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PAVEL KUDRIN

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LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following original publications, referred to by their Roman numerals.

I. Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. Shyp V, Tankov S, Ermakov A, **Kudrin P**, English BP, Ehrenberg M, Tenson T, Elf J, Hauryliuk V. EMBO Rep. 2012 Sep;13(9):835–9. doi: 10.1038/embor.2012.106. Epub 2012 Jul 20.

II. From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of *Enterococcus faecalis*. Gaca AO, **Kudrin P**, Colomer-Winter C, Beljantseva J, Liu K, Anderson B, Wang JD, Rejman D, Potrykus K, Cashel M, Hauryliuk V, Lemos JA. J Bacteriol. 2015 Sep;197(18):2908-19. doi: 10.1128/JB.00324-15. Epub 2015 Jun 29.

III. Molecular mutagenesis of ppGpp: turning a RelA activator into an inhibitor. Beljantseva J*, **Kudrin P***, Jimmy S, Ehn M, Pohl R, Varik V, Tozawa Y, Shingler V, Tenson T, Rejman D, Hauryliuk V. Sci Rep. 2017 Feb 3;7:41839. doi: 10.1038/srep41839.

IV. Subinhibitory Concentrations of Bacteriostatic Antibiotics Induce *relA*-Dependent and *relA*-Independent Tolerance to β-Lactams. **Kudrin P***, Varik V*, Oliveira SR, Beljantseva J, Del Peso Santos T, Dzhygyr I, Rejman D, Cava F, Tenson T, Hauryliuk V. Antimicrob Agents Chemother. 2017 Mar 24;61(4). pii: e02173-16. doi: 10.1128/AAC.02173-16. Print 2017 Apr

Author's contribution:

I. Performed several experiments for the stability of *E. coli* RelA.

II. Purified recombinant protein, purified nucleotides, performed most of the *in vitro* experiments, analyzed results and participated in writing the manuscript.

III. Performed most biochemical experiments, analyzed results and contributed in writing the manuscript.

IV. Performed most biochemical experiments, analyzed results and contributed in writing the manuscript.

Not included in current thesis:
Negative allosteric regulation of *Enterococcus faecalis* small alarmone synthetase RelQ by single-stranded RNA. Beljantseva J*, **Kudrin P***, Andresen L, Shingler V, Atkinson GC, Tenson T, Hauryliuk V. Proc Natl Acad Sci U S A. 2017 Apr 4;114(14):3726-3731. doi: 10.1073/pnas.1617868114.

***** **Denotes equal contribution**

LIST OF ABBREVIATIONS

SR	stringent response
c-di-GMP	cyclic diguanosine monophosphate
cAMP	cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA
Crp	cAMP receptor protein
(p)ppGpp	guanosine (penta)tetraphosphate
KO	knockout
MS	magic spot
ATP	adenosine triphosphate
GTP	guanosine triphosphate
GDP	guanosine diphoshpate
RNAP	RNA polymerase
mRNA	messenger RNA
IF2	initiation factor 2
EF-Tu	elongation factor, temperature unstable
EF-G	elongation factor G
r-protein	ribosomal protein
polyP	polyphosphate
LdcI	lysine decarboxylase, inducible
NTP	nucleoside triphosphate
MAR	methionine, alanine, arginine
cryo-EM	cryo-electron microscopy
IMP	inosine monophosphate
XMP	xanthosine monophosphate
HprT	hypoxanthine phosphoribosyltransferase
RSH	RelA/SpoT homologue
PVC	*Planctomycetes, Verrucomicrobia, Chlamydiae*
SAS	small alarmone synthetase
SAH	small alarmone hydrolase
SYNTH	synthesis domain
HD	hydrolysis domain
TGS	Threonyl-tRNA synthetase, GTPase, SpoT
CC	conserved cysteins
ACT	Aspartokinase, Chorismate mutase, TyrA
CTD	C-terminal domain
ZFD	zinc-finger domain
RRM	RNA recognition motif
SRL	sarcin-ricin loop
ACP	acyl-carrier protein

Mesh1	metazoan SpoT homolog 1
EHEC	enterohemorragic *Escherichia coli*
UPEC	uropathogenic *Escherichia coli*
WT	wild type
TA	toxin-antitoxin
SHX	serine hydroxamate
THF	tetrahydrofolate
Fus	fusidic acid
Cam	chloramphenicol
Tet	tetracycline
Ts	thiostrepton
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Ni-NTA	Ni nitrilotriacetic acid
TLC	thin layer chromatography
DMSO	dimethyl sulfoxide
TFE	2,2,2-trifluorethanol
v/v	volume/volume
w/v	weight/volume
DLS	dynamic light scattering
HPLC	high performance liquid chromatography

INTRODUCTION

Bacteria are the most abundant living organisms on Earth. Through billions of years of evolution they developed numerous adaptation mechanisms that allow them to survive in constantly changing environmental conditions. Bacteria protect themselves from various environmental challenges by entering a dormant state, by acquiring resistance to antibiotics, forming biofilms etc. All these varied adaptation mechanisms rely on the enzymatic activity of specific proteins that sense and response to stress – and that renders these proteins promising targets for the development of novel antibacterial agents.

The current work elucidates one of bacterial adaptive mechanisms called the stringent response that is orchestrated by RelA SpoT Homologue (RSH) enzymes in nutritionally poor environment, upon heat shock or cell wall damage. The varying level of effector-molecule of the stringent response – a highly charged nucleotide alarmone (p)ppGpp – is the key mediator of the survival program launched by bacteria during stringent response. In order to turn off the stringent response and increase the susceptibility of bacteria towards antibiotics, one can either target the RSH enzymes themselves or compromise the signaling nucleotide (p)ppGpp direclty. This dissertation discusses the possibilities of inhibiting the activity of *Escherichia coli* stringent factor RelA by re-examining the mechanism of action for the classical antibiotics and characterizing newly developed molecular tools based on a (p)ppGpp scaffold.

REVIEW OF LITERATURE

1. The stringent response, a core bacterial adaptation mechanism

1.1. Bacterial stress responses

Bacteria can be found everywhere across the planet including such extreme places as underwater volcanoes and ice-covered plains of Arctic. Their ability to adapt to almost every possible change in environmental conditions is the key for bacterial successful survival, proliferation and evolution. To adapt to changes, bacteria first have to sense it and therefore, they have numerous stress response mechanisms that can be differentiated by the types of stresses they are responsible of (Poole, 2012). Starting from nutrient limitation like the deprivation of Mg^{2+} ions (Groisman et al., 1997) to the oxidative stress, caused by reactive oxygen species (Gu and Imlay, 2011; Touati, 2000) and SOS response that is known to be activated as one of the last preventive measures upon DNA damage and exposure to drugs (Baharoglu and Mazel, 2014), the strategies for bacterial survival are highly diversified.

1.2. Discovery of the stringent response, SR

RNA synthesis in bacteria is regulated in different ways. A classical example is repression of stable RNA (i.e. ribosomal, rRNA, and transfer, tRNA, RNA) synthesis during amino acid deprivation (Neidhardt, 1964, 1966; Pardee and Prestidge, 1955). Such control was termed as “stringent” since RNA synthesis was stringently repressed in the case of WT (wild-type) bacteria (Neidhardt, 1964). Later it was shown that RNA synthesis cessation takes place in response to other cues, not just amino acid limitation (Cashel and Gallant, 1969; Haseltine and Block, 1973), i.e. limitation for other nutrients like iron (Vinella et al., 2005), phosphorus (Spira et al., 1995), carbon (Flärdh et al., 1994) and fatty acids (Battesti and Bouveret, 2006). In addition to nutritional deprivation heat shock also induces the stringent control response (Gallant et al., 1977).

As the “stringent control” is not a constitutive but is, rather, activated in response to certain stimuli, the term “stringent response” (SR) was coined as an alternative and quickly become the most commonly used one. Bacterial physiology changes dramatically upon the activation of the SR (Gallant and Harada, 1969). While the most pronounced consequence of SR induction is inhibition of synthesis of stable RNA (Cashel and Gallant, 1969), the SR also results in inhibition of carbohydrate synthesis (Sokawa et al., 1970), lipid (Sokawa et al., 1968) and phospholipid synthesis (Golden and Powell, 1972; Merlie and Pizer, 1973; Sokawa et al., 1972; Sokawa et al., 1970), membrane transport of nucleobases (Hochstadt-Ozer, 1972) and glycosides (Sokawa and Kaziro, 1969),

phosphate incorporation into glycolytic intermediates (Irr and Gallant, 1969), *de novo* nucleotide synthesis (Gallant, 1971) and synthesis of polyamines (Hölttä et al., 1974), increased rate of proteolysis (Sussman and Gilvarg, 1969). The activation of SR results not only in inhibition of cellular processes. Certain pathways conducting the survival of the cell are activated when bacteria are starving. These include the activation of transcription from certain promoters, biosynthesis of amino acids with consecutive synthesis of proteins necessary for survival etc (Hauryliuk et al., 2015; Potrykus and Cashel, 2008).

