

UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TECHNOLOGY

INSTITUTE OF CELL AND MOLECULAR BIOLOGY

INSTITUTE OF TECHNOLOGY

Katarina Beata Saltõkova

**ppGpp-based stringent response inhibitors:
characterization *in vivo* and *in vitro***

Bachelor's Thesis

Supervisor Dr. Vasili Hauryliuk

Co-supervisor MSc Vallo Varik

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Abbreviations

AMP	A denosine m onophosphate
ATP	A denosine t riphosphate
aa-tRNA	A minoacylated tRNA
CRP	C atabolite r egulation p rotein
EF	E longation f actor
FF	F ast F low
GDP	G uanosine d iphosphate
GPP	5' phosphorylase
GTP	G uanosine t riphosphate
HPLC	H igh p ressure l iquid c hromatography
IC	I nitiation c omplex
IF	I nitiation f actor
KO	k nock- o ut
LPS	L ipopolysaccharides
MIC	M inimal inhibitory c oncentration
MOPS	(3- N - m orpholino) p ropanesulfonic acid
MSI	M agic spot I
MSII	M agic spot II
ppGpp	g uanosine t etraphosphate

pppGpp	g uanosine p entaphosphate
PTC	P eptidyl t ransferase centre
QS	Q uorum s ensing
RF	R elease f actor
RRF	R ibosome r ecycling f actor
SAX	S trong a nion e xchange
SD	S hine- D algarno (sequence)
SF	S tringent f actor
TLC	T hin L ayer C hromatography

Abstract

The ability to adapt to environmental changes is essential for bacterial survival. One of the most important and most well-studied adaptive responses is the stringent response. When *Escherichia coli* experience starvation of amino acids or other nutrients the level of uncharged tRNA dramatically rises. This leads to a situation when a deacylated tRNA occupies the A-site of the ribosome. The enzyme called RelA or Stringent Factor (SF) binds to the ribosomal A-site and catalyzes synthesis of compounds guanine nucleotide tetra- and penta-phosphates, collectively known as (p)ppGpp alarmone. These nucleotides are produced from ATP and GDP or GTP. The ppGpp concentration is primary, although not exclusive, player of growth rate control correlating the amount of rRNA produced in *E. coli* with bacterial growth rate.

The accumulation of (p)ppGpp is important for bacterial virulence, differentiation and antibiotic resistance. Lately, chemical derivatives of (p)ppGpp were developed as competitive inhibitors of Rel proteins. One of these compounds which inhibits (p)ppGpp production *in vivo* and *in vitro* was purported as a novel antibacterial agent, Relacin. As Rel proteins are absent in mammals, this appears to be a very attractive approach of development of novel antibacterial agents.

In our work we test Relacin on Gram-negative *E. coli* cells following its effects on the growth rate. We try to develop a new method to measure nucleotide pools in living cells, since it is an important characteristic of bacterial physiological state. Using *in vitro* stringent response assay and translation assays we address the issue of Relacin's selectivity. Our investigations of Relacin are a first step towards developing more potent and specific ppGpp-based inhibitors of the stringent response.

Introduction

1. Protein biosynthesis

Proteins are biological molecules consisting of one or more poly-amino acid chains. They perform a various functions in living organisms, such as catalyzing metabolic reactions, responding to stimuli, replicating DNA, transporting molecules and so on. Proteins differ from one another in their amino acid sequence. The information contained in the DNA is transcribed into messenger RNA (mRNA). The four nucleotides (A, U, C or G) in the mRNA are translated into the 20 amino acids that make up proteins.

The molecular machine performing this translation is called the ribosome. It is highly conserved, with its functional cycle and basic structure conserved across all three domains of life: Archaea, Bacteria and Eukarya. The bacterial ribosome consists of two subunits – the small subunit (30S) and the large subunit (50S). The small subunit consists of 16S rRNA and 21 proteins whereas the large subunit consists of 23S rRNA, 5S rRNA and 31 proteins (Kaltschmidt & Wittmann, 1970). Together these subunits form the 70S ribosome. The cavity between two subunits where translational factors and tRNA bind is called the interface cavity (Lancaster *et al.*, 2002). Two subunits have different tasks during the translational process. The 30S subunit contains the decoding region, is responsible for binding to the Shine-Dalgarno sequence of mRNA and the interaction with the anticodon stem-loops of tRNAs in A-, P- and E-site (Schmeing & Ramakrishnan, 2009). 50S subunit contains peptidyl-transferase centre (PTC) which is located in 23S rRNA. PTC is responsible for peptide bond formation between incoming amino acid in the A-site tRNA and the nascent peptide chain attached to the P-site tRNA (Schmeing *et al.*, 2005). There is a GTPase associated region in the 50S subunit where elongation and release factors bind. Besides that, just as the 30S, the 50S also contains three binding sites for tRNA: the A-, P- and E-site (**Figure 1**).

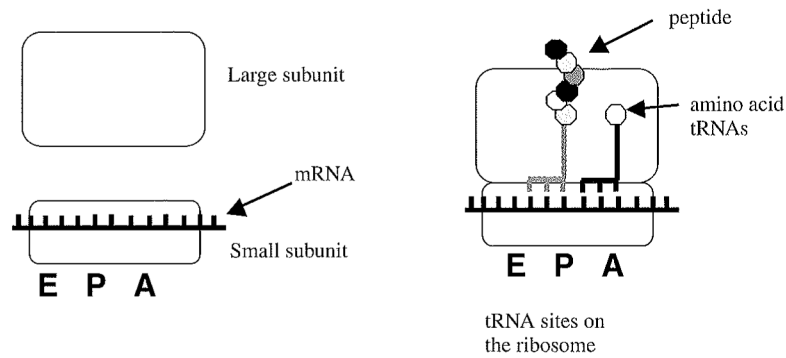


Figure 1. The general outline of the ribosomal structure. Ribosomes are large ribonucleoprotein complexes, composed of both RNA and proteins and are responsible for protein synthesis in all living cells. Ribosomes consist of the small subunit (30S) and the large subunit (50S). These subunits form the 70S ribosome. The large subunit contains peptidyl transferase centre for peptide bond formation between amino acids. The small subunit is responsible for binding the Shine-Dalgrarno sequence of mRNA, interaction with the anticodon stem-loops of tRNAs in A-, P- and E-site and decoding. A-site is where tRNAs with amino acids bind (exception is the first amino acid), P-site contains the nascent peptide and E-site is the ribosomal site harbouring deacylated tRNA on transit out of the ribosome. The mRNA is stalled to separated subunits on the small subunit. Amino acids are bound to tRNAs and the tRNA with the growing peptide chain is in the P-site. Adapted from (Liljas, 2004).

Since the experimental work presented in thesis was performed on bacterial system, I will focus on bacterial translational functional cycle.

1.2 Ribosomal functional cycle

The cycle is sub-divided in three stages: initiation, elongation and termination (**Figure 2**).

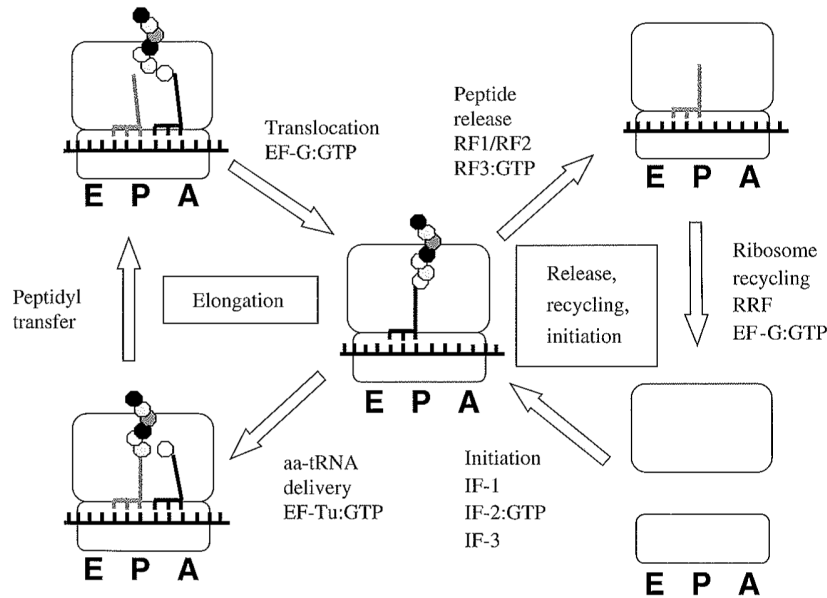


Figure 2. Functional cycle of the ribosome. The start point is on the lower right demonstrating the initiation of protein synthesis from separated subunits and mRNA binds to the small subunit. Bacteria have three Initiation Factors, IFs: IF1, IF2, IF3. IF3 binds to 30S and prevents premature association of subunits; IF1 stimulates activities of IF2 and IF3; IF2 is a GTPase that docks the initiator tRNA and catalyzes subunits association. In each of the elongation cycles one amino acid is added to the growing peptide chain. There are two main Elongation Factors, EFs, in bacteria EF-G and EF-Tu. EF-G is a GTPase and promotes the translocation of the A-site bound peptidyl-tRNA into the P-site; EF-Tu is also a GTPase and in complex with GTP catalyzes aminoacyl-tRNA (aa-tRNA) delivery to the A-site. When a stop codon is exposed in the A-site, the peptide is released. There are two class I Release Factors, RFs, in bacteria. RF1 is specific for codons UAG and UAA, RF2 for UGA and UAA. RF3 is a class II release factor and its function is to remove class I RF-s from the ribosome after they have completed the peptide release. Lastly, the ribosomes are recycled (split into subunits) for a new round of initiation by a concerted action of Ribosome Recycling Factor, RRF, and EF-G. Adapted from (Liljas, 2004).

