cellular growth rate by regulating ribosomal number, accumulation of ppGpp leads to DNA replication arrest [20], also it inhibits translation by binding to translational GTPases Initiation Factor 2 (IF2) and Elongation Factor G (EF-G) [21]. Unlike in the case of *E. coli* RNAP*,* in *B. subtilis* ppGpp does bind to the RNAP and the stringent response exerts its regulatory effects via reduction of the GTP levels during starvation [22]. Recently, the stringent response has been found to be linked to bacterial acid stress, via ppGpp-mediated regulation of lysine carboxylase – an enzyme crucial for survival in the acidic environment [23].

1.3 ppGpp-mediated regulation of bacterial virulence and antibiotic resistance

The stringent response has been implicated in bacterial virulence, antibiotic resistance and microbial persistence [24]. There is a growing evidence that ppGpp plays a key role for pathogens to successfully adapt to different microenvironments encountered during infection within the host organism [24] and for expression of virulence genes [25]. In several pathogenic bacteria (*Mycobacterium tuberculosis, Listeria monocytogenes* and many others) mutations in *rel* gene lead to diminished ppGpp accumulation and loss of long-term persistence ability during infection, also they show avirulent phenotype [26]. ppGpp is also

shown to be essential for pathogens to survive and replicate in the phagocytes. The stringent response is activated after uptake of *Staphylococcus aureus* in neutrophils and ppGpp activity is crucial for intracellular expression of genes, coding for factors mediating bacterial survival and escape after phagocytosis [27].

A growing body of evidence indicates that the stringent response contributes towards the antibiotic resistance and formation of non-dividing persister cells [28]. In the investigation of molecular determinants of antibiotic resistance and formation of small colony variant (SCV) phenotype in *S. aureus* it has been found that minor mutation in RelA coding gene is the cause of antibacterial tolerance and formation of persister cells. Mutation detected in *relA* partially impairs hydrolase function of the enzyme, leading to accumulation of ppGpp and result in reduced growth rate of pathogen, attenuated virulence, reduced antibiotic susceptibility – the key features of persistent infection [29]. In a different study it has also been found that attenuation of the ppGpp synthase RelA reduces the antibiotic tolerance, which also confirms importance of ppGpp in resistance mechanisms [30].

2. Role of Translational GTPases in Ribosomal cycle

GTPases is a large protein family with members involved in a variety of cellular processes including protein biosynthesis, various transport processes, as well as cell growth and differentiation [31]. The common feature of all GTPases is their ability to bind GTP nucleotide and hydrolyze it to GDP, releasing inorganic phosphate.

They are as molecular switches that cycle between inactive GDP-bound and active GTP-bound states, so called OFF (or D) and ON (or T) states (**Figure 3**). The GTPase cycle is regulated by a variety of modulators. GTP hydrolysis and consequent transition from ON to the OFF state is strongly activated by GTPase Activating Proteins (GAPs) [32]. Re-activation of the protein requires dissociation of GDP from the protein and its replacement with GTP. This is often a very slow process, which is accelerated by Guanine-nucleotide Exchange Factors (GEFs). Spontaneous exchange of GDP for GTP is inhibited by Guanine-nucleotide Dissociation Inhibitors (GDIs), which stabilize both GTPase:GTP and GTPase:GDP complexes [33].

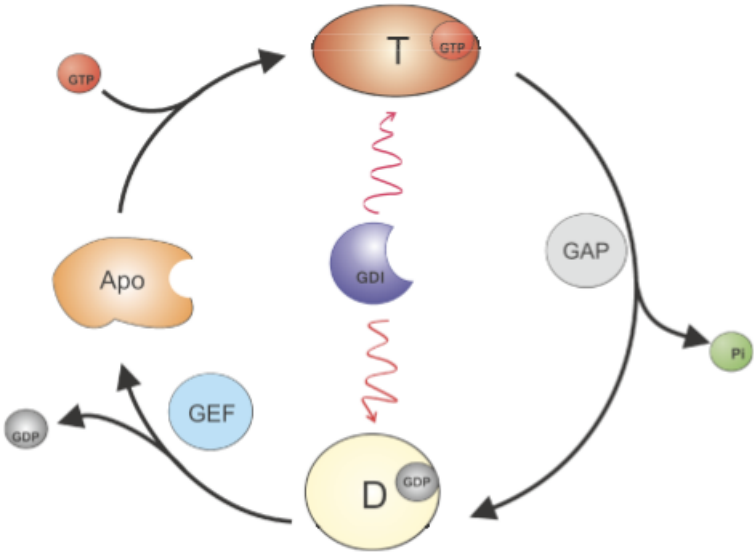

Figure 3. Functional cycle of translational GTPases. "T" and "D" represent the GTP and GDP bound forms of the GTPases, respectively. GAP stands for GTPase Activating Protein and GEF for Guanine-nucleotide Exchange Factor. Wavy arrows represent the stabilization effect of the GDI (Guanine-nucleotide Dissociation Inhibitor). Apo is nucleotide-free state of the GTPase.

GTPases that are involved in protein biosynthesis are referred to as translational GTPases (trGTPases). Their GTPase activity is induced by several elements of the large ribosomal subunit (see below) [34]. In bacteria the "classical" set of trGTPases comprises Initiation Factor 2 (IF2), Elongation Factor Tu (EF-Tu), Elongation Factor G (EF-G) and Release Factor 3 (RF3), which play crucial role in all stages of translation: initiation, elongation, termination and ribosome recycling (**Figure 4**) [35, 36].

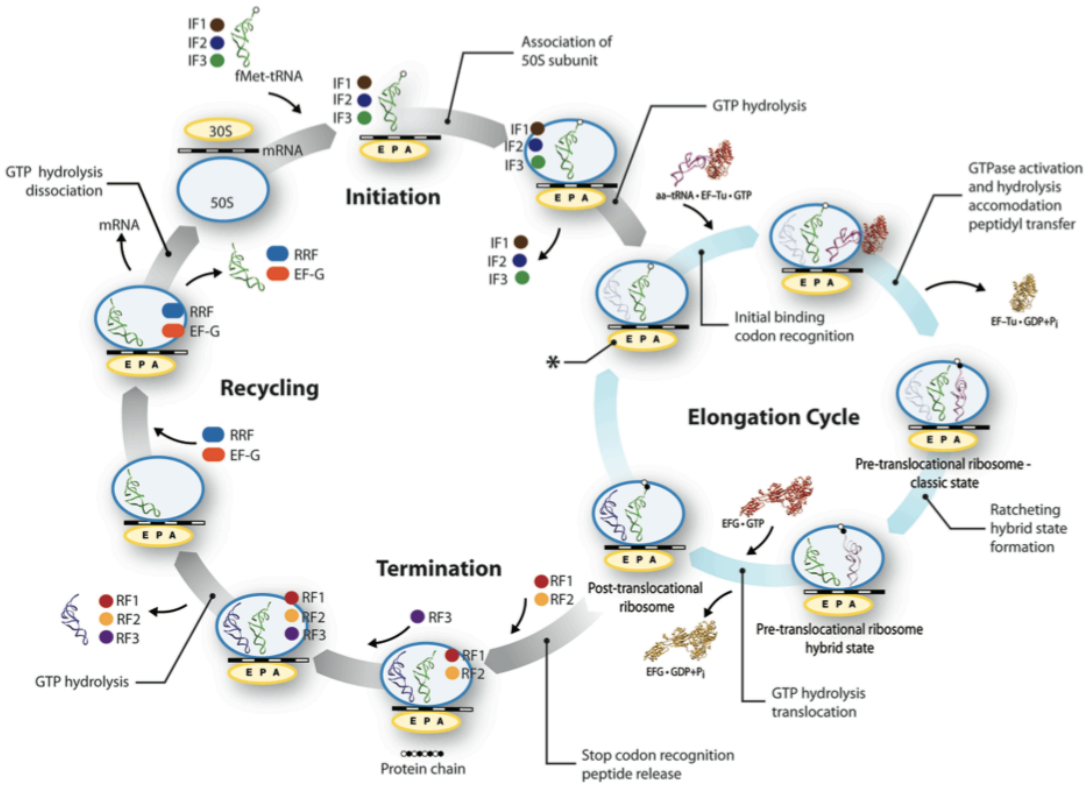

Figure 4. Bacterial translational cycle [37]. The four stages the ribosomal cycle are initiation, elongation, termination and recycling. During the initiation ribosomal subunits, mRNA and the initiator tRNA are brought together with the help of initiation factors IF1, IF2, IF3. During the elongation aa-tRNAs in complex with EF-Tu and GTP are delivered one by one to the ribosomal A-site and amino acids are added to the growing polypeptide chain. After codon-anticodon cognition EF-Tu hydrolyzes GTP and dissociates from the complex. This is followed by EF-G-catalyzed translocation, and post-translocational ribosome formation with peptidyl-tRNA in the P-site, deacylated tRNA in the E-site and empty A-site. Termination occurs when stop-codon enters the A-site of the ribosome and the completed polypeptide is released with the help of RF1 or RF2. RRF and EF-G:GTP complex catalyze the splitting of ribosome subunits in the process known as ribosome recycling. The figure is adapted from [37].