1.3. Pleotropic effects of the SR on bacterial physiology

Small secondary messenger molecules are the key chemicals mediating signaling in bacterial cells. One of the messenger classes is cyclic nucleotides. Changes in c-di-GMP (cyclic diguanosine monophosphate) cellular levels drive the switch between motile form and stable biofilm (Pesavento and Hengge, 2009; Romling and Simm, 2009; Ross et al., 1987). The universal alarmone cAMP (cyclic adenosine monophosphate) (Makman and Sutherland, 1965) is necessary for electrolyte homeostasis via modulating the activity of protein kinases and ion channels (Francis and Corbin, 1999). It also controls the activity of bacterial transcription regulator Crp (cAMP-receptor protein) responsible for the expression of genes under more than 100 different promoters (Busby and Ebright, 1999). Another cyclic nucleotide messenger, c-di-AMP, is important for sporulation delay upon DNA damage in *Bacillus subtilis* (Romling, 2008; Witte et al., 2008) and maintaining cell wall homeostasis (Luo and Helmann, 2012). The SR is also modulated by secondary messengers, alarmone molecules guanosine 5'-diphosphate 3'-diphosphate and guanosine 5'-triphosphate 3'-diphosphate, together termed as (p)ppGpp (Cashel and Gallant, 1969).

2. (p)ppGpp: 'Magic Spot' as a modulator of bacterial stringent response

2.1. Evolutionary distribution of (p)ppGpp-mediated signaling

Upon starvation of *E. coli* Cashel and Gallant have detected unusual nucleotides they called the "Magic Spots", MS, appearing on a thin layer chromatography plate (Cashel and Gallant, 1969). Analysis of the chemical structure of MSs have shown that the alarmones are guanosine 5'-diphosphate 3'-diphosphate (MSI, ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (MSII, pppGpp); collectively reffered to as (p)ppGpp (Cashel and Gallant, 1969; Cashel and Kalbacher, 1970). In addition to (p)ppGpp, there are several other related nucleotides: ppApp was detected in *B. subtilis*, though the function of the nucleotide is

unclear (Rhaese and Groscurth, 1974). Later MSs were found in *B. subtilis* (Swanton and Edlin, 1972) and photosynthesizing *Rhodopseudomonas spheroides* (Eccleston and Gray, 1973) suggesting universal distribution of MS nucleotides among bacteria. The search for (p)ppGpp in eukaryotes was mounted. (p)ppGpp was discovered in mice (Irr et al., 1974), but that was soon proved to be an artifact (Martini et al., 1977). Plastids, however, have functional SR system and (p)ppGpp (van der Biezen et al., 2000).

2.2. The (p)ppGpp cycle

Mutational analysis in *Escherichia coli* showed that bacteria can have two distinct phenotypes related to the SR. The one that is characterized by the arrest of stable RNA production in response to amino acid starvation is defined as "stringent" phenotype while the other one is defined as "relaxed" with stable RNAs being produced regardless in the decrease in nutritient levels (Alfoldi et al., 1962; Fiil and Friesen, 1968; Neidhardt, 1966). Detailed examination of "relaxed" mutants resulted in the discovery of *relA* (from "relaxed") gene the product of which is active in WT cells but either inactive or absent in the "relaxed" cells (Alfoldi et al., 1962; Stent and Brenner, 1961). Further studies revealed "relaxed" *E. coli* strains being deficient in (p)ppGpp, meaning *relA* gene product, stringent factor RelA in other words, is responsible for the SR activation and (p)ppGpp synthesis in *E. coli* (Cashel and Gallant, 1969). Another key player in the *E. coli* SR, SpoT, is known to have both the functions for (p)ppGpp hydrolysis and synthesis with the first being preferred (Laffler, 1974; Murray and Bremer, 1996). Both genes do not seem to be essential as their deletions result only in "relaxed" phenotype (Cashel, 1996; Laffler, 1974).

Upon amino acid starvation (p)ppGpp is synthesized in *E. coli* by stringent factor RelA (Potrykus and Cashel, 2008). The enzyme uses GTP or GDP and ATP as substrates (Cochran and Byrne, 1974; Haseltine et al., 1972b). Guanosine pentaphosphate or pppGpp is produced first by the transfer of β-γ-pyrophosphoryl group of ATP to the 3'-hydroxyl of GTP (**Fig. 1**) (Hogg et al., 2004a; Sy, 1973) but then mostly being dephosphorylated to ppGpp by the product of *gppA* gene, GppA exopolyphosphatase (Keasling, 1993; Somerville and Ahmed, 1979). Therefore, ppGpp can be produced in two ways – either by direct synthesis using GDP and ATP as substrates (Hogg et al., 2004a) or as degradation product of pppGpp (Weyer et al., 1976). It results in much higher levels of ppGpp as compared to pppGpp during stringent response (Cashel, 1996). Moreover, compared to guanosine penthaphosphate, ppGpp has much stronger regulatory effect on transcription (Mechold et al., 2013; Potrykus and Cashel, 2008).

During normal growth the basal intercellular concentration of (p)ppGpp is estimated to be around 50 μM (Kajitani and Ishihama, 1984) while it was estimated to reach up to 1 mM in starved bacteria (Cashel, 1974). The discoverer of (p)ppGpp, Mike Cashel defines micromolar variations in basal level as "growth

rate control" necessary to implement housekeeping functions. The changes on millimolar level refer to "stringent regulation" needed for the survival through harsh environmental conditions (Cashel, 1996). When nutritional stress is overcome and bacteria can return to normal growth i.e. upon addition of amino acids, (p)ppGpp levels should be decreased back to basis (Laffler and Gallant, 1974). SpoT degrades pppGpp to GTP and ppGpp top GDP (**Fig. 1**) with the half-life of pppGpp equal to 20 sec (Lund and Kjeldgaard, 1972b; Murray and Bremer, 1996).

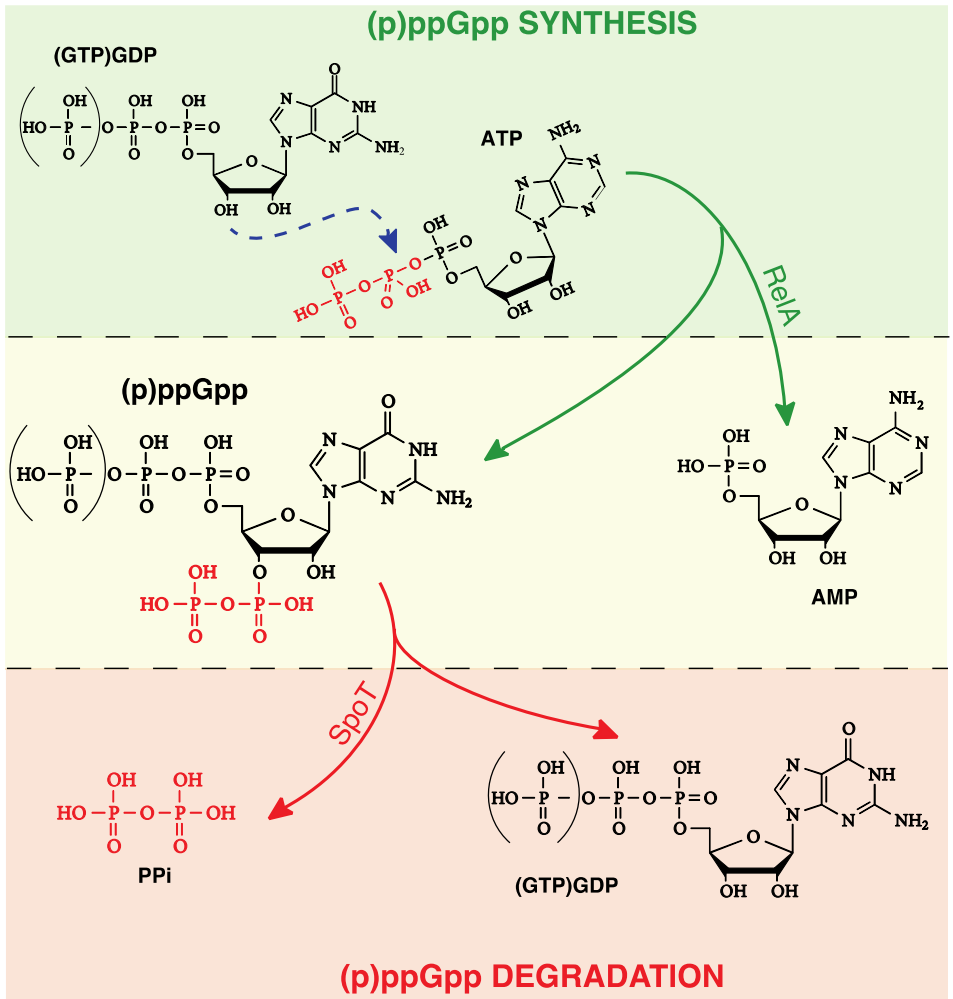

Figure 1. The lifecycle of (p)ppGpp in *E. coli*. Guanosine tetraphosphate, ppGpp, is synthesized from ATP and GDP. Guanosine pentaphosphate, pppGpp, is formed from ATP and GTP respectively. The synthesis step is performed by RelA (green arrow) while SpoT is responsible for degradation (red arrow). Dotted blue arrow indicates nucleophilic attack.