1.2.1 Translation initiation

Bacterial mRNAs are frequently polycistronic, this means that more than one protein can be translated from one mRNA chain. Translation is initiated by the binding of an mRNA to the free ribosomal small subunits. The initiation of prokaryotic translation requires the assembly of components of translational system, namely: ribosomal 50S and 30S subunits, the mRNA, the initiator tRNA (fMet-tRNA_i^{fMet}), energy source in the form of GTP and three Initiation Factors, IFs: IF1, IF2, IF3 (Gualerzi & Pon, 1990). Initiation factors provide the assembly the Initiation Complex (IC) (Malys & McCarthy, 2011) IF3 acts first and provides the assembly of ribosomal subunits (Gualerzi *et al.*, 1977). IF1 coordinates the activities of IF2 and IF3 (Pon & Gualerzi, 1984). IF2 GTPase delivers the initiator aminoacyl-tRNA and is coordinating the subunit joining (Antoun *et al.*, 2003).

Translational GTPases, such as IF2, are a large family of hydrolase enzymes that can bind and hydrolyze GTP (to use as an energy source). A-site of the ribosome is the point of entry for the aminoacyl-tRNA (except first aminoacyl-tRNA which binds to the P-site). The P-site is where aminoacyl-tRNA is formed in the ribosome and E-site is the exit site for the uncharged tRNA after it gives its amino acid to the growing peptide chain. Large subunit of the ribosome has an exit tunnel for the polypeptide chain.

During translation initiation, the initiation codon coding for methionine codon (AUG or GUG) has to be selected in the ribosomal P-site. In bacteria, the selection of the initiation methionine codon over elongation methionine codons or out of frame AUGs is done through a specific interaction of the so-called Shine-Dalgarno element (SD) of the mRNA with a specific element of the the small subunit ribosomal RNA – so-called anti-Shine-Dalgarno element (a-SD) (Shine & Dalgarno, 1974). In *E. coli* mRNAs typically have the SD sequence GGAGG located 7 ± 2 nucleotides upstream from the initiation codon (McCarthy & Brimacombe, 1994). As a result of the SD:a-SD is the complex formation of the initiation codon is positioned in the ribosomal P-site, ready for interaction with formulated methionine initiator tRNA. Once the pre-initiation complex is properly formed, the 50S subunit associates, GTP is hydrolyzed by IF2 and the initiation factors are released. The elongation phase of translation can begin.

1.2.2 Translation elongation

Elongation of polypeptide chain means addition of amino acids to the carboxyl end of the chain until it is incorporated into the nascent peptide.

At the start of the elongation cycle, the ribosome is in the post-translocation state with fMet-tRNA or a peptidyl tRNA in the P-site. Conformational change from closed form to open form in the A-site allows new aminoacyl-tRNA to bind. When a codon from mRNA binds a cognate tRNA in the A-site, the small subunit undergoes a conformational change from an open to a closed form (Stahl *et al.*, 2002). In the cell, most of the aminoacyl-tRNA is bound to complex EF-Tu GTPase in the complex with GTP which provides the binding to the A-site (Moazed & Noller, 1989). The binding and hydrolysis of GTP takes place in highly conserved G-domain (Scheffzek & Ahmadian, 2005). The P-site has the beginning of the peptide chain of the protein to be encoded and the A-site has the next amino acid to be added in the growing peptide chain (Valle *et al.*, 2003). The growing polypeptide connected to the tRNA is then detached from the tRNA in the P-site. Peptide bond is formed between last amino acid of the polypeptide and the amino acid which is still attached to the tRNA in the A-site (Rodnina & Wintermeyer, 2003). The whole assembly in the A-site is called dipeptidyl-tRNA and P-site is deacylated. The final stage of elongation, the deacylated tRNA and dipeptidyl-tRNA along with its corresponding codons move to the E- and P-sites respectively, and new codon moves to the A-site. This process is catalyzed by the elongation factor G (EF-G) (Spirin, 2002). The deacylated tRNA at the E-site is released from the ribosome as soon as new aminoacyl-tRNA occupies A-site (Schmeing *et al.*, 2003).

The ribosome continues to translate the remaining codons on the mRNA until it reaches a stop codon (UAG, UGA, UAA). This is the beginning of termination.

1.2.3 Translation termination

The termination of translation occurs when one of the three stop codons move into the A-site: UAG, UGA, UAA (Nakamura & Ito, 2003). The stop codon is recognized by specific protein factors called Release Factors, RFs. In bacteria, there are two class I release factors: RF1 and RF2. The Class I release factor RF1 recognizes stop codons UAA and UAG, while RF2

responds to UAA and UGA (Scolnick *et al.*, 1968). The release factors decode the stop codons and hydrolyze the completed peptide from the P-site tRNA. The hydrolysis may be induced directly or indirectly (Nakamura & Ito, 2003). Besides Class I RF proteins some bacteria also have a class II release factor, RF3 (Goldstein & Caskey, 1970). Class II RF removes class I RF-s from the ribosome after they have released a peptide chain from the ribosome. GTP is hydrolyzed on RF3 which helps RF1 and RF2 to dissociate and RF3 itself dissociates (Gao *et al.*, 2007). The recycling step of the translation may begin.

1.2.4 Ribosomal recycling

When the ribosome has reached a stop codon and the peptide is released, the extensive machinery of the ribosome has to be reused. This machinery consists of mRNA with the termination codon at the A-site, an uncharged tRNA at the P-site and the intact 70S ribosome. Since the mRNA is threaded through the tunnel between the subunits and is in intimate contact with the neck region of the small subunit and the deacylated tRNA has tight interactions in the P-site, the subunits would probably have to open up or probably even dissociate. For a new initiation, they have to be separated since the initiation is done with the small subunit alone. The Ribosome Recycling Factor (RRF) participates in this process together with EF-G. After the GTP hydrolysis the ribosomal subunits dissociate (Savelsbergh *et al.*, 2009). The initiation factor IF3 prevents subunits from re-association, releases the deacylated tRNA from the P-site and mRNA from the small subunit (Savelsbergh *et al.*, 2009). The ribosome is ready for the new round of the protein synthesis.

2. The stringent response

"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change" (C. Darwin)

The ability to adapt to environmental changes is the key component of the survival of bacteria and those who don't have that ability face the chance to be eliminated. Regulatory mechanisms which help bacteria to maintain their balanced and rather constant cellular composition mostly occur at the genetic level. One of the most important and the most studied adaptive response to stress conditions is the so-called stringent response.

2.1 General mechanism of the stringent response

One of the stressful conditions bacteria encounter is lack of amino acids or other nutrients in the environment. This, in turn, causes the condition called the stringent response.

During nutrient starvation, *Escherichia coli* elicits the stringent response to conserve energy and re-program its metabolism for survival. Repression of the transcription of stable RNA species like tRNA and rRNA (Deutscher, 2009) and the up-regulation of transcription of genes coding the enzymes involved in amino acid biosynthesis are some of the effects of stringent conditions (Deutscher, 2009).

The stringent response to amino acid starvation is mediated by a protein called the stringent factor or RelA (Chatterji & Ojha, 2001). RelA binds to a ribosome to an unacylated tRNA in the amino-acyl site (A-site) since there is a lack of amino acids. The RelA binds to blocked ribosomes and catalyzes synthesis of an unusual guanosine phosphate, an alarmone called (p)ppGpp. RelA utilizes ATP and GTP or GDP to produce AMP and pppGpp or ppGpp respectively (**Figure 3**) (Wendrich *et al.*, 2002). The alarmone interacts with the RNA polymerase that is responsible for transcription DNA to RNA in the cell and this results in a rapid down-regulation of stable RNA biosynthesis (tRNA and rRNA).

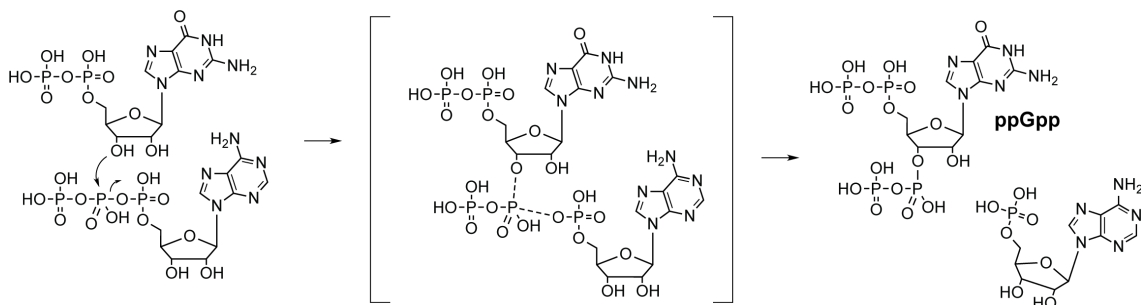


Figure 3. The chemical reaction catalyzed by RelA. The stringent factor RelA binds to stalled ribosomes and converts GTP and ATP into pppGpp by adding the pyrophosphate from ATP onto the

3' carbon of the ribose in GTP releasing AMP. pppGpp is converted to ppGpp by GPP, releasing extra phosphate group.