2.1 Initiation

Translation initiation is the starting phase of the protein biosynthesis which prepares the ribosome to enter the elongation cycle [38]. Initiation begins with positioning of the 30S ribosomal subunit and initiator tRNA at the start codon of the mRNA forming the Pre-Initiation Complex (PIC). Afterwards, joining of the 50S subunit and 30S PIC takes place to form the 70S Initiation Complex (70S IC).

In bacteria, this process involves three Initiation Factors – IF1, IF2 and IF3. Initiation factors IF1 and IF3 guide the assembly of PIC. IF1 binds to the aminoacyl site (A-site) of the 30S ribosomal subunit and guides the initiator tRNA to the peptidyl site (P-site) of the ribosome [39]. IF3 binds to the 30S subunit and prevents its premature association with the 50S subunit, as well as participates in selection of initiator tRNA [40]. IF2 recognizes the formyl group of the initiator tRNA and stabilizes its interaction with the 30S subunit [41]. In the next step IF2 accelerates the joining of 30S PIC and 50S ribosomal subunits. After 70S ribosome has been formed, IF2 hydrolyzes GTP, ribosome becomes capable of elongation and IF2 dissociates from the ribosome [42].

2.2 Elongation

During translation elongation amino acids are added one by one to the growing polypeptide chain in accordance with the information encoded in mRNA. In bacteria, this process is catalyzed by two trGTPases: EF-Tu and EF-G.

At the beginning of the cycle tRNA with nascent peptide chain linked to it is in the peptidyl-tRNA binding site of the ribosome (P-site). The incoming aminoacyl-tRNA (aa-tRNA) is docked to the empty aminoacyl-tRNA site (A-site) of the ribosome as a ternary complex (TC) with EF-Tu and GTP [43]. Base pairing between the codon on mRNA and the anticodon on tRNA leads to stabilization of the tRNA in the A-site and triggers the GTP hydrolysis by EF-Tu [44]. Hydrolysis of GTP dramatically decreases the affinity of EF-Tu for aa-tRNA and leads to subsequent release of the GTPase from the ribosome in GDP-bound form. Exchange of EF-Tu bound GDP with GTP is catalyzed EF-Tu's GEF – EF-Ts [45]. GTP hydrolysis by EF-Tu separates two steps in the correct codon-anticodon selection: initial selection and subsequent proofreading [46]. Initial selection is reversible process of ternary complex binding to the ribosome, where the non-cognate ternary complex usually dissociates from the ribosome and cognate one promotes GTP hydrolysis (irreversible process). Following the GTP hydrolysis, tRNA is either accommodated into the peptidyl transferase center or rejected - this is the second proofreading step [47]. After the tRNA accommodation, peptide chain is transferred from P-site tRNA to A-site tRNA. Following the peptidyl transfer, the ribosome has a deacylated tRNA in the P-site and peptidyl tRNA in the A-site [44], and now serves as a substrate for the second GTPase involved in elongation: EF-G.

To prepare the ribosome for the accommodation of next aa-tRNA, the assembly of tRNAs and mRNA must move in respect to the ribosome by one codon in a process known as translocation. Translocation completes the round of elongation and leaves ribosome with peptidyl-tRNA in P-site and deaminoacylated tRNA in the E-site. Hydrolysis of GTP by EF-G is required for catalyzing this movement [48].

2.3 Termination

Translation termination occurs when one of the three stop codons (UAG, UGA or UAA) enters the A-site of the ribosome and is specifically recognized by a class 1 Release Factor (in bacteria RF1 or RF2). Both factors recognize UAA codon, whereas UAG is recognized specifically by RF1 and UGA by RF2. Upon stop-codon recognition, class 1 RFs facilitate the hydrolysis of the ester bond that links the nascent polypeptide chain to P-site tRNA, and thereby induce the release of the completed peptide [49]. Subsequent dissociation of class 1 RF-s from the ribosome is driven by another ribosomal GTPase – RF3 [50]. By stimulating the release of RF1/RF2 from the ribosome RF3 also accelerates the transition from termination step to ribosomal recycling, while for the latter process required Ribosome Recycling Factor (RRF) and class 1 RFs have the overlapping binding site on the ribosome [51].

2.4 Ribosomal recycling

After release of the peptide chain, the ribosome is left in complex with mRNA and a deacylated tRNA in the P-site. This complex needs to be disassembled in order to prepare the ribosome for a new round of protein synthesis. Three proteins are responsible for this process: RRF, EF-G and IF3 [52]. RRF and EF-G serve to disassociate the ribosomal subunits in the reaction requiring GTP hydrolysis [53, 54]. This leaves 30S subunit with mRNA still bound to it containing as well the deacylated tRNA. IF3 then displaces the mRNA and tRNA and allows the 30S subunit to recycle. IF3 also prevents the ribosome from premature re-association [54].

3. Key structural elements of the ribosome

3.1 Regulation of translational GTPase

The activity of trGTPases is regulated by the ribosome. Structural studies showed that G-domains of all bacterial trGTPases interact with the ribosome at a common site near the sarcin-ricin loop (SRL) [48, 55, 56]. This conserved region of the large ribosomal subunit includes part of domain II of 23S RNA (the binding site of antibiotic thiostrepton), part of domain IV (SRL), and proteins L11 and L7/L12, and is responsible for activating trGTPases [36, 44].

The sarcin-ricin loop (SRL), a part of the ribosomal RNA, that is targeted by sarcin and ricin toxins, directly interacts with trGTPases [56] and is suggested to play a crucial role in activating the GTP hydrolysis [57]. However recent studies have shown that SRL acts solely as an anchoring point for GTPase binding and is not involved in the GTPase activation [58]. In vitro analysis demonstrated that SRL:EF-G or SRL:IF2 complex formation is inhibited by GDP, while being insensitive to GTP [59]. This leads to suggestion that SRL could play a role in discrimination between GTP- and GDP-bound forms of EF-G or IF2. It also provides a mechanism for selective destabilization of GDP-bound forms of trGTPases on the ribosome after GTP hydrolysis [59].

A prerequisite for binding of trGTPases to the ribosome is conformational change of N-terminal domain of L11 protein from inactive *trans* to the active *cis* configuration, which enables L11 NTD to connect to L12 CTD. This isomerization was suggested to be catalyzed by EF-G (or other trGTPases) [60]. N-terminal domain of L11 protein also forms along with the helixes 43 and 44 of 23S ribosomal RNA (rRNA) the target site for thiazole-family antibiotics such as thiostrepton and micrococcin. Thiostrepton is known to inhibit the EF-G dependent translocation, by causing the L11 NTD to become more rigid [61, 62].

Ribosomal protein L7/L12 promotes recruitment of trGTPases to the ribosome and stimulates the GTP hydrolysis by the ribosome-bound translation factors through stabilization of their active GTPase conformation. In *E. coli* there are four copies of L7/L12 proteins bound to the

ribosome via N-terminal domain. C-terminal domains of the protein are highly mobile and are crucial for the functional interactions with translation factors [63].