2.3. The molecular targets of (p)ppGpp

(p)ppGpp affects bacterial physiology on all the three levels of central dogma in molecular biology: DNA, RNA and protein synthesis (**Fig. 2**). Regulation of replication can occur either by modulating activity of IMP dehydrogenase that is crucial for the synthesis of GTP precursors (Gallant et al., 1971), mediating the transport of nucleotides (Hochstadt-Ozer, 1972) or modulating the activity of DnaG primase (Maciag et al., 2010; Wang et al., 2007). In *B. subtilis* inhibition of DnaG primase leads to the depletion of RNA primers needed for DNA polymerase activity followed by the arrest of replication fork (Maciag et al., 2010; Wang et al., 2007). ppGpp was co-crystalized with small GTPase Obg of *B. subtilis* (Buglino et al., 2002) implicated in interactions with several activators of stress transcription factor σ^B (Scott, 1999). In *E. coli*, ObgE (CgtA) is important for the stabilization of arrested replication forks (Foti et al., 2005) and *cgtA* gene deletion leads to disruption of cell cycle events (Foti et al., 2007). ObgE association with ribosomes and SpoT also supports putative ppGpp-ObgE interaction (Jiang et al., 2007).

As the major feature of active SR is ceased RNA synthesis (Cashel and Gallant, 1969), the main target for (p)ppGpp is transcription (Potrykus and Cashel, 2008). Interaction of the alarmone and RNAP will be discussed in the next section. Besides RNAP, (p)ppGpp interacts with other enzymes having a certain role in transcription or mRNA (messenger RNA) balance in the cell. In actinomycetes (p)ppGpp directly binds polynucleotide phosphorylase (crucial for RNA turnover (Siculella et al., 2010)) thus increasing the stability of mRNA (Siculella et al., 2010). Stabilization of mRNA by (p)ppGpp is also reported in *E. coli*, where (p)ppGpp inhibits transcription of *pcnB* gene necessary for poly(A) polymerase synthesis. In result, polyadenylation of mRNA that acts as a signal for degradation is limited (Dalebroux and Swanson, 2012).

Protein synthesis is as well regulated by (p)ppGpp as elevated levels of alarmone can inhibit translation up to 90 % *in vivo* (Svitil et al., 1993) while absence of (p)ppGpp (Cozzone, 1980) leads to increased rate of missense errors and frameshifting in relaxed cells (Edelmann and Gallant, 1977; Foley et al., 1981). Different steps in translation are affected by (p)ppGpp: it inhibits GDP binding by translational factors IF2 (Milon et al., 2006), EF-Tu (Arai et al., 1972; Legault et al., 1972) and EF-G (Mitkevich et al., 2010) therefore regulating the initiation and elongation of translation. Decreasing the number of ribosomes per cell by inhibiting rRNA and r-protein (ribosomal protein) synthesis (Dennis and Nomura, 1975; Lindahl et al., 1976) can also be considered as a way to regulate translation and growth rate.

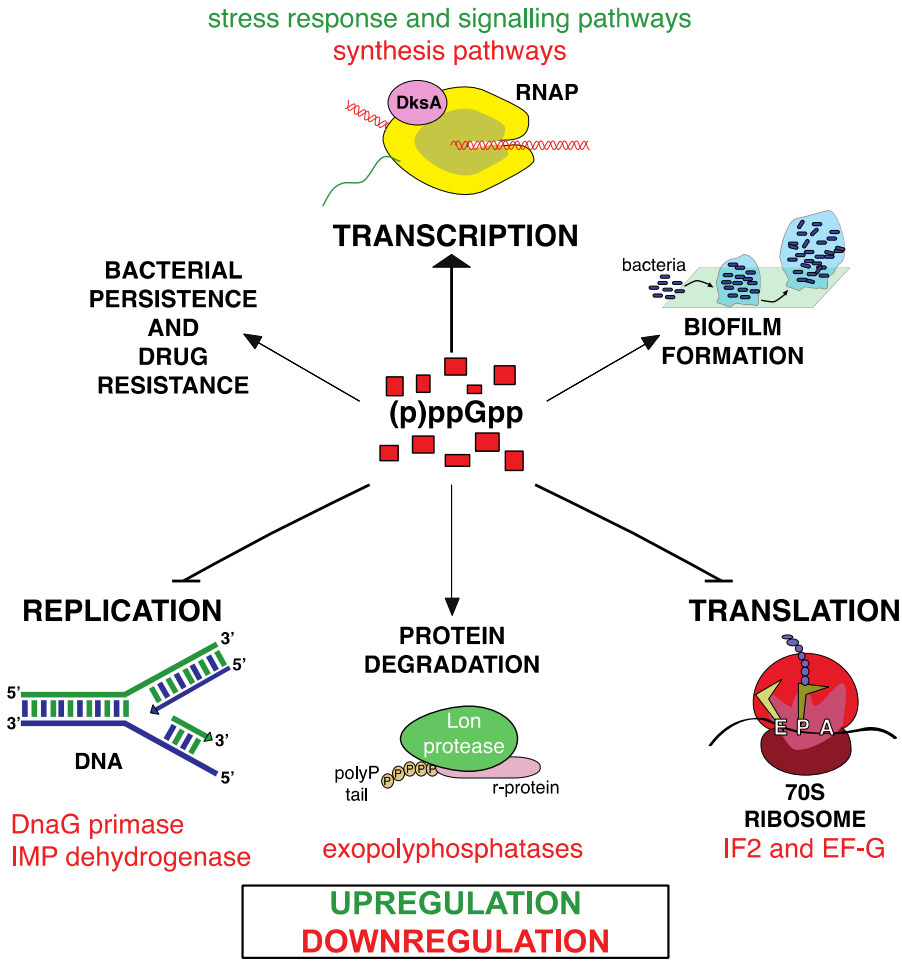

Figure 2. (p)ppGpp targets major steps of cellular activity including replication, transcription and translation. Enzymes and pathways downregulated by (p)ppGpp are marked with red and upregulated with green respectively.

Not only protein synthesis is under (p)ppGpp regulation; indirect control of protein degradation has also been reported (Kuroda et al., 2001). Polyphosphate (polyP) induces degradation of r-proteins by Lon protease (Kuroda et al., 2001; Maisonneuve et al., 2013). By inhibiting the activity of exopolyphosphatase enzymes, (p)ppGpp increases the level of polyP in the cell thus promoting r-protein degradation (Kuroda et al., 1997; Maisonneuve et al., 2013). Being major regulator, (p)ppGpp has pleiotropic, mostly inhibitory, effect on many other key enzymes during SR in order to spare as many cellular resources by preventing proliferation and growth and survive the environmental twist. Such synthesis pathways as fatty acid (Battesti and Bouveret, 2006; Polakis et al., 1973; Stein and Bloch, 1976), phospholipid (Lueking and Goldfine, 1975; Mer-

lie and Pizer, 1973), glycogen (Dietzler and Leckie, 1977), peptidoglycan (Ishiguro and Ramey, 1976), polyamine (Hölttä et al., 1974), certain amino acids (Kleeman and Parsons, 1977) are inhibited in the same way as glycolysis (Taguchi et al., 1978). On the contrary, physiological changes crucial for cell survival are upregulated by (p)ppGpp (**Fig. 2**). Amongst them are stress responses and signaling pathways: in *E. coli* (p)ppGpp regulates acid stress response through the modulation of LdcI (lysine decarboxylase, inducible) enzyme activity (Kanjee et al., 2011). The enzyme consumes protons during carboxylation of L-lysine to cadaverine and CO_2 thus increasing the cytoplasmic pH (Kanjee et al., 2011). Several studies indicate the role for (p)ppGpp in regulating the expression of genes important for quorum sensing in different bacteria species (Harris et al., 1998; van Delden et al., 2001; Zhang et al., 2004) and other signal nucleotides, namely c-di-AMP and c-di-GMP, dependent signaling pathways (Dalebroux and Swanson, 2012). Furthermore, *E. coli* and *Streptococcus mutans relA spoT* double knock out mutants were shown to be defective in biofilm formation (Balzer and McLean, 2002; Lemos et al., 2004) while the sporulation (development of fruiting bodies) in *Myxococcus xanthus* is regulated by (p)ppGpp on transcriptional level (Harris et al., 1998; Singer and Kaiser, 1995). Elevated levels of (p)ppGpp inhibit the processes of proliferation and growth bacteria cannot afford itself in given environmental conditions and activate stress defense genes and genes, responsible for minimal maintenance of cellular activity. It is done mainly through the modulation of RNA polymerase activity (Potrykus and Cashel, 2008).