Bacteria have evolved a range of cell-cell signaling mechanisms to determine population density (quorum sensing) and to initiate certain processes only when a certain number of bacteria is reached. This kind of signaling is referred to as bacterial social behavior and is related to such phenomena as biofilm formation. Biofilms consist of aggregates of multiple cells in close proximity. High population densities, necessary to reach “quorum”, correlate with the deficiency of nutrients and entry into the stationary phase. Therefore it is not surprising that quorum sensing stress- and starvation-pathway seem to be connected with (p)ppGpp regulation (Braeken *et al.*, 2006).

The stringent response has also been detected not only in bacteria but in plants. The best example is *Arabidopsis thaliana*. There were found a functional RelA-SpoT (major proteins in the stringent response) homologue as well as bacterial type RNA polymerase in chloroplasts. When plants were treated with plant hormones such as jasmonic acid and abscisic acid which are known to play a significant role in signal transduction network, an elevated level of ppGpp was noticed (Takahashi *et al.*, 2004). It was suggested that (p)ppGpp was mediating a stress-induced defense system (van der Biezen *et al.*, 2000). The findings propose that the stringent response has been conserved through evolution and thus contributes to the adaptation of plants to environmental changes analogous to that seen in bacteria. Observation suggest that the (p)ppGpp regulatory system is operative in chloroplasts of higher plants (Braeken *et al.*, 2006).

2.2 *E. coli* RelA- and SpoT-mediated metabolism of ppGpp

There are two pathways responsible for modulating the (p)ppGpp level in *E. coli* – RelA- and SpoT-dependent pathways.

Historically, first proteins which were involved in both synthesis and degradation of (p)ppGpp were the products of genes *relA* and *spoT*. Products of these genes proteins RelA and SpoT gave a name to the protein family RelA-SpoT-Homologue, RSH (Atkinson *et al.*, 2011). The *spoT* and *relA* genes have been suggested to evolve separately after gene duplication of an ancestral *rel*-like gene (Mittenhuber, 2001). It was shown that after the amino acid alignment of Rel and SpoT of *E. coli* the identity was only 29 % whereas after the

alignment of SpoT and bifunctional protein Rel of Gram-positive bacteria the results were higher. This suggests that bifunctional Rel is related to SpoT, both have synthetic and hydrolytic activities (Jain *et al.*, 2006).

The first gene encoding the amino acid starvation response in *E. coli* was discovered in sixties by Stent and Brenner (Stent & Brenner, 1961) and dubbed as *relA* – “relaxed” (i.e. unable to respond to amino acid starvation) phenotype A. It has been shown that the initiation of transcription from *relA* gene can occur at two promoters – *relAP1* and *relAP2*. One site is located 178 bp upstream of the RelA translational start site, the second is located 626 bp upstream. Both promoters are shown to be regulated by house-keeping sigma factor σ^{70} . The promoter *relAP1* was suggested to be active during all growth phases but the signal from *relAP2* was transiently induced at the transition state between the exponential growth phase and the stationary phase. It has also been shown that *relAP1* promoter is dependent on an UP-stream-like sequence. It is AT-rich sequence which locates around 40 bp upstream of the transcriptional start-site and enhance promoter recognition by RNA polymerase. *relAP2* seems also to be regulated by CRP (Catabolite Regulation Protein) region which is centered 61 bp upstream from the transcriptional start site (Nakagawa *et al.*, 2006, Metzger *et al.*, 1988).

The second pathway for ppGpp production in *E. coli* is the SpoT-dependent pathway which involves a product from the *spoT* gene. The *spoT* gene was first mentioned by Laffler and Gallant in “spotless” phenotypes where although large quantities of ppGpp accumulated the quantities of pppGpp stayed scarcely detectable (Laffler & Gallant, 1974) SpoT has a ppGpp hydrolysis activity. Although SpoT is responsible for degradation (p)ppGpp in the cell it has a low synthetic activity as well (Xiao *et al.*, 1991). For bacteria that also encode RelA, their bifunctional SpoT enzymes respond to variety of stimuli, including phosphate, carbon and iron starvation, as well as perturbations in fatty acid metabolism (Potrykus & Cashel, 2008). Unfortunately a little is known on molecular mechanisms which trigger the SpoT-dependent synthesis of (p)ppGpp (Magnusson *et al.*, 2005).

Recently two additional factors (YwaC and YjbM) were found to have (p)ppGpp-synthetase activity in *B. subtilis* (Nanamiya *et al.*, 2008).

2.4 (p)ppGpp

The alarmone nucleotides implicated in the stringent response were first called "magic spots" – MSI and MSII (Haseltine *et al.*, 1972). They were identified as guanosine tetra phosphate (ppGpp) and guanosine penta phosphate (pppGpp) (Cashel & Kalbacher, 1970), collectively called (p)ppGpp. After the activation of the stringent factor it triggers the synthesis of ppGpp by donation of β - and γ -phosphates from ATP to GTP or GDP, the major product *in vivo* being pppGpp (**Figure 4**).

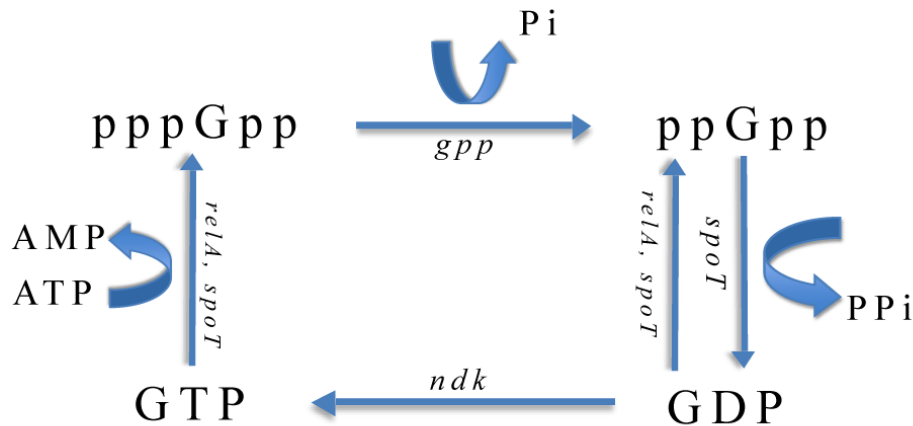


Figure 4. Cellular pathways of (p)ppGpp metabolism. The enzymes responsible for the metabolism are represented by their genes: *relA*, *spoT*, *gpp* and *ndk* (nucleoside 5' diphosphate kinase – catalyzes the exchange of phosphate groups between different nucleoside diphosphates).

5' phosphorylase (GPP) degrades penta phosphate to ppGpp and it binds to RNA polymerase, acting as an effector molecule. RNA polymerase consists of two α and two β subunits, altogether they form the RNAP core ($\alpha_2\beta\beta'$). Sigma factors (σ factors – in *E. coli* seven different) can bind to this core region and give specificity for particular promoter and transcription of specific genes. ppGpp is believed to bind to the β and β' subunits (Toulokhonov *et al.*, 2001). It can act both as positive and negative regulator of transcription. In general, σ^{70} -dependent genes involved in cell proliferation and growth are negatively regulated (Barker *et al.*, 2001), genes involved maintenance and stress defense are positively regulated by the alarmone (Nyström, 2004). The main cellular processes regulated by ppGpp are shown in Table 1.

Table 1. Summary of upregulated and downregulated cellular processes affected by (p)ppGpp

Downregulated	Upregulated
DNA replication	Amino acid biosynthesis
tRNA and rRNA synthesis	Long-term persistence and virulence
Protein synthesis	Universal stress protein synthesis
Ribosome biogenesis	Synthesis of sigma factors
Translation initiation and elongation	Virulence gene expression
Nucleotide biosynthesis	Antibiotic resistance
Phospholipid synthesis	Toxin/antitoxin systems
Oxidative metabolism	Carbohydrate metabolism
Cell division	Chaperones and proteolysis systems
Cell motility	Cyclopropane fatty acid synthesis
Metabolite transport	

3. The stringent response as a target for antibacterial compounds

The goal of every pathogen is to survive in the environment and replicate. To overcome the defense mechanisms of their hosts, bacteria have obtained traits associated with virulence, such as cell or surface invasion, surface attachment and transmission. Numerous pathogens couple their common virulence pathways with more general adaptations, like stress resistance, by integrating dedicated regulators with global signaling networks. Many bacteria rely on alarmones to cue metabolic disturbances and coordinate survival and virulence programs.

It has been shown that response controlled by the alarmone (p)ppGpp is widespread and involves many features important for cell physiology and not only during growth but also during stationary conditions (Mouery *et al.*, 2006).