3.2 Regulation of RelA

Besides the assumable functions of binding and activation of trGTPases, ribosomal protein L11 is also implicated in the stringent response by regulating the activity of RelA [64]. Ribosomes that are lacking L11 are deficient in activating the ppGpp synthesis, at the same time being capable of binding RelA [65].

L11 protein consists of dynamic N-terminal domain connected to an RNA-binding C-terminal domain. N-terminal domain has been shown to be involved in the interaction with EF-G, RF1, and antibiotics thiostrepton and micrococcin [64]. NTD has a highly conserved proline-rich helix that lies on the surface of L11 facing away from the ribosome. Mutations in this region or its absence lead to resistance to above mentioned antibiotics as well as relaxation of the stringent response phenotype. Mutational analysis showed that a point mutation of the proline 22 of L11 protein renders it unable to stimulate ppGpp synthetic activity of RelA (see below) [66].

4. Inhibitors of the stringent response

The stringent response is an important mechanism for pathogens to express their virulence genes and to survive in the host organisms (see section 1.3). However, *rsh* genes, encoding the stringent factor are widespread only in Bacteria and plants, but have not yet been found in animals. Therefore the stringent response is considered possible therapeutic target and inhibition of the stringent response is of great medical interest [26]. Several antibiotics targeting translation and ppGpp analogue molecules are acting as inhibitors of the stringent response.

4.1 Antibiotics

The most well-characterized examples of antibiotics inhibiting the stringent response are thiostrepton and tetracycline. They have been shown to interfere with the ribosome-dependent

ppGpp synthesis [67] and the mutations in the L11 protein, which confer resistance to thiostrepton lead also to inhibition of ppGpp synthesis [68].

Tetracycline and thiostrepton are both A-site specific antibiotics inhibiting the protein synthesis. Tetracycline exerts its activity by blocking tRNA binding to the A-site of the ribosome [69]. As the unaminoacylated tRNA in the A-site of the ribosome is a prerequisite for activation of the stringent response, and tetracycline blocks binding of any tRNA, tetracycline has been shown to inhibit the ppGpp synthesis [65].

Thiostrepton on the other hand perturbs the function of the elongation factors by binding to the GTPase center of the ribosome (GAR). Two sites of the GAR are found to be essential for thiostrepton binding: region around residue A1067 of the 23S rRNA [70], which is also the binding site for L11, and proline rich area of L11 protein. There are several lines of evidence that thiostrepton binds strongly and primarily to rRNA in the 1067 region. First, *Streptomyces azureus*, the producer of thiostrepton, possesses the methylase specific for 2'O of A1067, conferring total and selective resistance to the thiostrepton [71]; second, ribosomes containing adenine to uracil substitution at residue 1067 are as well thiostrepton resistant and show weakened thiostrepton binding [72]. Mutations in conserved proline residue of L11 protein on the other hand did not affect the binding affinity of protein to the rRNA nor the binding of thiostrepton. But mutation does result on thiostrepton-resistant phenotype [73]. It has been proposed that thiostrepton by binding in the close vicinity of L11 [74], interferes with conformational transitions in L11, which are important for its functioning [75]. Therefore, it was suggested that thiostrepton inhibits ppGpp synthesis by blocking the function of L11.

Unlike in the case of translational GTPases, effects of thiostrepton on the stringent response where never investigated using thiostrepton-resistant ribosomes, therefore specificity of the inhibition was never established. All of the reported experiments were performed at fixed concentration of thiostrepton (commonly 10 μM), prompting more quantitative analysis.

4.2 ppGpp analogues

As traditional antibiotics target essential cellular components, which makes them effective mostly during bacterial growth, a series of novel ppGpp analogue antibacterial compounds were synthesized. These compounds impede bacterial long term survival pathways as they

inhibit RelA activity, which is responsible for bacterial alternative survival strategies. For the mechanism of inhibition has been suggested that ppGpp analogues bind to the catalytic site of the Rel proteins, competing with the GTP/GDP for the same binding site [76, 77].

The only of these compounds that was shown to have effects not only in vitro, but also when added to live cells is Relacin [78]. Relacin has been shown to inhibit RelA and reduce ppGpp production as well as affect entry into stationary phase in Gram positive bacteria and disrupt formation of multicellular biofilms, making it a promising lead compound for developing a specific inhibitor of the stringent response interfering with bacterial long-term survival mechanisms [78].

Aims of the project

1. Validation of the specificity of thiostrepton-mediated inhibition of the stringent response.
2. Characterization of the stringent response inhibition by ppGpp-based inhibitors.

Materials and Methods

1. Preparation of *E. coli* ribosomes

1.1 Wild type ribosomes

E. coli strain MRE600 was grown at 37°C in a liquid Luria-Bertani (LB) medium. Cells were allowed to grow until OD_{600}=1.1. The bacteria were then collected by low speed centrifugation (4500 g, 20 minutes) and resuspended in cell opening buffer (20 mM Tris-HCl pH 7.5, 100 mM NH_4Cl, 15 mM Mg-acetate, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 20 U/ml DNase I and 0.1 mM PMSF). The suspension was lysed using EmulsiFlex-C3 High Pressure Homogenizer. The lysate was diluted with one volume of 1x overlay buffer (20 mM Tris-HCl pH 7.5, 60 mM NH_4Cl, 15 mM Mg-acetate, 2.5 mM EDTA and 3 mM β-merkaptoethanol (β-Me)). The cell debris was pelleted by centrifuging the lysate for 50 min at 18 000 rpm in SS 34 Sorvall rotor. Supernatant was loaded on sucrose cushion buffer (20 mM Tris-HCl pH 9.0, 500 mM NH_4Cl, 15 mM Mg-acetate, 0,5 mM EDTA, 3 mM β-merkaptoethanol, 1.1 M sucrose) and centrifuged through cushion in Ti 50.2 rotor 17 – 19h at 28000 – 30000 rpm at 4°C. Pellet was dissolved in washing buffer (20 mM Tris-HCl pH 7.5, 500 mM NH_4Cl, 15 mM MgOAc, 0.5 mM EDTA, 7 mM β-Me) and washed again through sucrose cushion buffer in Ti 50 rotor at ω2t = 3,85*1011 rad2s, 4 °C. The precipitate was redissolved in 30 mL of 1x overlay buffer and loaded to a 15-30% sucrose gradient in overlay buffer. 70S ribosomes and free subunits were then fractionated by centrifugation in zonal Ti-14 rotor at $\omega^2t=4.67x10^{11}$ rad^2/s at 4°C. Fractions containing 70S were collected and pelleted by centrifugation at $\omega^2t=1.12x10^{12}$ rad^2/s at 4°C in Ti-45 rotor. The pelleted 70S subunits were redissolved in 1x Polymix buffer (25 mM Hepes pH 7.5, 15 mM MgCl2, 0.5 mM CaCl, 95 mM KCl, 5 mM NH_4Cl, 8 mM putrescine, 1 mM spermidine, 5 mM K_3PO_4 pH 7.3 and 1 mM 1,4-dithioerytreithol), shock-frozen in liquid nitrogen and stored at -80°C. Concentration was estimated by measuring OD at the wavelength 260 nm considering that 1 OD_{260} unit equals 24 pmols.

1.2 Mutant ribosomes: A1067U and ΔL11

For preparation of A1067U mutant ribosomes *E. coli* strain MC315 containing pKK3535 (ampicillin resistant) plasmid was used. This strain was a gift from Dr. Aivar Liiv [79]. Strain for ΔL11 ribosomes was provided by Dr. Scott Blanchard [80]. The process of mutant ribosomes' preparation was the same as for the wild type ribosomes, except the cells were harvested at OD_{600} 0.8.