3. RNA polymerase as a main target for (p)ppGpp

3.1. Molecular architecture of bacterial RNAP

RNA polymerase is the enzyme that conducts RNA synthesis. Although there are several types of RNA polymerases in eukaryotes, bacteria have only one being responsible for the transcription of all the RNA species. The RNAP core enzyme consists of 5 subunits; 2 α subunits, responsible for core enzyme assembly and interaction with transcription factors, β and β' subunits, containing the active center of the enzyme and ω subunit, important for the stability of the core enzyme (**Fig. 3A**) (Ebright, 2000; Gross et al., 1992). In order to become active, *E. coli* RNAP needs to bind one of 7 σ-factors that help RNAP choose which genes to transcribe in given environmental conditions. During normal exponential growth the housekeeping σ^{70} takes its place in RNAP holoenzyme (Hengge-Aronis, 2002) while stress σ-factors have higher affinity towards RNAP apoenzyme under starvation, heat-shock (Hengge-Aronis, 2002; Merrick, 1993) etc.; in other words, when (p)ppGpp levels are high. Two ways of transcription regulation by (p)ppGpp were proposed – indirect and direct respectively (Krasny and Gourse, 2004). The direct regulation implies (p)ppGpp

binding to RNAP and is typical for most proteobacteria (Krasny and Gourse, 2004; Ross et al., 2016). In the case of indirect regulation the alarmone exerts its influence through mediating GTP levels (Krasny and Gourse, 2004). Excluding proteobacteria, most other bacteria adhere to this way of regulation (Krasny and Gourse, 2004).

3.2. (p)ppGpp directly interacts with *E. coli* RNAP

It was said in the late 70's that (p)ppGpp can possibly act via RNA polymerase (Lindahl et al., 1976; Travers, 1976). More of that, several years later, RNAP was claimed to be the main target for (p)ppGpp (Little et al., 1983; Tedin and Bremer, 1992). Considering the structure of the molecule, the following model for RNAP inhibition by (p)ppGpp was proposed: ppGpp binds into the RNAP secondary channel, necessary for substrate entry, where it competes with NTPs (Jores and Wagner, 2003; Wagner, 2002). Numerous studies based on mutational analysis (Hernandez and Cashel, 1995; Reddy et al., 1995) and crosslinking experiments (Chatterji et al., 1998; Toulokhonov et al., 2001) gave controversial results on ppGpp binding pocket. X-ray structural study of *Thermus thermophilus* RNAP-ppGpp complex suggested ppGpp binding between β and β' subunits in close proximity to the active center (Artsimovitch et al., 2004). The proposed binding site was, however, disproved and claimed to be an artefact (Vrentas et al., 2008). Recent Cryo-EM study on RNAP-ppGpp complex, this time with *E. coli* RNAP, suggests absolutely different location for ppGpp binding on RNAP. According to Zuo and colleagues, the putative binding site is 28 Å away from the polymerase active center, in a cavity between α, β' and ω subunits with both pyrophosphate moieties interacting mostly with ω and guanosine base with β' subunit (**Fig. 3B**) (Zuo et al., 2013). In another study, MAR sequence (Met, Ala, Arg) at the N-terminus of ω subunit was proposed to be a marker for the possibility of ppGpp-RNAP interaction (Mechold et al., 2013; Ross et al., 2013). Mutations in the MAR sequence and residues involved in RNAP-ppGpp interaction (Zuo et al., 2013) significantly decrease ppGpp ability to affect RNAP activity confirming the suggested positioning of ppGpp on RNA polymerase (Ross et al., 2013). The MAR sequence is found to be conserved in α-, β-, γ- and δ-proteobacteria but not in other bacterial phyla (Ross et al., 2013), leaving a possibility for alternative (p)ppGpp binding sites like it was shown for *Thermus thermophilus* (Artsimovitch et al., 2004). Recently additional (p)ppGpp binding site was identified at the interface of β' subunit of RNAP and transcription factor DksA (Ross et al., 2016). It is proposed that at low concentration (p)ppGpp primarily binds into the binding pocket described in (Zuo et al., 2013) whereas upon stringent response both sites get filled with (p)ppGpp (Ross et al., 2016).

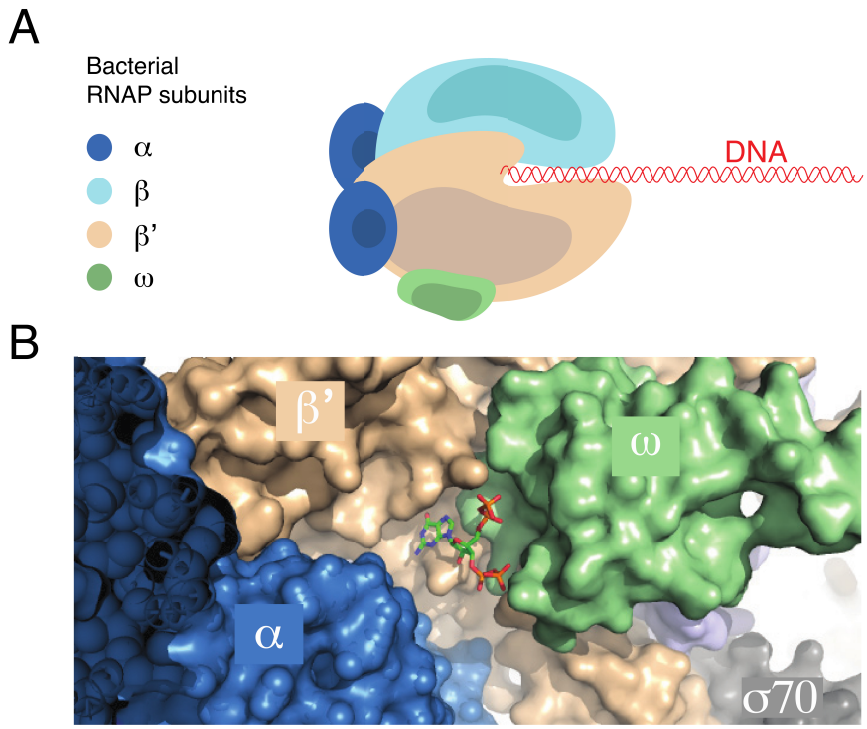

Figure 3. Bacterial RNA polymerase constitution (A) and its interaction with ppGpp (B). RNAP subunit α is in blue, β' in beige, ω in green and σ70 in grey. Nucleobase of ppGpp contacts β' and phosphate moieties ω subunits of RNAP respectively. Adapted from (Zuo et al., 2013), PDB accession code 4JKR.

Considering the tremendous influence of changes in cellular ppGpp levels onto activity of σ-factors and the choice of promoters to be transcribed, the following model of RNAP activity inhibition by (p)ppGpp was suggested (Potrykus and Cashel, 2008). The model is based on discoveries that (p)ppGpp: a) mostly negatively regulates σ^{70}-dependent promoters (Bernardo et al., 2006; Chang et al., 2002; Kvint et al., 2000b), b) affects RNAP-promoter DNA open-complex (necessary for translocation step) formation during transcription initiation (Ohlsen and Gralla, 1992; Raghavan et al., 1998). (p)ppGpp directly inhibits transcription from various promoters (Barker et al., 2001; Cashel et al., 1996a; Kajitani and Ishihama, 1984; Potrykus et al., 2002) what results in an impaired transcription initiation, thus causing breakage of already very unstable open-complexes (Barker et al., 2001; Choy, 2000; Paul et al., 2004; Paul et al., 2005). The open-complex breakage, in turn, results in the increased concentration of free RNAP enzyme that can again bind either σ^{70}-dependent promoters (Kvint et al., 2000b; Xiao et al., 1991a) or, what is more possible, positively regulated promoters that depend on alternative σ-factors (Bernardo et al., 2006; Hernan-

dez and Cashel, 1995; Jishage et al., 2002; Laurie et al., 2003; Magnusson et al., 2003).

Another model of RNAP activity regulation represents the mechanism completely opposite to the one described previously (Potrykus and Cashel, 2008). It states that (p)ppGpp can possibly increase the pausing during transcriptional elongation causing the decreased availability of free RNAPs (Bremer et al., 2003; Jensen and Pedersen, 1990; Jores and Wagner, 2003; Krohn and Wagner, 1996; Wagner, 2002). This model is called the "trapping mechanism" suggesting that RNAP, modified by (p)ppGpp binding, is trapped in RNAP-DNA closed-complex (Heinemann and Wagner, 1997a) compromising further formation of open-complex (Maitra et al., 2005; Potrykus et al., 2006).