3.1 Role of the stringent response in bacterial virulence

The appearance of (p)ppGpp in starved cells is correlated with cessation of rRNA synthesis and is referred to as the stringent response (Potrykus & Cashel, 2008). Bacterial and plant cells that experience nutritional stress synthesize ppGpp to initiate global physiological changes. The alarmone generally functions to promote the adaptation and flexibility of bacterial cells faced with adversity.

In heterogeneous environments which are mammalian and plant hosts bacteria have to alter their metabolism and protein repertoire in response to local conditions. Bacterial adaptation can be triggered by changes in nutrient supply, alternations in immune responses or contact with new surfaces. To gain an advantage in changing environmental conditions bacteria can activate specialized secretion systems, motility organelles or adhesins. Adhesins are cell-surface components of bacteria that help bacterial adhesion or adherence to other cells or inanimate surfaces. All these factors help bacteria to get access to nutrients, modulate host cell immune system or migrate to more favorable locations. Altogether they are called virulence factors or regulators. The expression and activity of many virulence regulators are integrated into a global response mediated by ppGpp, thereby coupling pathogenesis to metabolic status (Dalebroux *et al.*, 2010).

Bacteria have evolved a number of cell-cell signaling mechanisms to determine the population density – quorum sensing (QS). This kind of phenomena is related to biofilm formation and is interpreted as bacterial social behavior. QS and biofilms formation are often associated with virulence and infections. Very persistent biofilms which form from bacteria in their stationary phase are a huge problem in medical world, because antibiotics can't target inactive cells (Anderl *et al.*, 2003).

The effect of (p)ppGpp level on virulence determinants have been studied mostly in pathogenic bacteria belonging to the γ -proteobacteria, revealing that the importance of the RelA and SpoT homologue when they occur together in a signal bacterium may differ between the species. For example, RelA fulfills an important role in modulating the ToxR regulon (transcription of transcriptional activator ToxR, as a result production of cholera toxin and the transcription of toxin-coregulated pilus are severely reduced) in *Vibrio cholerae* (Haralalka *et al.*, 2003), SpoT but not RelA regulates virulence in *Salmonella typhimurium* mainly through expression of *hilA* (transcriptional regulator of pathogenic gene expression) and the expression of plasmid-born virulence in response to environmental changes (Pizarro-Cerda & Tedin, 2004) (Song *et al.*, 2004). Moreover, the expression of RelA in *Legionella pneumophila* was shown to induce expression of several virulence traits (Hammer & Swanson, 1999).

In some bacteria such as *Clostridium* and *Bacillus spp.* The response to starvation is to form metabolically inactive endospores which are quite effective form of resistance to starvation

and stress conditions such as heat and dehydration. It is a measure for a long-term survival for Gram-positive bacteria (Piggot & Hilbert, 2004). Entry into sporulation is triggered by the decrease in intracellular GTP nucleotide pools which is converted to (p)ppGpp by RelA (Lopez *et al.*, 1979).

The precise mechanism used by the alarmone (p)ppGpp to regulate virulence has still remained unstudied but it has been noted that it resembles to one found in *E. coli* – biofilm formation is decreased in *spoT relA* mutants (Balzer & McLean, 2002).

3.2 ppGpp analogues as stringent response inhibitors

At present, most of the drugs administered to treat bacterial infections are aimed to protein synthesis, nucleic-acid synthesis, cell wall synthesis or folate synthesis (Walsh, 2003). The stringent response is a potential target for antibiotics because it is crucial for activation of survival strategies, such as sporulation, stationary phase and biofilm formation (Jain *et al.*, 2006). It has been shown that the stringent response mediates antibiotic tolerance in nutrient-limited bacteria (Nguyen *et al.*, 2011). As the stringent response is induced by the accumulation of (p)ppGpp recently Wexselblatt and colleagues started to synthesize compounds which chemical structure is close to (p)ppGpp (Wexselblatt *et al.*, 2008). They have used the crystal structures of RelA analogues. In their experiments they use full-length RelA from *E. coli* which N-terminal domain has (p)ppGpp-synthetase activity and C-domain is ribosome binding domain and the RelA homologue Relseq385 which has a bifunctional N-terminal domain of *Staphylococcus equisimilis*; (p)ppGpp synthetase and hydrolase activities are in two separate catalytic sites and the recombinant protein construct lacks of C-terminal ribosome binding site (Wexselblatt *et al.*, 2012). One of their latest works is the novel antibacterial agent called Relacin (**Figure 5**) which they claim to inhibit *E. coli* RelA *in vitro* and reduce (p)ppGpp production *in vivo* in *Bacillus subtilis*. Ultimately Relacin must perturb the switch into stationary phase and lead to bacterial death (Wexselblatt *et al.*, 2012).

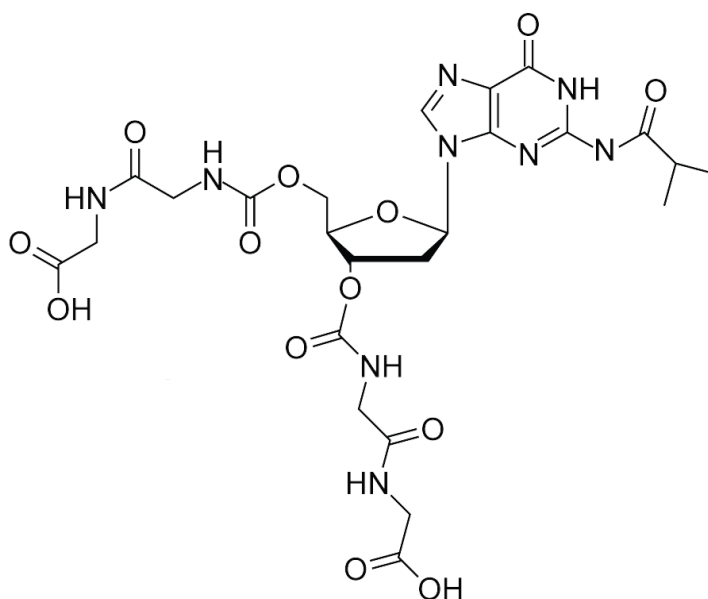


Figure 5. The structure of Relacin. Based on the Rel/Spo crystal structure Wexselblatt and colleagues designed Relacin a 29-deoxyguanosine-based analogue of ppGpp, in which the original pyrophosphate moieties at position 5' and 3' were replaced by glycyl-glycine dipeptides linked to the sugar ring by a carbamate bridge. Modeling the binding of Relacin to the Rel/Spo synthetase site shows that it occupies a considerable volume of the binding pocket and forms a range of hydrogen bonds and hydrophobic interactions, providing structural basis for the inhibitory effect of Relacin (Wexselblatt et al., 2012).

Aims of the project

In our research we focus on defining the molecular mechanisms of the bacterial-mediated RelA stringent response using a combination of *in vivo* and *in vitro* techniques. The stringent response plays a formative role in bacterial physiology, especially in bacterial virulence, biofilm formation and antibiotic tolerance. Besides, we try to complete well-known techniques and elaborate the techniques being still in the process of development.

The specific aims are:

(i) to elucidate of the mechanism of RelA by its product ppGpp, to characterize the RelA mediated stringent response *in vivo* by specifying quantities of nucleotide pools (especially intracellular ppGpp levels) in living cells;

(ii) to characterize RelA mediated stringent response *in vitro* in the presence of the compound Relacin; to confirm the theory that Relacin works as RelA inhibitor by occupying its reactive center and ppGpp synthesis decreases;

(iii) to test Relacin on bacterial culture and define its effect on bacterial growth and status in metabolic changes;

(iiii) to develop, complete and learn new methods in following the RelA mediated stringent response in cell-free system and on bacterial culture to test novel stringent response inhibitors.

The defining mechanisms of SR have two major fields of application: biotechnological and clinical. In the clinical context, an efficient stringent response inhibitor would be a highly desired anti-virulence antimicrobial. In the biotechnological context it could be used for modulation of various stringent response biotechnologically relevant cellular processes like antibiotic and protein production.

Experimental part

1. Materials and methods

1.1 Measuring nucleotide pools in *E. coli*

Bacteria and culture conditions. *E. coli* strain AS 19 was used. It is a strain with defective lipopolysaccharides and is therefore more permeable to antibiotics (Good *et al.*, 2000). Bacteria were grown in MOPS media (Neidhardt *et al.*, 1974) that consisted of 0.4% glucose, 1xMOPS concentrate (**Table 2**), 9.52 mM NH₄Cl and 1.32 mM K₂HPO₄ (the concentrate is made without ammonium and phosphate to induce nitrogen and phosphate starvation if necessary). Antibiotic mupirocin was added at 0.3 µg/ml [that is 3 times of our determined Minimal Inhibitory Concentration (MIC) for AS19].