2. Preparation of recombinant proteins, nucleotides

2.1 *E. coli* 6His RelA

E. coli 6His RelA protein was purified from BL21 (DE3) cells transformed with pET24b plasmid encoding C-terminally 6His-tagged RelA [81]. Cells were grown in liquid LB medium containing 50 μg/ml kanamycin. Protein expression was induced with 0.5 mM isopropyl thio-β-Dgalactosidase (IPTG) at OD_{600} = 0.5 and grown additional 3 h at 37 °C. Cells were harvested by centrifugation 15 minutes at 3000 rpm and diluted in cell opening buffer (25 mM Tris pH 7.5, 1 M Na Cl, 2 mM $MgCl_2$, 1 mM β-Me, 5 mM Imidazole) with addition of 1 μg/ ml DNase-1 and 1 mM PMSF. Cells were lysed by Avestin EmulsiFlex-C3 High Pressure Homogenizer after which cell debris was removed by centrifugation for 20 minutes at 15 000 rpm. Supernatant was then diluted 3 times by Opening buffer without salt (25 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM bME, 5 mM imidazole) and loaded on the 1 ml His-trap Ni^{2+} column (GE Healthcare) equilibrated with Washing buffer: 25 mM Tris (pH 7.5-8), 350 mM NaCl, 2 mM $MgCl_2$, 5 mM imidazole, 1 mM bME. Finally the protein was eluted, by washing the column with Elution buffer (25 mM Tris pH 7.5, 350 mM NaCl, 2 mM $MgCl_2$, 300 mM imidazole, 1mM bMe). Collected fractions were ran on 10% SDS-PAGE and then the fractions showing the pure protein were concentrated against RelA Storage buffer (0.7M KCl, 25 mM Hepes, 2mM DTT, 5mM MgCl2, 10% Glycerol) using Amicon Ultra Centrifugal Filters. Protein concentration was measured by Bradford protein assay. Protein was aliquoted, shock-frozen in liquid nitrogen and stored at -80 °C.

2.2 *E. coli* 6His EF-G

E. coli 6His EF-G protein was purified from BL21 (DE3) cells transformed with pRSET plasmid encoding N-terminally 6His-tagged EF-G [82]. Cells were grown in a liquid LB medium with 50 μg/ml Kanamycin. Protein expression was induced with 0.5 mM IPTG at OD_{600} = 0.6 and cells were grown additional 2 h at 30 °C. Cells were harvested by centrifugation at 3500 rpm at 4 °C. The cell pellet was dissolved in lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 200 mM NaCl), 1 μg/ ml DNase-1 and 1 mM PMSF were added and cells were lysed with Avestin EmulsiFlex-C3 High Pressure Homogenizer. Cell debris was removed by centrifugation at 15 000 rpm for 20 minutes 4 °C and supernatant was loaded to 1 ml His-trap Ni^{2+} column (GE Healthcare) equilibrated with loading buffer (50 mMTris-Hcl pH 7.5, 200 mM NaCl). Finally the protein was eluted from the column by elution buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 300 mM Imidazole). For purity check the protein was ran on 12% SDS-PAGE and then concentrated in 1x Polymix through Amicon Ultra Centrifugal Filters (Merck KGaA) by centrifuging at 4000 rpm. Protein concentration was estimated by Bradford protein assay. Finally the concentrated protein was aliquoted, shock-frozen in liquid nitrogen and stored at -80 °C.

2.3 ppGpp

ppGpp was prepared using the Rel_{Seq} enzyme from *Streptococcus equisimillis* essentially as described by Mechold and colleagues [9], with minor modifications.

For ppGpp preparation, a 500 μl reaction mixture containing 30 mM Tris pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM ATP, and 6 mM GDP is initiated by adding 50 μl of purified Rel*Seq* [9] (final concentration of 100 μM) and, incubated for at least 30 minutes at 37°C. Reaction was stopped with the equal volume of phenol and centrifuged at 16000 g for 20 minutes. After centrifugation the aqueous phase was loaded on the MonoQ 5/50 GL (GE Healthcare Bio-Sciences AB) column equilibrated with Buffer A (0.5 mM LiCl, 0.5 mM EDTA, 2.5 mM TrisCl pH 7.5). For ppGpp purification, nucleotides were eluted with Buffer B (2 M LiCl, 0.5 mM EDTA, 2.5 mM TrisCl pH 7.5). Nucleotides were monitored in fractions by following the UV absorption at 253 nm and verified by TLC mobilities relative to GTP. Fractions containing ppGpp were collected and precipitated by adjusting LiCl to 1 M, then adding 3 volumes of 95% EtOH. This was kept 1 h at -80 °C (or -20 °C overnight) and

then centrifuged 16000g for 20 minutes. The pellet was dried using MAXI dry plus Heto Vacuum Centrifuge. After, the pellet was dissolved in H_2O. Final concentration was determined spectrophotometrically at 254 nm using molar extinction coefficient of 13600.

2.4 thio-ppGpp

For 6-thio-ppGpp preparation was used the same procedure as for ppGpp with the exception of used substrate, 6-thio-GDP (Jena Biosciences) instead of GDP.

2.5 ATP

ATP (Amersham Biosciences) was dissolved in water until concentration of 20 mM and pH was adjusted with NaOH to 7.5.

3. In vitro assays

All biochemical reactions were carried out in high-fidelity 1x Polymix buffer for in vitro translation mimicking in vivo conditions [83]: 25 mM Hepes pH 7.5, 15 mM MgCl2, 0.5 mM CaCl, 95 mM KCl, 5 mM NH_4Cl, 8 mM putrescine, 1 mM spermidine, 5 mM K_3PO_4 pH 7.3 and 1 mM 1,4-dithioerytreithol. High concentrations of magnesium ions where used for efficient non-enzymatic tRNA binding to the ribosome.

3.1 The stringent response system

The RelA mediated ppGpp production in the presence of different RelA inhibitors was investigated by monitoring the ppGpp production from GDP and ATP, using the 3H-labeled GDP. Process of reaction was quantified as 3H-GDP to 3H-ppGpp conversion, ranging from 0 (no 3H-ppGpp is produced) to 1 (all the 3H-GDP is converted to 3H-ppGpp).

The stringent response system experiments were performed with 30-100 nM RelA, 0.5 μM 70S (wild type or ΔL11), 0.3 mM 3H-labeled GDP (purchased from American Radiolabelled Chemicals), 1 mM ATP, 100 μM ppGpp and titration by different concentrations of RelA inhibitors.

RelA inhibitors tested:

1) Antibiotic thiostrepton (Tocris Bioscience)

2) ppGpp analogue molecules: thio-ppGpp, ppApp, Relacin (the latter two compounds were synthesized by Dominik Rejman at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic)

Reactions were performed at 37 °C and reaction mixes were preincubated for 2 minutes before the reaction was started by addition of ATP. After activation, to monitor ppGpp production (from ATP and GDP) in time, reactions were stopped at 0, 5, 10 and 15 minute timepoints by Kill Mix solution (71% Formic Acid, 4 mM GDP, 4 mM GTP). The concentration of formic acid after addition of Kill mix to the reaction is 30 %. In the reaction with ΔL11 ribosomes due to considerably lower activity of RelA in this system timepoints were taken at 0, 10, 20 and 40 minutes. Stopped reaction mixes were loaded on POLYGRAM CEL 300 PEI/UV_{254} Pre-coated Thin Layer Chromatography (TLC)-sheets (MECHEREY-NAGEL). After the samples have been eluted, separated nucleotides were visualized by UV_{254}-light and GDP and ppGpp spots were cut out from the TLC sheets. Cut out peaces were subjected to ScintiSafe 3 Liquid Scintillation Cocktail (Fischer Scientific) and with the usage of Perkin Elmer Liquid Scintillation Analyzer the production of 3H-ppGpp from 3H-GDP was counted.

3.2 EF-G GTPase reaction

GTP hydrolysis by EF-G was monitored by using 3H-labeled GTP and quantified as conversion of 3H-labeled GTP to 3H-labeled GDP.