Recent structural study considers the possibility that both models are true. Based on a cryo-EM structure of *E. coli* RNAP-ppGpp complex, Zuo and coallegues divide RNAP enzyme onto modules and propose that core and shelf modules of RNA polymerase undergo cyclic ratcheting movements to switch between open (translocation) and closed (catalysis) states (Zuo et al., 2013). Subsequently, ppGpp, when bound, can stabilize one of the states thereby slowing down transcription (Zuo et al., 2013).

The stabilization of RNAP:DNA complexes by (p)ppGpp is to some extent dependent on transcription factors. Although (p)ppGpp is a major regulator of RNAP activity by itself, its ability to conduct the transcription is usually tightly coupled with the activity of transcription factor DksA (Paul et al., 2005) that is said to strengthen the effect of (p)ppGpp independently of either the effect is positive or negative (Krasny and Gourse, 2004; Paul et al., 2004).

3.3. Cooperation between DksA and (p)ppGpp in regulation of RNAP

Numerous studies report the importance of DksA for stringent regulation of transcription (Hauryliuk et al., 2015). Thus, the rise of (p)ppGpp level had no effect on rRNA transcription in DksA deficient strain (Paul et al., 2004). And on opposite, addition of DksA increases (p)ppGpp inhibitory impact on σ^{70}-dependent promoters (Paul et al., 2004) as well as positive effect on transcription of amino acid biosynthetic promoters (Paul et al., 2005) and (p)ppGpp-dependent production of σ-factors in DksA knock-out *E. coli* strain (Bernardo et al., 2006; Brown et al., 2002; Laurie et al., 2003) .

At the same time DksA is important not only during stringent regulation; the deletion of the respective *dksA* gene, the homologs of which are widely distributed among bacteria (Perron et al., 2005; Sharma and Payne, 2006; Viducic et al., 2006), resulted in pleiotropic effect on numerous processes including chaperon function, quorum sensing (Branny et al., 2001; Kang and Craig, 1990; Webb et al., 1999) etc. The essentiality of DksA for expression of virulence genes in *Shigella flexneri* (Sharma and Payne, 2006) and *Salmonella typhimurium* (Webb et al., 1999) has also been confirmed.

Although there is an evidence for *Pseudomonas aeruginosa* DksA binding to DNA (Perron et al., 2005); as a transcription factor, DksA directly interacts with RNA polymerase, binding into the secondary channel where NTP enters the complex to become a part of a new RNA molecule (Perederina et al., 2004). Structurally DksA is quite similar to transcription elongation factors GreA and GreB; they also bind at the same positions on RNAP (Perederina et al., 2004; Potrykus et al., 2006). Both GreA and GreB act by inserting their N-terminal coiled-coil finger domain into RNAP secondary channel (Borukhov et al., 2005) suggesting DksA may do the same (Perederina et al., 2004). Functionally, while GreB may have the same effect on certain promoters as DksA (Rutherford et al., 2007), GreA usually acts in opposite manner (Potrykus et al., 2006). Overall structural and functional similarity of DksA with the better studied GreA/B gives good opportunity for prediction of DksA role on RNAP.

It is nowadays suggested that the main role for DksA is the stabilization of ppGpp binding to RNAP (Paul et al., 2004; Vrentas et al., 2005). It is strongly supported by the fact that together with RNAP DksA comprises a binding pocket for (p)ppGpp (Ross et al., 2016). Similarly to GreA/B, the N-terminal domain of DksA resembles a long helical hairpin structure that, by protruding into RNAP active center (Perederina et al., 2004; Zuo et al., 2013), modifies the transcription initiation complex so that certain promoters become responsive towards changes in (p)ppGpp level (Rutherford et al., 2009). What is interesting, there is a negative feedback loop between DksA and its promoter which is also regulated by (p)ppGpp (Chandrangsu et al., 2011) meaning (p)ppGpp is able to regulate its own effect on RNAP.

As opposed to the γ–proteobacterium *E.coli*, deletion of most homologous to DksA ORFs in firmicute *B. subtilis* had no effect on rRNA transcription (Krasny and Gourse, 2004). Moreover, increase in (p)ppGpp levels also remained uninfluential (Krasny and Gourse, 2004) suggesting other ways for rRNA transcription regulation than direct contact between (p)ppGpp, DksA and RNA polymerase (Krasny and Gourse, 2004).

3.4. rRNA transcription in *Bacillus subtilis* is regulated through the modulation of GTP level by (p)ppGpp

In the beginning of 1980s it was stated that GTP/ATP ratio is the main effector of the stringent response in *B. subtilis* (Lopez et al.), which is logical, considering that GTP and ATP are the substrates for (p)ppGpp production. Taking together previously written, the fast decrease in GTP levels during stringent response (Gallant and Harada, 1969), the importance of GTP for rRNA transcription activation (Krasny and Gourse, 2004) and inability of (p)ppGpp to directly affect RNA polymerase activity in *B. subtilis* (Krasny and Gourse, 2004) it ends up with the possibility that RNA polymerase activity is mainly regulated through the modulation of GTP levels.

(p)ppGpp synthetase of *B. subtilis*, RelBsu, can regulate GTP levels (Inaoka and Ochi, 2002; Inaoka et al., 2003). (p)ppGpp, produced by the enzyme, diminishes GTP levels directly since the more product ((p)ppGpp) is produced, the less substrate (GTP) is left. In *B.subtilis* (p)ppGpp is reported to reduce the activity of IMP dehydrogenase GuaB that converts IMP into XMP, the precursor of GMP (Krasny and Gourse, 2004; Kriel et al., 2012; Liu et al., 2015). HprT, hypoxanthine phosphoribosyltransferase, that makes GMP out of guanine and xanthine (Jensen et al., 2008) and Gmk or GMP kinase that catalyzes GMP to GDP conversion were also shown to be inhibited by (p)ppGpp in different bacterial species (Gaca et al., 2013; Kriel et al., 2012; Liu et al., 2015). This leads to suppression of GTP synthesis and thereby contributes to GTP depletion. GTP depletion, in turn, leads to decrease in rRNA production in *B. subtilis*, where all the rRNA promoters were suggested to initiate with GTP (Krasny and Gourse, 2004), and *T. thermophilus* (Kasai et al., 2006). On the other hand, expression of genes necessary for adaptation is induced; transcriptional regulator CodY represses them in GTP-bound state (Handke et al., 2008; Ratnayake-Lecamwasam et al., 2001) but when GTP levels are low CodY target genes become eligible for transcription (Handke et al., 2008; Lemos et al., 2008; Ratnayake-Lecamwasam et al., 2001).

3.5. Cross-talk between (p)ppGpp and transcriptional σ-factors

Structural studies did not show any possible direct interactions between (p)ppGpp and σ-factors. However, influence of the alarmone on σ-factor synthesis and activity was reported decades ago. The product of *rpoS* gene, σ^S factor, is most common to replace σ^{70} on RNAP to direct the enzyme in stress conditions (Hengge-Aronis, 2002). Not unexpectedly, both the synthesis and activity of σ^S are strictly regulated by (p)ppGpp (Gentry DR, 1993; Klauck et al., 2007; Kvint et al., 2000a; Lange et al., 1995). One of the indirect pathways for σ^S activity regulation by (p)ppGpp includes adaptor protein RssB that in normal conditions directs σ^S to the ClpXP proteasome for degradation (Bougdour et al., 2006; Hengge-Aronis, 2002). In starved bacteria high (p)ppGpp levels induce the synthesis of anti-adaptor proteins IraP and IraD with the task to block the activity of RssB. This ensures the increase in the concentration of σ^S within the cell (Bougdour and Gottesman, 2007; Merrikh et al., 2009). Other alternative sigma factors, heat-shock σ^{32} (Grossman et al., 1985; VanBogelen and Neidhardt, 1990) and nitrogen-limitation sensitive σ^{54} (Carmona et al., 2000; Sze and Shingler, 1999) are also regulated by (p)ppGpp, as well as extracytoplasmic stress factor σ^E (Costanzo and Ades, 2006; Costanzo et al., 2008). σ^E activity is under negative control of regulatory protein RseA. RseA degradation, in turn, is mediated by (p)ppGpp meaning the higher level of (p)ppGpp the more active is σ^E (Ades et al., 2003).

(p)ppGpp directly and indirectly regulates the production and activity of σ-factors, the main role for whom is to navigate RNAP on what genes under which promoters to transcribe (Ebright, 2000). Subsequently, (p)ppGpp is as well able to regulate the expression of genes being under promoters with certain structural features; several research groups have stated that GCGC discriminator motif (around -10 promoter element) downstream to TATA box (Riggs et al., 1986; Shand et al., 1989; Travers, 1980) is important for negative stringent regulation. Later this importance, however not absolute necessity, has been confirmed for certain promoters (Davies and Drabble, 1996; Mizushima-Sugano and Kaziro, 1985; Travers, 1984; Zacharias et al., 1989). Another study, still confirming the negative effect of GCGC discriminator, at the same time detracts its significance by mutational analysis. Thus modified upstream promoter sequences or even promoters lacking them give similar response to the ones with the intact discriminator (Josaitis et al., 1995). Nevertheless, the significance of the discriminator sequence is also reported to be dependent on the length and the sequence of the linker between -35 and -10 upstream positions (Park et al., 2002). Negatively regulated promoters have 16 bp linker and GC-rich discriminator while positively regulated promoters have longer linkers and AT-rich discriminator (Park et al., 2002).