Table 2. 10xMOPS concentrate

Components	Concentration
K ₂ HPO ₄	1.32 mM
NH ₄ Cl	9.52 mM
MgCl ₂	0.523 mM
K ₂ SO ₄	0.276 mM
FeSO ₄	0.010 mM
CaCl ₂	5*10 ⁻⁴ mM
NaCl	50 mM
MOPS	40 mM
Tricin	4 mM
Microelements	
(NH ₄) ₆ (MO ₇) ₂₄	3*10 ⁻⁶ mM
H ₃ BO ₃	4*10 ⁻⁴ mM
CoCl ₂	3*10 ⁻⁵ mM
CuSO ₄	10 ⁻⁵ mM
MnCl ₂	8*10 ⁻⁵ mM
ZnSO ₄	10 ⁻⁵ mM

Growing and collecting cells for nucleotide extraction. We started an overnight (O/N) culture in 3 ml of 0,4% Glu MOPS transferred to 37 °C shaker (180-220 rpm). Next day, we inoculated 200 ml prewarmed MOPS in 1 l flask from O/N culture; dilution was 1:100 (so we added 2 ml of O/N culture). Culture was grown at 37°C shaker until OD600 was 0.5. Usually it took up to 5 hours.

The next step was to collect cells. We poured cell cultures onto formic acid (final concentration 1 M) and transferred the tubes immediately into liquid nitrogen. The samples were transferred to -80 °C to store the samples for further preparation and analysis.

Nucleotide extraction and sample preparation for HPLC. Samples from -80 °C were thawed at 37 °C water bath for 10 minutes. It was important not to let samples to get too much heat, as nucleotides were stable only in cold formic acid. After thaw, nucleotides were extracted for 30 minutes on ice with frequent vortexing. Insoluble material was then pelleted at 7000 G for 7 minutes at 4 °C (Sigma 4K15C centrifuge) and supernatants were filtrated with 0.2 µm syringe-filter.

For the enrichment of nucleotides, BioLogic LP (BioRad) system at 4°C refrigerator was used with a 1 ml Q-sepharose Fast Flow (GE Healthcare) column matrix. Column was equilibrated with cold mQ H₂O at highest flow rate in purge regime (6-7 ml/min). Sample was diluted 20 times in cold mQ water and loaded onto the column at 5-6 ml/min. After sample was loaded, column was washed with about 15 mL of water. Next, column-bound material was eluted with 2 M LiCl in 25 mM Tris pH 8 with a flow rate of 1 ml/min. Elution was followed at 254 nm, after about 3 minutes a peak appeared, we waited for another minute for the fraction to reach the sample collector and collected the fraction for 4-5 minutes. After collection, column was washed with 5-10 ml of 2 M LiCl in 25 mM Tris pH 8. When not in use, column was stored in 20 % ethanol.

For the precipitation of nucleotides, 4 volumes of 96 % ethanol (-20 °C) and 4 µl of ice-cold 1 M K₂HPO₄ were added to collected fraction. The salt was included to help the formation of precipitate and to ease the handling of precipitate. Sample was left to precipitate over night at -20 °C. Next day, sample was centrifuged for 20 minutes at 5525 G at 4 °C using a swing-out rotor (centrifuge Sigma 4K15C) to collect the precipitate. Supernatant was discarded and precipitate was washed with 5 ml of 70 % cold ethanol and centrifuged for another 20 minutes at 5525 G at 4 °C. Supernatant was again discarded and precipitate was first dried by short lyophilization (about 20 min) and then dissolved in 400 µl of cold mQ with heavy vortexing. The sample was transferred to 1,5 ml Eppendorf and centrifuged at maximum RPM (about 14,000-16,000 G) for 20 minutes at 4 °C to remove extra salt and other insoluble material, as

salt may cause additional peaks when running HPLC. The supernatant (about 350 μ l) was collected to new tube that was pre-chilled on ice (**Figure 6**).

HPLC (High Pressure Liquid Chromatography) assay of nucleotides. HPLC was performed by anion-exchange chromatography on Agilent 1100 system with a diode array detector. Nucleotides were separated on 4.6 by 150 mm S5 SAX column either from Phenomenex (Sphereclone S5 SAX) or from Waters (Spherisorb S5 SAX) with latter one giving slightly better resolution. Analytical column was equipped with a Phenomenex SAX guard column and chromatography was performed at 27 °C. Guanosine nucleotides were detected at 252 nm and adenosine nucleotides at 259 nm with a bandwidth of 4 nm. At a flow rate 1 ml/min, a linear gradient of 0:100 to 100:0 (A:B) was run for 30 min, from 30 to 45 min 0:100 was held. Buffer A was 0.05 M ammonium phosphate (pH 3.4) and buffer B was 0.5 M ammonium phosphate (pH 3.4). Injection volume was mostly 100 μ l.

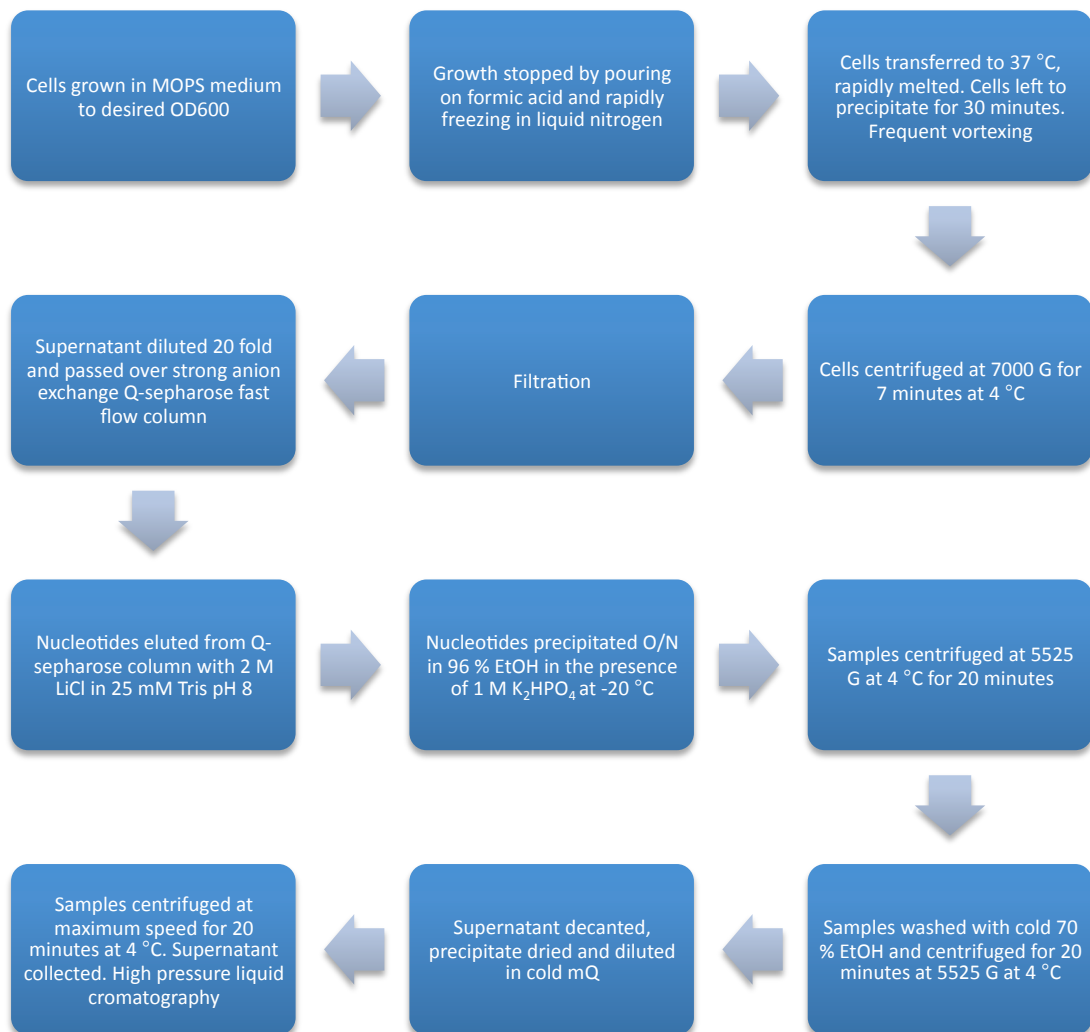


Figure 6. The main steps of the nucleotide sample preparation. Those steps include growing the bacteria in MOPS minimal medium adding 0.4 % glucose, filtration and dilution the sample 20 fold and passing over SAX Q-sepharose FF column. The samples are eluted from column with 2 M LiCl in 25 mM Tris pH 8, precipitated O/N in 96 % ethanol in the presence of 1 M K_2HPO_4 at $-20\text{ }^\circ\text{C}$. Samples are centrifuged, washed with cold 70 % ethanol. Finally samples are collected in mQ. Then run on HPLC.

1.2 *In vitro* stringent response assay

To test Relacin effect on stringent response we monitored how it affects the RelA mediated ppGpp synthesis. Several reaction mixes were prepared with $0.5\text{ }\mu\text{M}$ 70S ribosomes, $100\text{ }\mu\text{M}$ ppGpp, $300\text{ }\mu\text{M}$ $(^3\text{H})\text{GDP}$ (American Radiolabelled Chemicals), 0.1

μ M RelA (purified from *E. coli* strain BL21 (DE3) cells containing pET24b plasmid and kanamycin resistance) and different concentrations of Relacin (from 0 to 5 mM) in 1x Polymix (PM) buffer (25 mM Hepes pH 7.5, 15 mM MgCl₂, 0.5 mM CaCl, 95 mM KCl, 5 mM NH₄Cl, 8 mM putrescine, 1 mM spermidine, 5 mM K₃PO₄ pH 7.3 and 1 mM 1,4-dithioerythritol).