EF-G GTPase reactions were performed with 0.1 μM EF-G, 0.5 μM 70S, 0.5 mM 3H-labeled GTP (AMERSHAM) diluted by cold GDP. This reaction mix was titrated by different concentrations of GTPase inhibitor compounds (the same inhibitors as in stringent response system). Reactions with thiostrepton were performed in the presence of 0.1 μM Pluronic F-127 (Sigma) for dissolving the water-insoluble thiostrepton.

Following procedures were similar to the stringent response system reaction:

1. Reaction preincubation for 2 minutes at 37 °C
2. Activation of the reaction by 3H-GTP dilution
3. Stopping reaction by taking 0, 5, 10 and 15 minute timepoints
4. Loading the samples on TLC and eluting
5. Cutting out GDP and GTP spots and subjecting them to scintillation cocktail
6. Counting the 3H-GDP production from 3H-GTP by Liquid Scintillation Analyzer

Results

1. Effects of thiostrepton

1.1 Inhibition of EF-G GTPase

As a specificity control of thiostrepton-mediated inhibition of RelA we decided to use a well-studied inhibition of in vitro EF-G GTPase, taking advantage of well-characterized ribosomal mutants resistant to thiostrepton [72, 84]. Since thiostrepton has a tendency for precipitation, which can lead to artifacts [85], we have performed experiments in the presence and absence of 0.1% of Pluronic F-127 – a nonionic surfactant polyol used for solubilizing hydrophobic compounds [86].

As a first control we showed that at 0.1% Pluronic F-127 does not affect EF-G GTPase activity, both in the presence and absence of *E. coli* 70S ribosomes (**Figure 5**). Next we performed a titration of thiostrepton following EF-G GTPase in the presence of thiostrepon-sensitive (wild type) as well as thiostrepton-resistant (A1067U rRNA mutant and a mutant missing ribosomal protein L11, ΔL11) *E. coli* ribosomes **(Figure 6).**

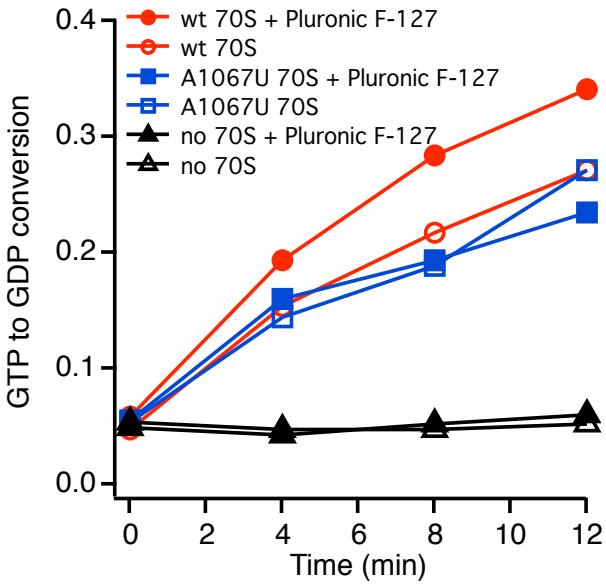

Figure 5. 0.1% Pluronic F-127 does not affect EF-G GTPase activity. The reaction mixture contained 0.5 µM 70S ribosomes (wild type or A1067U), 0.1 µM EF-G, 300 µM 3H-GTP, Polymix, 0.1 % Pluronic F-127.

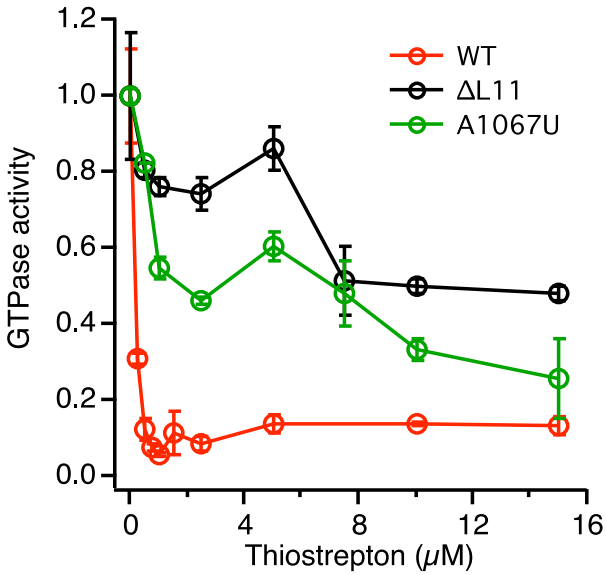

Figure 6. Thiopstrepton effect on EF-G GTPase activity. EF-G GTPase activity on the wild type ribosomes is inhibited by thiostrepton already at 1 μM. On the other hand mutant ribosomes show considerably lower sensitivity to the antibiotic. The reaction mixture contained 0.5 μM 70S ribosomes (wild type, ΔL11 and A1067U), 0.1 μM EF-G, 300 μM 3H-GTP, 0.1 % Pluronic F-127, Polymix and variable concentrations of thiostrepton.

Our results are consistent with previously shown data [72, 85]: A1067U and ΔL11 ribosomes are resistant to thiostrepton inhibition of EF-G GTPase, while EF-G GTPase activity in the presence of wild type ribosomes is completely inhibited at already 1 μM thiostrepton.

1.2 Inhibition of RelA

After establishing the EF-G GTPase system as a control, we proceeded to investigation of thiostrepton-mediated inhibition of RelA. Again, just like in the case of EF-G, we first demonstrated that in the case of RelA addition of 0.1% Pluronic F-127 does not affect the system (**Figure 7**).

Next we performed a thiostrepton titration in stringent response system with wild type and ΔL11 ribosomes in the presence and absence of Pluronic F-127 (**Figure 8**). In the case of ΔL11 ribosomes the turnover rates where considerably lower then in the case of wild type ribosomes, consistent with L11 being crucial for RelA-activation [65, 66, 87, 88]. However, since inhibition curves are normalized on RelA activity in the absence of the antibiotic, this decrease in activity is not apparent on the figures.

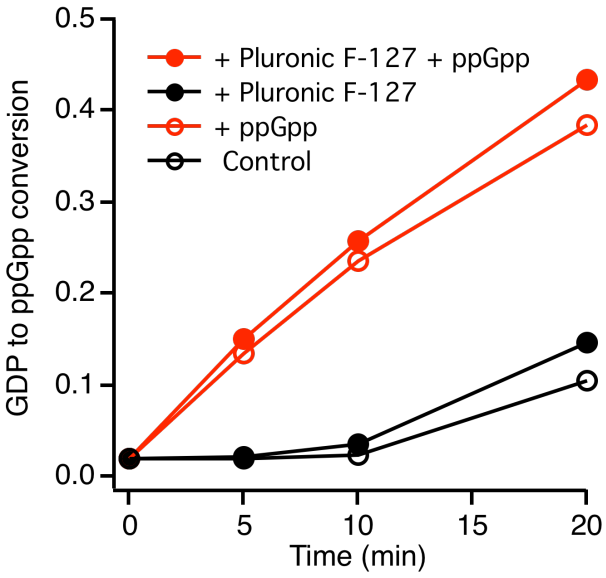

Figure 7. Pluronic F-127 does not affect the activity of RelA. The reaction mixture contained 100 nM RelA, 0.5 μM 70S, 0.3 mM 3H-labeled GDP, 1 mM ATP, 0.1 % Pluronic F-127, Polymix.

Surprisingly, in the presence of Pluronic F-127 we observed no inhibition of RelA in the presence of as much as 10 μM of thiostrepton (**Figure 8A**). In the absence of Pluronic F-127 we observed ΔL11-insensitive inhibition at concentrations at least ten times higher than these required for the inhibition of EF-G GTPase in the presence of thiostrepton-sensitive ribosomes (**Figure 6**). The extent of RelA inhibition was consistent with that reported in the literature [67, 81].