4. Stringent factor RelA and RSH superfamily

4.1. The evolutionary distribution of RSH superfamily

The enzymes responsible for the rise or drop in (p)ppGpp levels within the cell comprise a superfamily of RSH proteins. The abbreviation comes from RelA/SpoT Homologue based on the sequence similarity between the representatives of the superfamily with *E. coli* RelA and SpoT proteins (Atkinson et al., 2011), the original stringent factor (Cashel and Gallant, 1969) and its opposing hydrolase (Sy, 1977) respectively. RSH enzymes are absent in archaea (Mittenhuber, 2001) but widely present in bacteria; the only exclusions are parasitic bacteria with reduced genome like *Treponema pallidum*, *Rickettsia prowazekii*, representatives of PVC (*Planctomycetes*, *Verrucomicrobia*, *Chlamydiae*) superphylum and obligate intracellular endosymbionts (**Fig. 4**) (Atkinson et al., 2011; Mittenhuber, 2001). The enzymes of the superfamily are mainly divided onto long – RelA, SpoT, Rel, and small RHSs – SAS or small alarmone synthetases and SAH or small alarmone hydrolases (Atkinson et al., 2011).

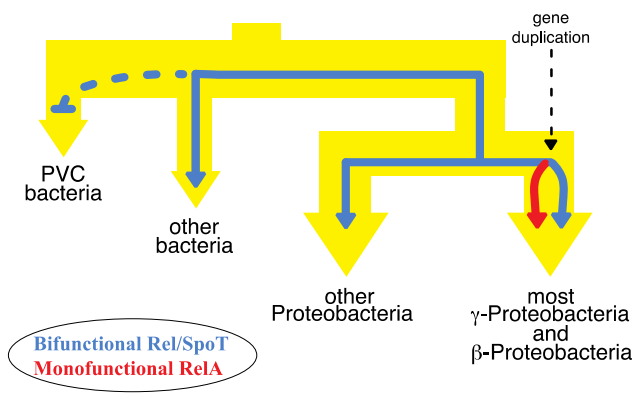

Figure 4. The distribution of long RSHs in bacteria. Monofunctional (p)ppGpp synthetase is indicated with red arrow, bifunctional enzymes with blue arrows. Dashed arrow indicates the gene duplication event. Adapted from (Atkinson et al., 2011)**.**

While Gram-negative bacteria of β- and γ-subdivisions (including the best studied *E. coli*) of proteobacteria usually have 2 distinct enzymes for (p)ppGpp turnover, namely RelA and SpoT (Atkinson et al., 2011; Mittenhuber, 2001), single bifunctional enzyme Rel is typical for most of bacteria with studied examples from *B. subtilis* (Wendrich and Marahiel, 1997), *Streptococcus equismilis* (Mechold et al., 1996) and *Mycobacterium tuberculosis* (Avarbock et al., 1999).

In addition to long RSH proteins many bacteria have one or several small RSHs, mainly SASs (Atkinson et al., 2011; Gaca et al., 2013; Steinchen et al., 2015). SASs or SAHs have only one, either synthetase or hydrolase domain.

Long RSH are canonically divided onto 6 domains arranged in the following order starting from N-terminus: (p)ppGpp hydrolysis domain (HD), (p)ppGpp synthesis domain (SYNTH), TGS (Threonyl-tRNA synthetase, GTPase, SpoT (Wolf et al., 1999)), Helical, Conserved Cysteins (Eccleston and Gray, 1973) and ACT (**Fig. 5**) (Aspartokinase, Chorismate mutase, TyrA (Chipman and Shaanan, 2001)) where four last are thought to regulate the catalytic activity of the enzyme (Atkinson et al., 2011). Through the phylogenetic analysis four more paralogous groups of enzymes, Rsh A-D, have been distinguished (Atkinson et al., 2011).

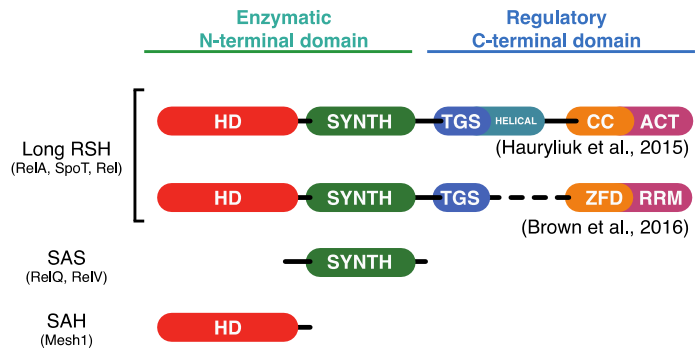

Figure 5. Domain structure of most studied RSH enzymes. In 2016 Brown with colleagues suggested to call the domains of long RSHs in a different way than they were used previously (Brown et al., 2016; Hauryliuk et al., 2015).

4.2. Domain organization and regulation in RSH enzymes

The best studied representative of RSH enzymes, RelA has 6 domains (Atkinson et al., 2011). Looking at the protein sequence it can be suggested that the enzyme is bifunctional, both SYNTH and HD domains are present; RelA, however, has very strong synthetic activity while hydrolytic activity is absent (Hauryliuk et al., 2015; Hogg et al., 2004b; Potrykus and Cashel, 2008). Conserved EXDD/RXKD motif in SYNTH domain of RSH enzymes is said to be of importance for the choice between mono- and bifunctionality of the protein where the EXDD sequence is typical for monofunctional RelA and RXKD for bifunctional Rel proteins respectively (Sajish et al., 2007). Another region crucial for activity regulation in RSH proteins is C-terminal region uniting TGS, Helical, CC and ACT domains (Hauryliuk et al., 2015). Recent cryo-EM study, however, suggests domain organization that differs in CTD; while TGS remains intact, zinc-finger domain (ZFD) and RNA recognition motif (RRM), previously defined as ACT, were revealed (**Fig. 5**) (Brown et al., 2016). Long RSH enzymes have two activity states: hydrolase OFF/synthetase ON, specific for RelA and other long RSHs (Potrykus and Cashel, 2008), and hydrolase ON/ synthetase OFF, specific for bifunctional Rel enzymes and SpoT, where binding

of respective substrates may trigger the switch between conformations (Hogg et al., 2004b). Loss of putative C-terminal regulatory domains activates synthetase and completely abolishes hydrolytic activity of bifunctional Rel of *S. equismilis* (Mechold et al., 2002) and impedes the regulation of Rel's activity in *M. tuberculosis* (Avarbock et al., 2005). CTD was suggested to form intramolecular interaction with SYNTH domain thus blocking synthetic activity; binding of deacylated tRNA, in turn, disturbs the "intra-domain crosstalk" and triggers SYNTH domain activity (Jain et al., 2007; Mechold et al., 2002). Deletion of CTD in *M. tuberculosis* also resulted in inability of Rel_{Mtb} to form homooligomers (trimers in this case) as it was observed with full enzyme (Avarbock et al., 2005). Several more studies reported on RelA's ability to form oligomers through CTDs (Gropp et al., 2001; Yang and Ishiguro, 2001a) leading to a suggestion that RelA is inactive in a dimer state and the monomeric state is needed for binding to the ribosome (Gropp et al., 2001; Yang and Ishiguro, 2001a).

4.3. *E. coli* RelA and activation of stringent response

Shortly after the discovery of (p)ppGpp and its importance the studies to reveal the mechanism of its production started. First of all it was confirmed that RNA is involved in activation of stringent response (Wong and Nazar, 1970) followed by the evidences that only uncharged (Haseltine et al., 1972a) cognate tRNA is able to activate it (Haseltine and Block, 1973; Pedersen et al., 1973). Another crucial player in stringent response activation was confirmed to be the ribosome (Haseltine and Block, 1973; Heinemann and Wagner, 1997b; Krohn and Wagner, 1995; Pedersen et al., 1973). In 1973 Block and Haseltine showed that the stringent factor responsible for (p)ppGpp production in *E. coli*, RelA, precipitates with ribosome during centrifugation (Block and Haseltine, 1973) and year later it was first purified together with the ribosome (Cochran and Byrne, 1974). The molecular machinery called a ribosome is responsible for protein synthesis. It consists of two subunits; in bacteria these are 50S and 30S, named so according to their sedimentation coefficients. Each subunit is composed of ribosomal r-proteins, 21 in 30S and 31 in 50S subunit respectively, and ribosomal rRNA, 23S and 5S rRNA in 50S and 16S rRNA in 30S subunit. mRNA molecule binds onto 30S subunit with consecutive binding of the large subunit. On the surface of the subunits of associated, 70S, ribosome three tRNA binding sites are formed: A-site that stands for aminoacylated tRNA and can be transformed into A/T-site upon binding of ternary complex of aa-tRNA;GTP: EF-Tu (Connell et al., 2008), P-site for peptidyl tRNA and E-site for deacylated tRNA. Nevertheless, in terms of the SR the most interesting is r-protein L11 of 50S subunit that was shown to be crucial for the activation of (p)ppGpp synthesis (Parker et al., 1976; Ramagopal and Davis, 1974; Stark and Cundliffe, 1979; Wendrich et al., 2002).