After incubating mixes for two minutes at 37 °C, the reaction was started by adding 1 mM ATP and carried out at 37 °C. After the timepoints had been taken, the reaction was stopped with the addition of Killmix (2 parts of 10 mM GDP, 10 mM GTP to 5 parts of 100% Formic acid). One sample should not contain more than 30% Formic acid in the end. To separate nucleotides 2D-TLC (Thin Layer Chromatography) method was used. The samples were loaded onto Polygram Cel 300 PEI/UV₂₅₄ precoated TLC sheets (Mecherey-Nagel) and the chromatography ran in 0,5 K₂HPO₄ TLC buffer. The positions of ppGpp and its substrate, GDP, were located and those parts of the TLC sheets were cut out. Then they were added to scintillation cocktail ScintiSafe3 (Fischer Scientific) and counted for (³H) radioactivity using Perkin Elmer Tri-Carb 2810TR Scintillation Analyzer.

1.3 Effect of Relacin on *E. coli* growth

On the first day we harvested three *E. coli* strains – AS19 (with hyper-permeable membrane), *E. coli* wild-type strain BW25113 and the mutant strain missing RelA protein Δ RelA, and grew them over-night at 37 °C. We started three *E. coli* cultures in 3 ml of M9 medium (CaCl₂, MgSO₄, M9 salts (**Table 3**), glucose as a carbon source), from LB plates to grow for 17 hours in 37 °C shaker to prolong their stationary phase. Next day cultures were diluted to final concentration OD₆₀₀ 0.05. For every strain we did three separate dilutions. To AS19 we added 1 mM and 2 mM Relacin. We didn't add anything to wild-type and mutant strain. We put samples to 96 well-plate, 100 μ l of every dilution. Borders of the plate were filled with the medium M9 to measure "blank" sample and later count results taking "blank" measurement into account. Plate was closed, properly wrapped with parafilm and placed into the microplate reader (FLUOstar Omega). Basic settings for the protocol are given in Table 4.

Table 3. 10xM9 salts

Components	
KH ₂ PO ₄	22
NaCl	9
NH ₄ Cl	19
Na ₂ HPO ₄	42
MgSO ₄	1
CaCl ₂	0,09

Table 4. Basic settings for micro-plate reader FLUOstar Omega

Basic settings	
Measurement type	Absorbance
Microplate name	GREINER 96 F-BOTTOM (light wave path)
Number of cycles	130
Cycle time	7 minutes
Number of flashes per well	30
Excitation	600
Shaking frequency (rpm)	700 (maximum)
Shaking mode	orbital
Additional shaking time	300 seconds after each cycle
Reading direction	→
Target temperature (°C)	37
Absorbance path length correction volume (μl)	100

2. Results

The experimental work is mainly based on description of function of the stringent factor, the protein called RelA. RelA is responsible for incorporating main nucleotides ATP and GTP or GDP into the alarmone called (p)ppGpp during nutrient starvation of bacteria (Chatterji & Ojha, 2001). Therefore, RelA plays an important role in formation of quantities of nucleotide pools during the stringent response.

During the experiments we complemented the method of measuring the quantity of nucleotide pools in *E. coli* by Buckstein and colleagues, we found ways to elaborate it considerably. Next, we applied our new method by testing a novel antibacterial agent, ppGpp analogue Relacin on *E. coli in vivo*. Relacin effect on RelA was also studied *in vitro*.

Wexselblatt and colleagues showed that Relacin inhibits *E. coli* RelA *in vitro* and prevent *B. subtilis* sporulation . In current work, for *in vitro* studies, we used *E. coli* RelA but *in vivo* studies were carried out with *E. coli* strain AS19 that has defective LPS. AS19 was used because *E. coli*, as all Gram-negative bacteria, has an outer membrane and periplasmic structures that prevent many compounds to enter the cell.

2.1 Measurement of nucleotide pools *in vivo*

In order to study stringent response *in vivo* one has to follow nucleotide levels, for that, we started from a method developed by Buckstein and colleagues (Buckstein et al., 2008) and elaborated on that considerably. Most notably, we saved sample processing time and increase selectivity for nucleotides by LiCl-ethanol precipitation strategy with K_2HPO_4 as co-precipitate.

To test out our new bacterial nucleotide pool quantification method, we sought to observe the effects on RelA with added antibiotic mupirocin (3 fold minimal inhibitory concentration) which inhibits tRNA-synthetase and protein synthesis (Reiss *et al.*, 2012). We could follow the level of the alarmone ppGpp elevated in cells (**Figure 7**).

We found that 3 times MIC mupirocin causes very fast ppGpp accumulation in AS19 cells, so that ppGpp levels reached plateau already in five minutes.

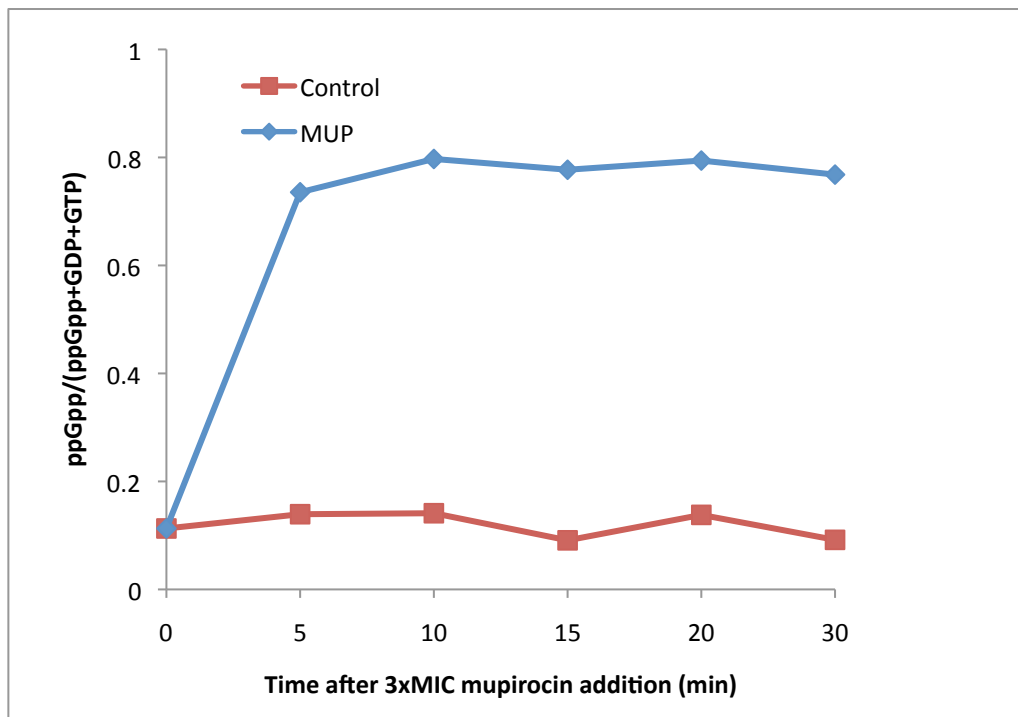


Figure 7. Induction of ppGpp synthesis in AS19 *E. coli* in the presence of mupirocin. Cells were grown in 0,4 % glucose MOPS at 37 °C and 3xMIC of mupirocin was added when cells reached OD₆₀₀ 0.5. As a result, the level of ppGpp elevated rapidly and acquired a plateau in the concentration of ppGpp. As a control we used cells where we didn't add any mupirocin.

2.2 Effects of Relacin on *E. coli* RelA in vitro

The RelA mediated stringent response is at the heart of bacterial adaption to starvation and stress, producing the alarmone ppGpp which globally reprograms transcription, translation and replication. To investigate the biological activity of the compound Relacin Wexselblatt and colleagues evaluated its inhibitory potential on the (p)ppGpp synthetase activity of RelA. We tried to repeat the experiment to test the results presented in the article (Wexselblatt et al., 2012) (Shyp *et al.*, 2012).

The protein RelA was purified from *E. coli* strain BL21 (DE3), using the plasmid pET24b, the strain had the kanamycin resistance. To inhibit RelA we used a ppGpp analogue antibacterial agent Relacin. The *in vitro* assay was developed by Hauryliuk and colleagues (Shyp et al., 2012). The main reaction was carried out in polymix. We had a stock of relacin which was diluted in mQ, we re-diluted it in polymix as the main reaction was carried out in polymix to avoid pH misbalance. We used liquid scintillation analyzer to count the conversion of GDP to ppGpp. Process of reaction was quantified as $^3\text{H-GDP}$ to $^3\text{H-ppGpp}$ conversion, ranging from 0 (no $^3\text{H-ppGpp}$ is produced) to 1 (all the $^3\text{H-GDP}$ is converted to $^3\text{H-ppGpp}$).

We calculated the conversion of GDP to ppGpp and plotted it against the reaction time (**Figure 8**). Next we calculated turnover of ppGpp per RelA molecule per minute and displayed it against Relacin concentration (**Figure 9**).

As a result displayed on the chart we can see that conversion of GDP to ppGpp does decrease over time as concentration of Relacin increases in the reaction mixture. There is almost no conversion of GDP to ppGpp in the presence of 5 mM Relacin. This means that Relacin binds to RelA and does not allow the synthesis of ppGpp.