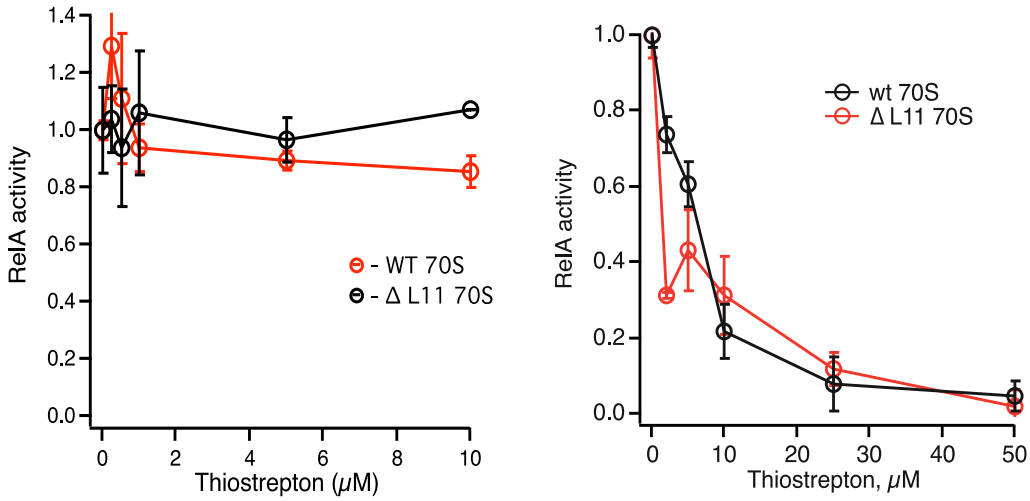

Figure 8. **Nonspecific effect of thiostrepton on RelA activity in the presence (left) and absence of Pluronic F-127 (right).** In the presence of Pluronic F-127 there is virtually no inhibitory effect of thiostrepton on EF-G GTPase activity, both in the case of wild type ribosomes and ΔL11 ribosomes. In the absence of Pluronic F-127 thiostrepton nonspecifically inhibits RelA activity at high

concentrations, independently of L11 protein. The reaction mixture contained 100 nM RelA, 0.5 μM 70S, 0.3 mM 3H-labeled GDP, 1 mM ATP, Polymix and variable concentrations of thiostrepton.

2. Effects of ppGpp analogues

2.1 Inhibition of EF-G GTPase

As a specificity control for RelA inhibition we used one of the ppGpp targets - GTPase EF-G (**Figure 9**). Titration with Relacin [78] demonstrated no significant effects on GTPase activity of EF-G, indicating good specificity of the compound towards inhibition of the stringent response.

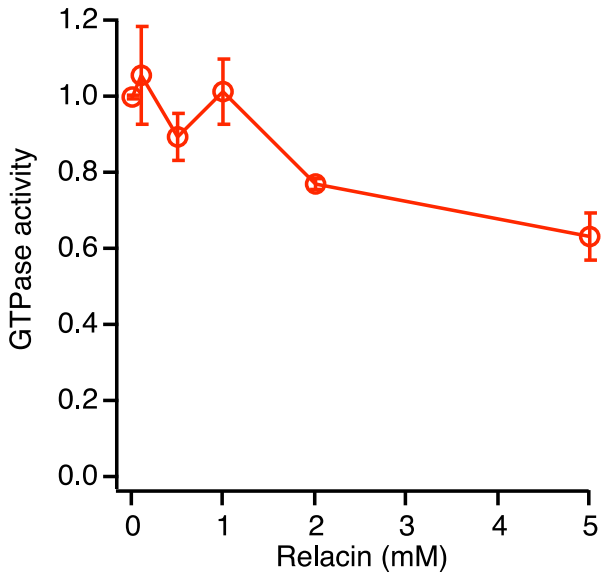

Figure 9. Relacin does not significantly affect EF-G GTPase activity. The reaction mixture contained 0.5 μM 70S ribosomes, 0.1 μM EF-G, 300 μM 3H-GTP, Polymix and variable concentrations of Relacin.

2.2 Inhibition of RelA

Recently our laboratory has shown that ppGpp regulates its own production by directly activating RelA [88], which is seemingly contradictory to RelA inhibition by ppGpp-based compounds [76-78]. We carried out a reaction in a stringent response system increasing

ppGpp concentration up to 1 mM **(Figure 8)**, resolving the contradiction: ppGpp activates RelA at low concentrations (up to 200 μM) and inhibits it at higher concentrations.

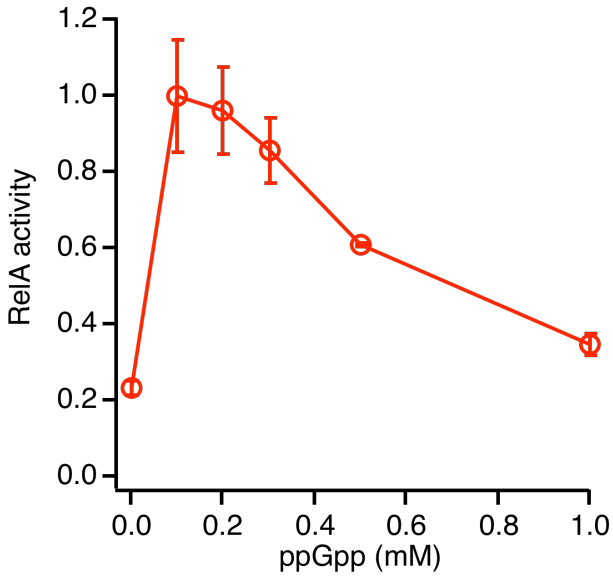

Figure 10. ppGpp is activating RelA protein at low concentrations and inhibiting it at higher concentrations. The reaction mixture contained 100 nM RelA, 0.5 μM 70S, 0.3 mM 3H-labeled GDP, 1 mM ATP, Polymix and variable concentrations of ppGpp.

Next, we proceeded to characterization of Relacin (**Figure 11**), a ppGpp analogue has been shown to specifically inhibit RelA activity and reduce ppGpp production both in vitro and in vivo [78]. Results from our in vitro stringent response system experiment are consistent with the previously showed data **(Figure 12)**: Relacin does inhibit RelA in vitro, and the efficiency of inhibition is low – lower than that of the parental compound, ppGpp. The probable cause of this is substitution of highly charged phosphate groups with less charged residues. Note that all the following experiments (with exception of that presented on **Figure 15**) are performed in the presence of 100 μM ppGpp resulting in activation of the in vitro stringent response system.

Since Relacin is effective in bacterial cells only at very high concentrations [78], consistent with its low efficiency in vitro (**Figure 12**), we have proceeded to testing several other ppGpp derivatives in the search for a more efficient stringent response inhibitor. Namely, we investigated the effect of ppApp and 6-thio-ppGpp **(Figure 13)**. 6-thio-ppGpp is a UV-dependent crosslinkable derivate of ppGpp recently used to map the ppGpp binding site on E. coli RNAP [89], and a similar approach could potentially be employed to investigate the

structural basis for the ppGpp-mediated activation of RelA. In comparison with the parental molecule – ppGpp - these molecules have modifications in the nucleotide base.

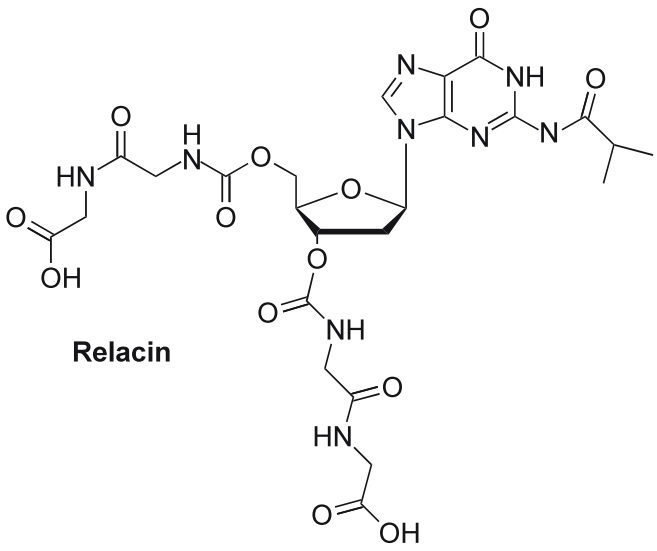

Figure 11. Chemical structure of Relacin. In comparison to ppGpp, in Relacin phosphate groups are substituted with less charged peptide-like moieties, and guanine base is carrying a iBu protection group.