But how exactly the activation of stringent response occurs? Amino acid deprivation leads to increased levels of deacylated tRNAs which stall the

ribosome by binding into ribosomal A-site. The exact mechanism of the SR activation is unknown but, according to one of the hypotheses, RelA, sitting on a ribosome in an inactive mode (Wendrich et al., 2002), is suggested to directly inspect the CCA end of A-site tRNA and intact –OH group on terminal adenosine is what promotes the activation of stringent response (**Fig. 6**) (Sprinzl and Richter, 1976). As immunoprecipitation assay showed that there is only one molecule of RelA per 200 ribosomes (Pedersen and Kjeldgaard, 1977), one molecule of RelA should somehow interact with many ribosomes to ensure the appropriate response to the stress i.e. synthesize enough (p)ppGpp. Therefore, according to "hopping" model (Wendrich et al., 2002) activated RelA hops off the ribosome and produces one molecule of (p)ppGpp followed by binding to next stalled ribosome, thus triggering next synthesis event. In a recent single-molecule tracking study the model was transformed into "extended hopping" model suggesting RelA remains active off the ribosome for several rounds of (p)ppGpp production without binding to ribosome after every synthesis event (English et al., 2011; Hauryliuk et al., 2015).

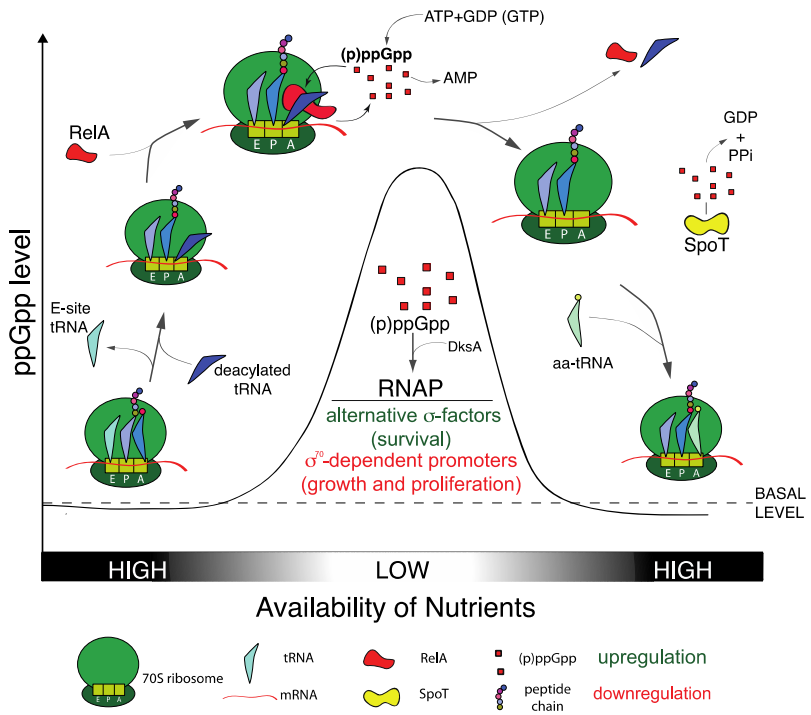

Figure 6. Schematic view of the stringent response mechanism in *E. coli*. Key steps in stringent response activation and regulation are shown as solid black arrows. Dashed line represents the basal level of (p)ppGpp in unstressed cells. The major effect of the elevated (p)ppGpp level is underlined either in red (negative regulation) or green (positive regulation).

4.4. Structural features of RelA-ribosome interactions

The importance of ribosomal protein L11, especially its N-terminus (Yang and Ishiguro, 2001b), for RelA activation have been proven biochemically (Cashel et al., 1996b; Potrykus and Cashel, 2008) suggesting that RelA can bind in the vicinity of L11. Later the RelA interaction with L11 was confirmed by cryo-EM studies where contact with sarcin-ricin loop (SRL) and direct interaction with A-site tRNA were also unveiled (Agirrezabala et al., 2013). Later study, however, reports on observing no direct contacts between RelA and L11 protein (Brown et al., 2016). Upon binding of RelA A-site tRNA was shown to acquire unusual A/T-like (A site/EF-Tu) conformation (Agirrezabala et al., 2013). More recent cryo-EM study enables to look into RelA-70S-tRNA interactions with more details; it supports the unusual conformation of deacylated tRNA in the A-site and claims highly extended conformation of RelA that lets it wrap around tRNA, positioning TGS domain right at the CCA end of tRNA thus examining the acylation state of tRNA (**Fig. 7**) (Brown et al., 2016). While HD and SYNTH domains are on the surface of the ribosome, ZFD and RRM of RelA lie along the anticodon arm of tRNA and all domains are connected via flexible helical elements (Brown et al., 2016).

Besides RelA there is one more RSH in *E. coli*; compared to RelA SpoT has poor synthetic activity and does not need to cooperate with ribosome to perform one of its main tasks, (p)ppGpp hydrolysis (Gentry and Cashel, 1995; Raue and Cashel, 1975).

4.5 Hydrolytic activity of SpoT balances the synthetic activity of RelA in *E. coli*

A decade after *rel* gene discovery another gene's product has been implemented in being important for stringent response regulation (Laffler, 1974). The enzyme termed SpoT (as *spoT* locus) was shown to be able to degrade (p)ppGpp with GDP (GTP) and pyrophosphate as products (Heinemeyer and Richter, 1977; Laffler, 1974; Sy, 1977; Xiao et al., 1991b). Although *spoT* gene is well-characterized (Sarubbi et al., 1989), SpoT, the *E. coli* (p)ppGpp hydrolase, remains to be studied due to difficulties with its purification. In fifty years after its discovery a lot of aspects concerning the protein remain elusive. Moreover, to our knowledge, no successful attempts of getting pure and active SpoT have been documented.

Unlike RelA, SpoT is extremely sensitive towards cellular concentration of certain divalent metallic ions. It was noticed that (p)ppGpp degradation depends on concentration of Mn^{2+} ions (Raue and Cashel, 1975). Later this dependence was explained by the presence of conserved His-Asp (HD) motif in SpoT protein sequence (Aravind and Koonin, 1998). Through the presence of HD motif Aravind and Koonin suggested to distinguish a superfamily of metal-dependent phosphohydrolases with SpoT being one of the most striking representatives (Aravind and Koonin, 1998).