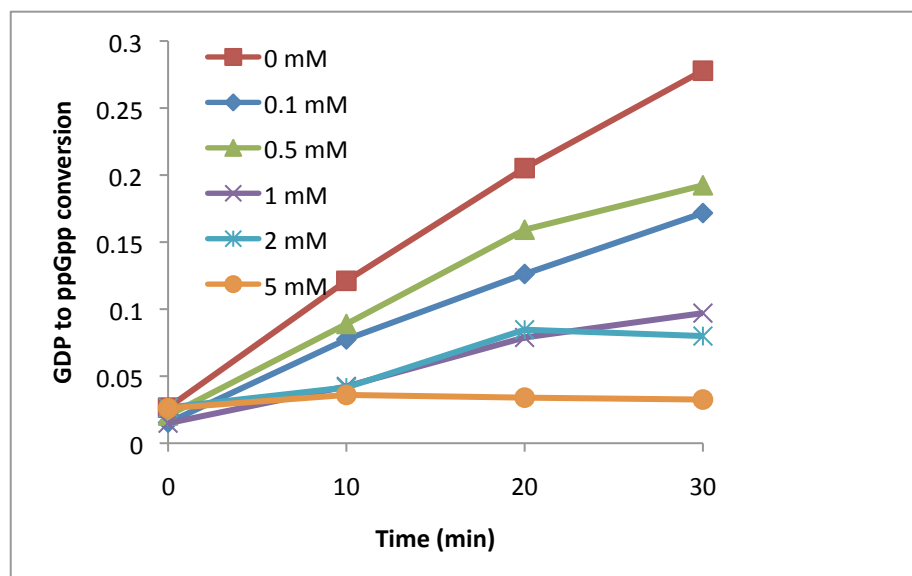


Figure 8. Conversion of GDP to ppGpp in the presence of Relacin. The main reaction was carried out in polymix, where 70 S ribosomes, ppGpp, 6His RelA, and tritiated GDP at 37 °C. We activated reaction with ATP and stopped reaction with killmix. The time points were taken at 5, 10, 20 and 30 minutes and concentrations of Relacin were 0, 0.1, 0.5, 1, 2 and 5 mM. There is a correlation between concentration of Relacin and conversion of GDP to ppGpp. The strongest effect is shown in the presence of 5 mM Relacin and lowest is without Relacin. ppGpp analogue Relacin does inhibit RelA *in vitro* and prevents the synthesis of the alarmone ppGpp where GDP is used as a substrate. Both GDP and ppGpp are tritiated.

To show that inhibition of ppGpp synthesis is in fact caused by Relacin binding to RelA we calculated the turnover of ppGpp per RelA molecule per minute. Following chart shows that as the concentration of Relacin increases the activity of reaction – synthesis of ppGpp by RelA decreases. These results support the claim of Wexselblatt that Relacin in fact binds to the stringent factor and disturbs RelA to produce ppGpp (Wexselblatt et al., 2012).

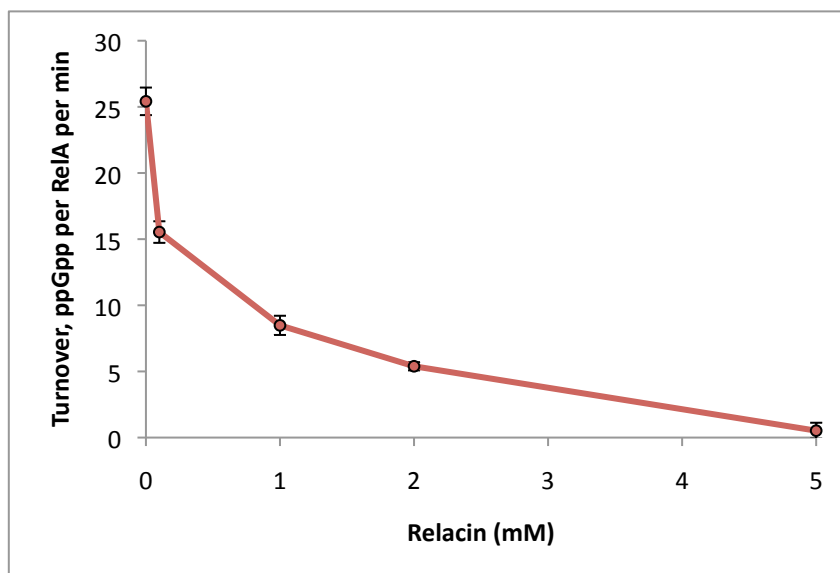


Figure 9. Inhibition of RelA by Relacin in vitro. The main reaction was carried out in polymix, where 70 S ribosomes, ppGpp, 6His RelA and tritiated GDP at 37 °C. We activated reaction with ATP and stopped reaction with killmix. The time points were taken at 5, 10, 20 and 30 minutes and concentrations of Relacin were 0, 0.1, 0.5, 1, 2 and 5 mM. In growing concentrations of ppGpp analogue Relacin the RelA activity decreases and synthesis of ppGpp per minute slows down. In the presence of 5 mM Relacin the reaction is almost stopped and no ppGpp is synthesized. Errors bars stand for standard deviation.

2.3 Effect of Relacin on *E. coli* growth

For this experiment instead of *B. subtilis* we used *E. coli* strain AS19. As a control we used *E. coli* wild-type strain BW25113 and *E. coli* mutant strain Δ RelA which is missing protein RelA. We used M9 minimal medium.

The hypothesis was that the strain with missing RelA enters exponential phase later than wild-type strain but grows as fast as wild-type strain. The same effect we hoped to see on AS19 in presence of Relacin, because Relacin should inhibit the protein RelA.

At the same time we observed the growth curves of AS19 in the presence of Relacin. Relacin inhibits synthesis of ppGpp, the lower is the concentration of ppGpp the faster it grows. Therefore, the higher is the concentration of Relacin the lower is the concentration of ppGpp and bacteria grows faster. Moreover, Relacin should prevent bacteria to go to stationary phase.

The results weren't those we expected (**Figure 10**). Even though the mutant strain Δ RelA did enter exponential phase later than wild-type strain, the strain AS19 in the presence of Relacin didn't show any drastic changes comparing to the sample without Relacin, especially comparing the concentrations of 1 mM and 2 mM. So we conclude that Relacin doesn't have any effect on *E. coli in vivo*. There may be many explanations but the most likely one is that Relacin simply didn't enter the cell. Mutant strain Δ RelA and wild-type strain BW25113 didn't reach stationary phase.

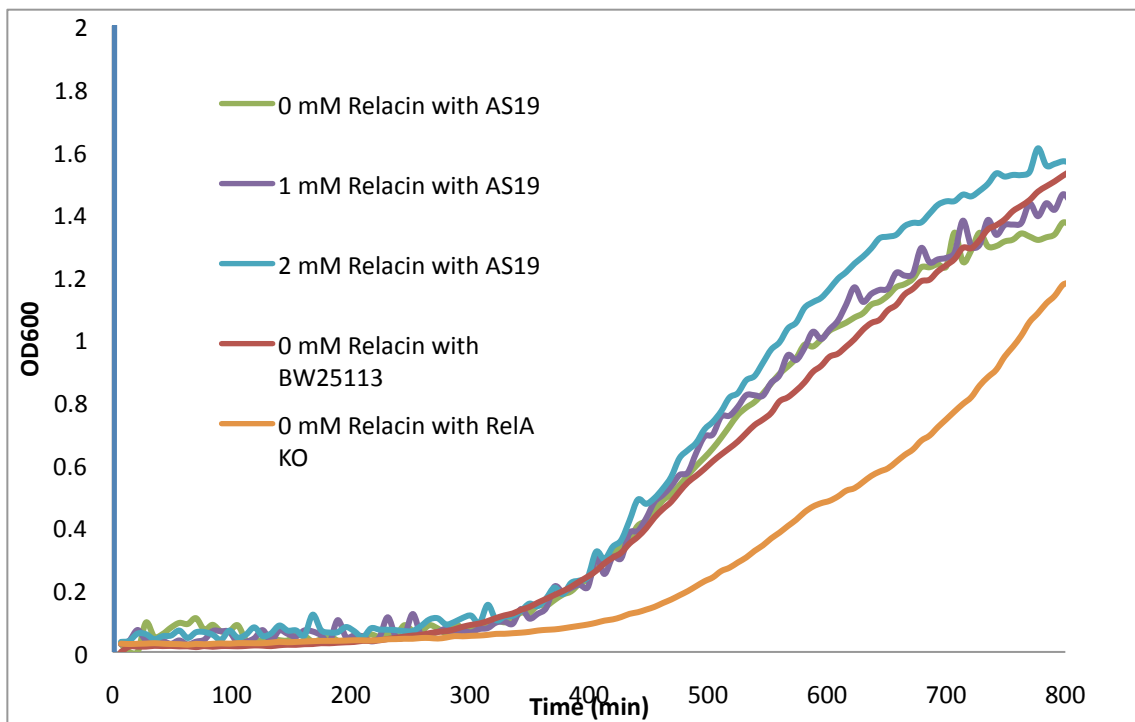


Figure 10. Effect of Relacin the growth of *E. coli* AS19. Cells were grown in M9 minimal medium at 37 °C. Strain AS19 was grown in the presence of Relacin – 1 mM and 2 mM. As a control we used *E. coli* wild-type strain BW25113, mutant strain missing protein RelA Δ RelA (or RelA KO) and strain AS19 without Relacin. The wild-type strain and mutant strain didn't enter the stationary phase. AS19, on the contrary, had entered the stationary phase. There are no drastic changes in growth speed of cells in the presence of Relacin (1 mM and 2 mM). The Relacin doesn't affect the growth of *E. coli* strain AS19.