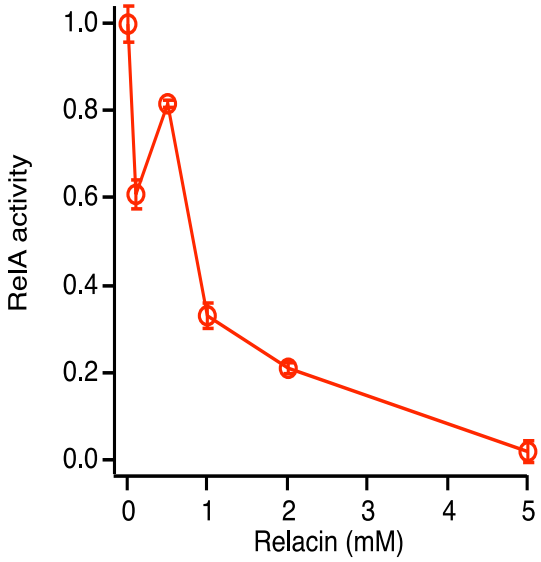

Figure 12. Inhibitory effect of Relacin on RelA activity. The reaction mixture contained 100 nM RelA, 0.5 μM 70S, 0.3 mM 3H-labeled GDP, 1 mM ATP, Polymix and variable concentrations of Relacin.

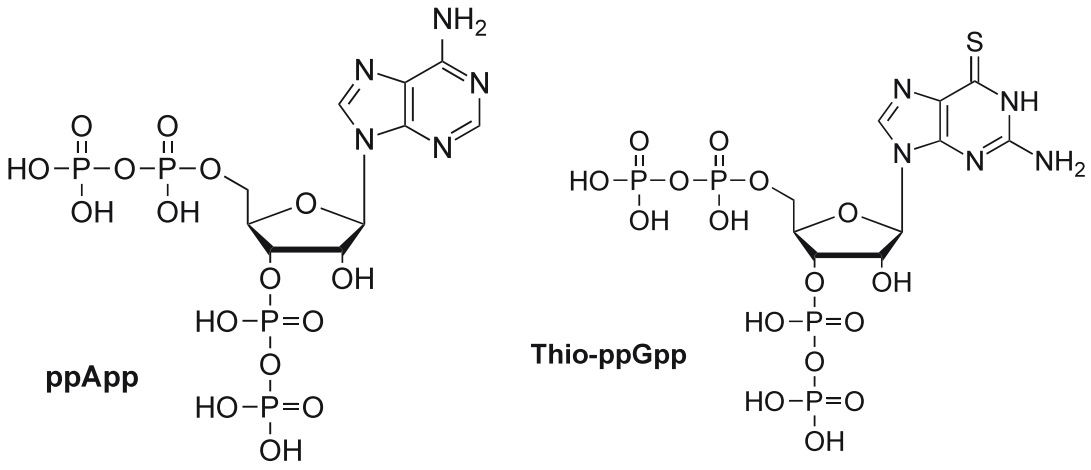

Figure 13. Chemical structures of ppApp (left) and thio-ppGpp (right). In comparison to the parental ppGpp, ppApp molecule has Adenine nucleobase instead of Guanine and thio-ppGpp has a sulfur atom at the sixth position of Guanine base.

Surprisingly, both of these compounds turned out to be more efficient stringent response inhibitors than both Relacin and ppGpp, significantly inhibiting RelA already at a concentration of 100 μM (**Figure 14**).

We have also tested ppApp-mediated activation of RelA (**Figure 15**). Unlike ppGpp (**Figure 10**), even at low concentrations ppApp did not activate the in vitro stringent response system, suggesting that the identity of the nucleotide base is essential.

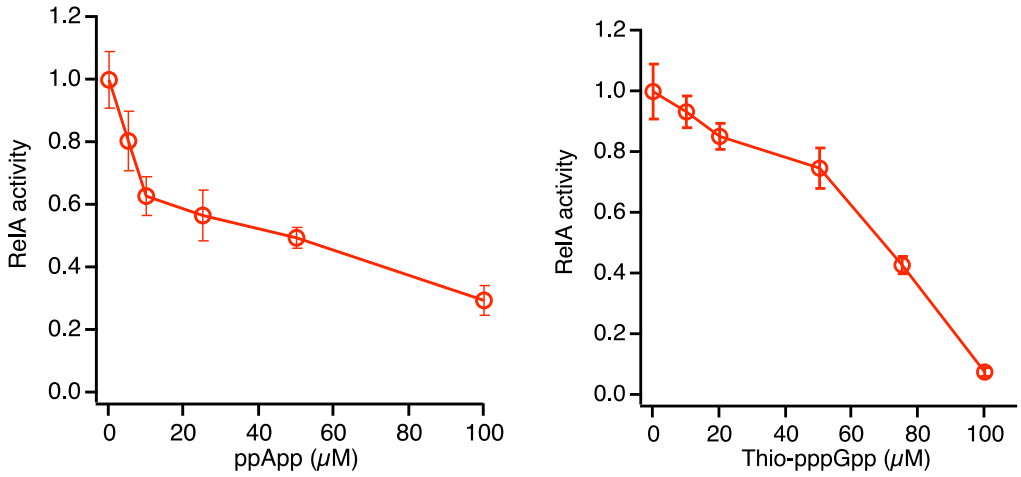

Figure 14. ppApp and thio-ppGpp efficiently inhibit RelA at already 100 μM concentration. The reaction mixture contained 100 nM RelA, 0.5 μM 70S, 0.3 mM 3H-labeled GDP, 1 mM ATP, Polymix and variable concentrations of ppApp (left) or 6-thio-ppGpp (right).

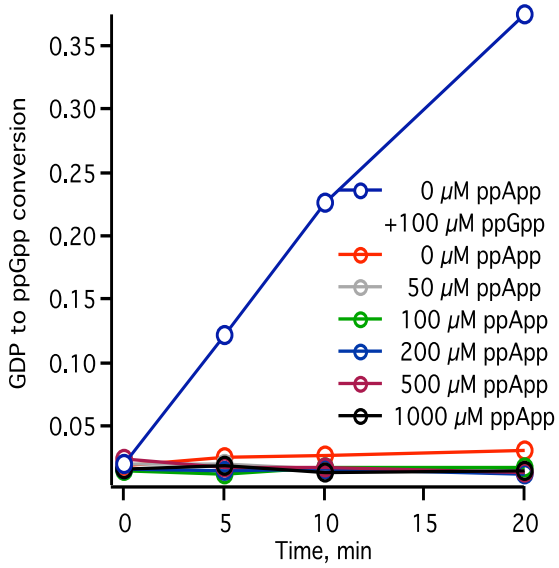

Figure 15. Unlike ppGpp, ppApp does not activate stringent response, even at low concentrations. The reaction mixture contained 100 nM RelA, 0.5 μM 70S, 0.3 mM 3H-labeled GDP, 1 mM ATP, Polymix and variable concentrations of ppApp.

Discussion and outlook

Inhibition of the stringent response has been recently recognized as promising target for developing novel antibacterials [90], since this global regulatory mechanism is implicated in bacterial virulence, antibiotic tolerance and biofilm formation [24, 25, 28]. In this thesis I have investigated two potential stringent response inhibitors: antibiotic thiostrepton and three ppGpp analogues: Relacin, 6-thio-ppGpp and ppApp.

Inhibition of RelA by thiostrepton is seemingly a well-documented phenomenon: it was demonstrated in vitro in numerous publications [67, 81, 91, 92], starting with a pioneering work by Haseltine and colleagues [93]. However, all of the reports used only one – and relatively high – concentration of the antibiotic and never tested selectivity of the effects using thiostrepton-resistant ribosomes. Therefore we have investigated this phenomenon in more detail.