Discussion and outlook

The main targets for antibiotics in bacterial cells are protein synthesis, folate acid synthesis, nucleic acid synthesis and cell wall synthesis (Walsh, 2003). In the last 40 years a limited number of new antibiotic classes have been introduced and dramatical increase in antibiotic resistance is compromising the efficiency of available compounds. One promising direction is targeting bacterial systems responsible for bacterial virulence. The main advantage of this strategy is lower selectivity pressure due to non-essentiality of the target and thus, slower development of resistance. The stringent response seems to be the perfect target being one of the central bacterial regulatory mechanisms. Several ppGpp analogues have been shown to inhibit the stringent response in cell-free assays; the ppGpp analogue Relacin was shown to inhibit ppGpp-dependent sporulation in bacterial cultures of *Bacillus subtilis* and ppGpp production by the central molecule of the stringent response – RelA.

We tried to repeat experiments done with Relacin *in vitro* and *in vivo*. For *in vitro* stringent response assay we used elevating concentrations of Relacin on purified RelA protein by activating the reaction with ATP and stopping with Killmix, later counting the production of ppGpp on Liquid Scintillation Analyzer. Indeed, in higher concentrations of Relacin the synthesis of ppGpp almost stopped and RelA activity almost stopped. This confirms that Relacin inhibits RelA in a cell-free system. We can conclude that ppGpp analogue which was synthesized using RelA homologues occupies the active center of the protein RelA and prevents the synthesis of ppGpp.

Original experiments with Relacin *in vivo* were done on Gram-positive bacteria *Bacillus subtilis*. We used *E. coli* strain with a hyper-permeable membrane hoping the compound will penetrate cells. We didn't see any drastic effects on bacterial growth in the presence of Relacin. The problem may be addressed to the fact that Relacin didn't enter cells and small fluctuations may be caused by the treatment of samples.

Above the experiments described in the original paper we repeated (Wexselblatt et al., 2012), we managed to improve a method on collecting nucleotides and measuring the nucleotide pools in living cells. We used an antibiotic mupirocin which has been shown to elicit the

stringent response, thus the levels of ppGpp must elevate. The results showed the rapid accumulation of ppGpp. Our pre-liminary results has shown that in wild-type strain the accumulation of ppGpp is slower and plateau is reached later.

Those experiments are a cornerstone for the whole system of developing novel antibacterial agents since the inhibitors of the stringent factor cause the decrease in the concentration of ppGpp in the cell which in turn causes the effects on other nucleotide pools. The effects must be studied on single proteins and on bacterial physiology *in vivo*. Changes in levels of even standard nucleotides (for example GDP or ATP) may affect bacterial physiology.

The aim of our project is to develop new stringent response inhibitors and because of that our main target is now investigation of effects of Relacin on *B. subtilis* in bacterial culture and determination of nucleotide quantities *in vivo* in the presence of Relacin.

There are several new ppGpp derivatives which are in process of testing on Rel-A mediated stringent response, for example an adenine analogue of ppGpp ppApp and pGp, a ppGpp analogue with reduced phosphate groups. A 6-G-thio derivative of ppGpp was identified as a strong inhibitor for RelA.

Summary

The stringent response is central bacterial regulatory pathway mediated by the alarmones collectively called (p)ppGpp. In the cell the levels of (p)ppGpp are controlled by the RelA-SpoT Homologue enzymes which in turn synthesize or degrade the alarmone in response to different stress stimuli, like nutrient starvation, fatty acid deficiency, heat shock etc. ppGpp modulates a number of various enzymes: RNA polymerase, translational GTPases and more importantly it activates ppGpp-synthetic activity of RelA itself.

Since the stringent response regulates bacterial virulence and antibiotic tolerance, it is promising to develop specific inhibitors which may lead to designing novel antibacterial agents. For example, the stringent response is bound to biofilm formation which is a big problem in a medical world since antibiotics can't target non-dividing cells.

In this work we investigated the effects of the novel antibacterial agent, ppGpp analogue, Relacin which was synthesized using the crystal structure of the RelA protein. The original work was done using *B. subtilis* bacterial culture and the RelA purified from *E. coli*. We found that Relacin inhibits work of the RelA *in vitro*. In the growing concentrations Relacin did inhibit the synthesis of ppGpp. For *in vivo* system we used a *E. coli* strain with hyper-permeable membrane and didn't see any changes in bacterial growth in cultures with Relacin. In addition, we managed to complete a method for extracting nucleotides from cells to measure nucleotide pools on high pressure liquid chromatography.

The outlook of our project is to test Relacin on *B. subtilis* bacterial culture and try to collect and measure nucleotides in the presence of Relacin. Broader aim is to try testing synthesized ppGpp analogues on the stringent factor RelA and develop efficient and specific ppGpp stringent response inhibitors with good pharmacokinetic features. This work may be a threshold for developing a new group of drugs.

ppGpp-sarnased poomisvastuse pärssijad: *in vivo* ja *in vitro* iseloomustamine

Katarina-Beata Saltõkova

Resümee

Efektiivsemaks toimimiseks ja ellujäämiseks peavad bakterid olema suutelised tunnetama muutusi ümbritsevas keskkonnas ja kohanema muutustega kiiresti. Toitainete puuduses on mõistlik lülitada geeniekspressiooni ümber hädavajalikele mehhanismidele või kohasust tõstvatele füsioloogistele reaktsioonidele. Üks tähtsamaid vastuseid näljastressitingimustele on poomisvastus ehk *stringent response*. Seda vahendavad GDP ja GTP derivaadid, guanosiin tetra- ja pentafoostaat, (p)ppGpp. (p)ppGpp metabolismiga tegelevad Rel/Spo homologide perekonda kuuluvad valgud, mis sünteesivad ja lagundavad (p)ppGppd. (p)ppGpp ise mõjutab mitmeid ensüüme rakus, näiteks RNA polümeraasi, translatsioonilisi GTPaase ja ka RelA valku ennast, olles iseenda sünteesi positiivseks regulaatoriks.

Poomisvastus on tihedalt seotud bakterite virulentsusega ja antibiootikumide taluvusega, mis on üha kasvav probleem meditsiinimaailmas; bakterite resistentsete spooride teke on ppGpp-sõltuv protsess, poomisvastus on tihedalt seotud biofilmide tekkimisega. Antibiootikumidel ei ole mõju mittejagunevatel ehk statsionaarses faasis olevatel rakkudel. Seepärast on poomisvastuse mehhanismi uurimine esmatähtsal kohal just selle pärssimise seisukohalt. Viimasel ajal on hakatud looma uusi aineid, mis struktuurilt sarnanevad ppGpp molekuliga. Üheks selliseks aineks on uus antibakteriaalne mõjur Relacin. Relacin sünteesiti kasutades RelA valgu kristallstruktuuri. Suuremates kogustes inhibeerib Relacin RelAd rakuvabas süsteemis ja ppGpp sünteesi elusates kultuurides. Originaalartiklis kasutati *E. colist* eraldatud RelA valk ja *in vivo* katsed viidi läbi *B. subtilise* tüvel. Antud töös kordasime osasid katseid, bakteritüvena kasutasime *E. coli* tüve defektse lipopolüsahhariidse membraaniga, mis võimaldab ainetel võimalikult kergesti rakku pääseda. *In vitro* süsteemis inhibeeris Relacin RelA valku; bakterikultuuris ei olnud Relacini mõjutused märgatavad, mille põhjuseks võib olla aine mitte pääsemine rakku. Antud katsete kõrval suutsime täiendada nukleotiidide eraldamise meetodit rakkudest. Lihtsamate nukleotiidide (nagu näiteks ATP või GDP) reservuaarid rakus võivad oluliselt mõjutada bakterite füsioloogiat, seepärast on nende mõõtmine tähtsaks teguriks kirjeldamiseks bakterites toimuvaid füsioloogilisi muutusi.

Meie edasine eesmärk on testida Relacini *B. subtilise* tüvel ning võrrelda Gram-positiivsete ja Gram-negatiivsete bakterite vastust antud ainele, jälgides bakterite kasvu ja nukleotiidide reservuaaride muutumist. Relacin pole ainus sünteesitud aine oletatavalt RelA valgu inhibeerivate omadustega, hiljuti on nimekirjale lisandunud adenosini analoog ppApp ja ppGpp analoog, millel puuduvad kaks fosfaatrühma, pGp. Kavas on testida ka neid aineid nii rakuvabas süsteemis, rakukultuuris jälgida bakterite kasvu ja mõju nukleotiididele. Pikemas perspektiivis loodetakse selliste katsete käigus luua heade farmakokineetiliste omadustega antibakteriaalsed ained, mis võiksid tulevikus levida edasi ravimitööstusesse ning luua uue antibiootikumide mõjuspektri.

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