Since thiostrepton has a tendency for precipitation, we have used a nonionic surfactant Pluronic F-127 to stabilize the solution. Surprisingly, in the presence of Pluronic F-127 we observed no inhibition of RelA by thiostrepton (**Figure 8A**), while control experiments with EF-G GTPase demonstrated efficient and specific inhibition (**Figure 6**). In the absence of Pluronic F-127 we have recreated RelA inhibition reported in the literature (**Figure 8B**), however it required considerably higher concentrations of the antibiotic as compared to inhibition of EF-G and was insensitive to ribosomal mutations leading to thiostrepton resistance. We concluded that inhibition of the stringent response by thiostrepton is, most likely, an artifact caused by the precipitation of thiostrepton and is, therefore, unlikely to serve as a route for developing specific stringent response inhibitors.

Our second direction – investigations of ppGpp analogues – demonstrated that these compounds are more likely to lead to the development of a specific stringent response inhibitor. First we characterized Relacin [78] and showed that this compound has very little effect on EF-G GTPase (**Figure 9**), and inhibits RelA with efficiency similar to that of the parental compound, ppGpp (compare **Figure 10** and **Figure 12**). However, even though

seemingly specific, Relacin is not very efficient requiring concentrations of about 1 mM for significant inhibition of RelA in vitro.

Next, we examined two ppGpp analogues – ppApp and 6-thio-ppGpp – which turned out to be considerably more efficient inhibitors of stringent response than ppGpp and Relacin, inhibiting RelA already at 100 μM concentration. Unlike Relacin, which in comparison with ppGpp has alterations in phosphate moieties, ppApp and 6-thio-ppGpp have modifications at the nucleotide base, suggesting that modification of nucleobase is a promising way to development of a specific and potent stringent response inhibitor.

Our future plans for development of ppGpp analogues as promising antimicrobial drugs are as follows:

1) To set up an in vitro transcription system for testing the effects of ppGpp based compounds on the RNAP – the main ppGpp target, and the most likely candidate for the off-target effects. Since ppApp has been shown to have no effect on the RNAP from *Thermus thermophilus* [67], ppApp-based inhibitors are likely to be more specific than ppGpp-based.

2) To synthesize and test a new generation of ppGpp analogues containing modifications of both phosphates and nucleobase thus improving pharmacokinetics and specificity of the compounds respectively. The two compounds planned for the next step are 6-thio-Relacin and A-Relacin **(Figure 16)**. 6-thio-Relacin has a sulfur atom at the sixth position of Guanine base and does not have iBu protection group of the parental compound. A-Relacin has a Guanine base replaced by Adenine.

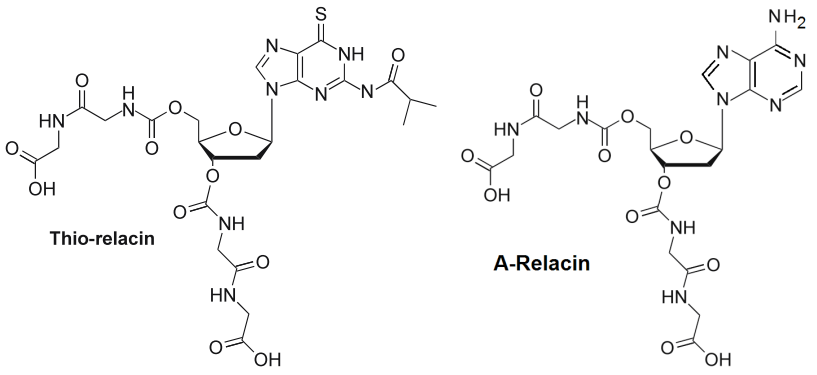

Figure 16. Chemical structures of Thio-Relacin (left) and A-Relacin (right).

3) To investigate the crosstalk between ppGpp-based RelA inhibitors and ppGpp. Specifically, we would like to determine if ppGpp potentiates or protects from the effects of the inhibitory effects of the ppGpp analogues. This in vitro experiment will indicate whether stringent cells are either more or less sensitive to the inhibitors compared to cells with low ppGpp levels.

Conclusions

1) The inhibitory effect of the thiostrepton on the stringent response is most likely an artifact caused by precipitation of thiostrepton at high concentrations and thus interfering with the RelA activity nonspecifically.

2) ppGpp-based stringent response inhibitor Relacin is a weak, but potentially specific inhibitor with almost nondeductible off-target effect on EF-G GTPase.

3) Modification of the nucleotide base of the ppGpp molecule is a promising route for developing more potent inhibitors of the stringent response: ppApp and 6-thio-ppGpp have potency at least ten times higher then that of Relacin and ppGpp.

4) ppApp, unlike ppGpp, does not activate RelA in vitro, suggesting that identity of the nucleotide base is crucial for the activation.

Bakteriaalse poomisvastuse inhibiitorite, tiostreptooni ja ppGpp analoogide, iseloomustamine

Jelena Beljantseva

Resümee

Bakteriaalne poomisvastus on alarmoonimolekuli ppGpp poolt vahendatud bakteri üks põhilisi adaptatsioonimehhanism stressitingimustele. ppGpp avaldab oma aktiivsust moduleerides erinevaid rakusiseseid ensüüme, sh RNA polümeraasi, translatsioonilisi GTPaase, DnaG primaasi, ja nagu meie laboris hiljuti avastati, ka RelA valku, mis on ribosoomist sõltuv ppGpp-d produtseeriv ensüüm. Kuna poomisvastus on seotud bakterite virulentsusega, antibiootikumresistentsusega ja biofilmi tekkega, omab spetsiifiliste poomisvastuse inhibiitorite leidmine suurt kliinilist tähtsust.

Antud töös uurisin ma kaht tüüpi potentsiaalseid poomisvastuse inhibiitoreid: antibiootikumi tiostreptoon ja ppGpp-analoogseid molekule. Tiostreptoon pidurdab ribosoomi võimet aktiveerida translatsioonilisi GTPaase. Tiostreptooni inhibeeriv mõju RelA valgule on küll põhjalikult kirjeldatud, kuid seda ei ole detailselt uuritud, mis loob vajaduse antud uuringu järele. Praeguseks väljaarendatud ppGpp-analoogsed RelA inhibiitorid on madala efektiivsuse ja biosaadavusega.

Võrreldes tiostreptooni mõju RelA valgule ja translatsioonilisele GTPaasile EF-G-le metsiktüüpi või tiostreptoon-resistentsete ribosoomide juuresolekul, leidsime, et tiostreptooni inhibeeriv mõju RelA valgule on mittespetsiifiline, tingitud antibiootikumi väljasadenemisest kõrgetel kontsentratsioonidel. Seega võib järeldada, et tiostreptooni modifitseerimine ei vii spetsiifilise poomisvastuse inhibiitori leidmiseni. Teisalt näitasime, et kaks ppGpp-analoogset molekuli, ppApp ja 6-tio-ppGpp, on vähemalt ühe suurusjärgu võrra efektiivsemad poomisvastuse inhibiitorid kui seni kirjeldatud ppGpp analoogid. Antud töö tulemused näitavad, et lämmastikaluste keemiline modifitseerimine on potentsiaalne strateegia arendamaks spetsiifilisi ja efektiivseid poomisvastuse inhibiitoreid.

Acknowledgments

I would like to thank collaborators of our group for sharing the ideas and providing the necessary compounds for this project. Dr. Sergei Kopanchuk for suggesting using Plurinic F-127 for stabilizing of thiostrepton solutions, Dr. Aivar Liiv for sharing the *E. coli* strain for production of A1067U ribosomes, Dr. Dominik Rejman for synthesis of Relacin and ppApp.

I'd like to thank my supervisor Dr. Vasili Hauryliuk for his contribution to make my master thesis as practical as possible, for his support and help. I express my gratitude to Pavel Kudrin, Stoyan Tankov and Dr. Viktoriya Shyp for sharing their knowledge and laboratory skills. I am very thankful to Prof. Tanel Tenson for accepting me to his group and for generous financial support.

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