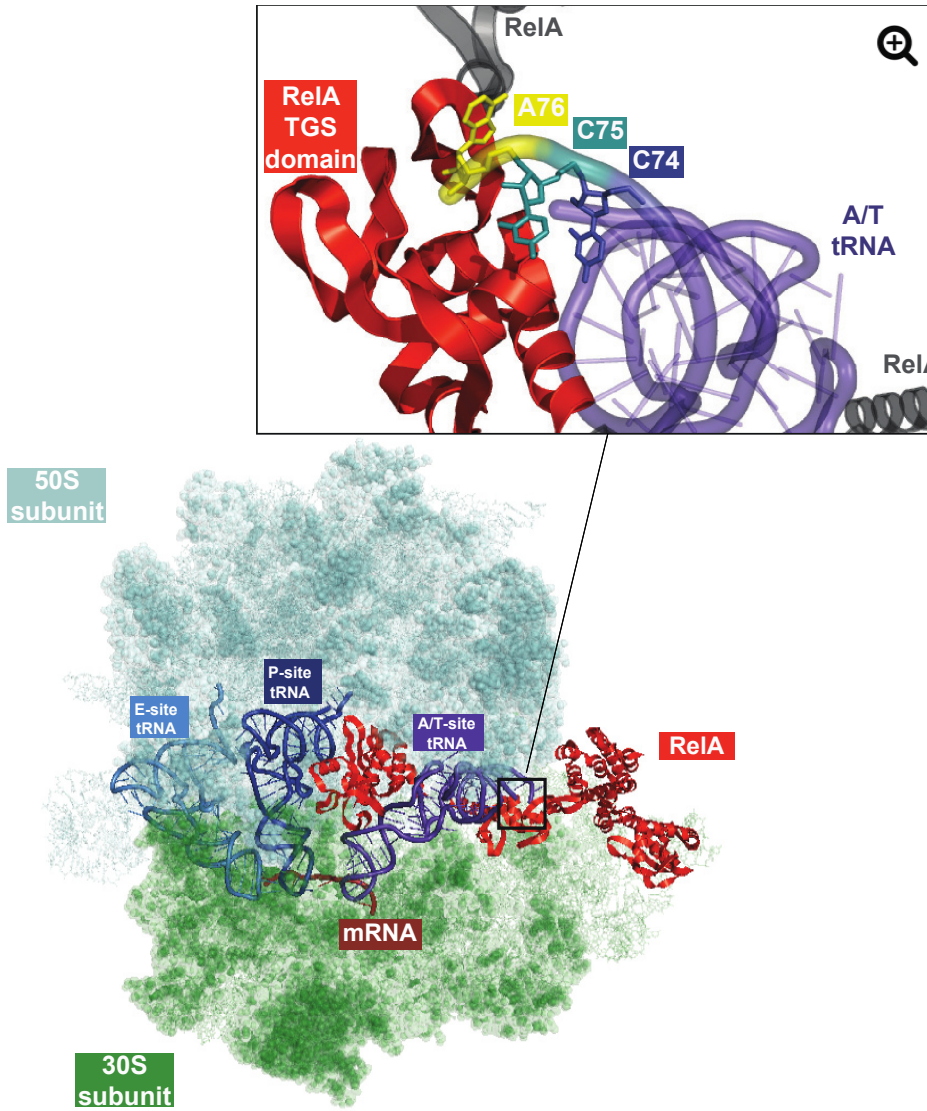

Figure 7. On a starved 70S ribosome RelA wraps around A/T-site tRNA and directly investigates its CCA-end. Ribosomal 30S subunit is shown in green, 50S subunit in cyan, E-site tRNA in light blue, P-site tRNA in dark blue, A/T-site tRNA in purple, mRNA in dark red and RelA in red. A/T-site tRNA residues C74, C75 and A76 are in purple, cyan and yellow respectively. Adapted from (Brown et al., 2016), PDB accession code 5IQR.

SpoT is also involved in activation of stringent response. While RelA is responsible to react on limitation of aminoacids, SpoT starts (p)ppGpp production upon different challenges, independently of RelA (Gentry and Cashel, 1995; Hernandez and Bremer, 1991; Xiao et al., 1991b). Deprivation in iron (Vinella et al., 2005), phosphorus (Spira et al., 1995), carbon (Murray and Bremer, 1996) and fatty acid sources (Battesti and Bouveret, 2006) leads to SpoT-dependent activation of stress response. Here acyl carrier protein (ACP) that plays a central role in fatty acid biosynthesis (Byers and Gong, 2007) and binds SpoT TGS domain was shown to be important (Battesti and Bouveret, 2006). With limited access to fatty acid sources ACP adopts a certain conformation that may act as a mediator allowing SpoT to sense the fatty acid starvation (like deacylated tRNA does for RelA) and switch onto the synthesis mode (Battesti and Bouveret, 2006). The fact that ACP cannot bind RelA, despite high similarity in sequence with SpoT, supports ACP role in regulation of SpoT activity (Battesti and Bouveret, 2006). This theory has been confirmed only for the bacteria that have two distinct long RSHs the same way *E. coli* does (Battesti and Bouveret, 2009; Dalebroux et al., 2009).

The data on SpoT positioning within the cell is controversial. There are reports stating the protein does not bind the ribosome or any intracellular membranes and is considered to be a cytosolic protein (Gentry and Cashel, 1995) as well as the ones claiming SpoT is associated with ribosomal 50S subunit (Jiang et al., 2007). It is also well documented that 50S ribosomal subunit assembly factor, GTPase CgtA (ObgE) (Sato et al., 2005), directly binds SpoT (Wout et al., 2004) and can control its activity during exponential phase helping to maintain the basal levels of (p)ppGpp (Jiang et al., 2007). Thus, mutations in CgtA result in highly increased intracellular (p)ppGpp levels (Jiang et al., 2007).

The control over basal levels of (p)ppGpp in bacteria that instead of two long RelA and SpoT RSHs have one bifunctional Rel enzyme and one or several SASs is possibly carried out in a different way where the main role belongs to SASs.

4.6. Biological functions of SAS enzymes

In addition to bifunctional Rel enzymes many bacteria, including most pathogenic *Firmicutes*, encode one or several small alarmone synthetases (SAS) (Lemos et al., 2004; Nanamiya et al., 2008). The presence of these small (p)ppGpp synthetases, often termed RelQ/RelP (Lemos et al., 2007) or RelV (Das et al., 2009), was reported in *B. subtilis* (Nanamiya et al., 2008), *Listeria monocytogenes* (Okada et al., 2002b), *Streptococcus mutans* (Lemos et al., 2007), *Vibrio cholerae* (Das et al., 2009) etc. SASs and SAHs (stands for small alarmone hydrolase) are united by the complete lack of CTD domain and putative regulation by different factors such as ribosome, tRNA and others (Atkinson et al., 2011). Depending on the function of the protein they also miss either SYNTH or HD domain (Atkinson et al., 2011). A structure of SAS YjbM (RelQ) from *B.*

subtilis was recently obtained with X-ray crystallography method (Steinchen et al., 2015). In its active form RelQ comprises a homotetramer with allosteric regulation by (p)ppGpp (Steinchen et al., 2015).

The role of SASs remains unclear; it is possible that SASs respond to stresses that bifunctional long RSHs do not sense, such as alkaline shock (Nanamiya et al., 2008). The other possibilities are the supportive role in rapid stringent response induction (Gaca et al., 2012; Lemos et al., 2007) and maintenance of the basal (p)ppGpp levels (Gaca et al., 2013). RelQ of *Enterococcus faecalis* is also implemented in the synthesis of alarmone molecule pGpp, which role, however, is obscure (Gaca et al., 2015).

While SASs are being extensively studied, not much is known about SAH enzymes. One of the SAH representatives is reported to be Ndx8 - a member of Nudix pyrophosphatase family in *T. thermophilus* (Ooga et al., 2009). The putative function of Ndx8 is in modulating the transition between the growth phases via (p)ppGpp degradation (Ooga et al., 2009). SpoT ortholog was also identified in such eukaryotes as *Drozhophila melanogaster*, *Caenorhabditis elegans* and even human (Sun et al., 2010). The name of this protein is Mesh1 (metazoan SpoT homolog 1) and it possesses both HD domain and His-Asp motif crucial for Mn-ion binding (Sun et al., 2010). Mesh1 is able to hydrolyze (p)ppGpp both in vitro and vivo while the deletion of the corresponding gene results in retarded body growth and reduced starvation resistance in *D. melanogaster*. The role of the enzyme *in vivo* remains, however, elusive (Sun et al., 2010).

4.7. Stringent response in phototrophs

For a long time stringent response regulatory pathway has been considered unique for bacteria. In early 2000s this hypothesis was proved to be wrong as RSHs were found in plants (van der Biezen et al., 2000). The confirmations of all photosynthetic bacteria having RSHs (Atkinson et al., 2011; Braeken et al., 2006; Masuda and Bauer, 2004) and discovery of high (p)ppGpp concentrations in pea chloroplasts (Takahashi et al., 2004) supported the possibility of (p)ppGpp signaling pathway presence in plants. Although the presence of RSH enzymes and (p)ppGpp in plants is restricted to plastids, stringent response system is very important for plant physiology (Givens et al., 2004; Masuda et al., 2008a; Masuda et al., 2008b). The most popular model organism in plant biology, *Arabidopsis thaliana*, was shown to have four RSH enzymes: RSH1, RSH2, RSH3 (van der Biezen et al., 2000) and CRSH (Tozawa et al., 2007). The domain conservation in RSH1 suggests lack of synthetase activity (Atkinson et al., 2011) positioning RSH1 as (p)ppGpp hydrolase (Takahashi et al., 2004). In *A. thaliana* RSH1 is as well reported to interact with R-protein RPP5 that is encoded by plant disease resistance genes and is involved in pathogen detection (van der Biezen et al., 2000). Whereas RSH2 and RSH3 are possibly bifunctional long RSH enzymes (Atkinson et al., 2011; Masuda et al., 2008a),

CRSH seems to be the most interesting of *A. thaliana* RSHs. CRSH is a Ca-dependent enzyme that possesses Ca-binding EF-hand domain. The (p)ppGpp synthetase activity of the enzyme can be activated by Ca ions and, similarly to *E. coli* RelA, CRSH does not have hydrolase activity (Tozawa et al., 2007). Similar RSH distribution was also documented in *Nicotiana tabacum*, *Oryza sativa* and *Physcomitrella patens* suggesting it can be universal amongst plants (Givens et al., 2004; Masuda, 2012; Tozawa et al., 2007).

In plants stringent response is involved in protection from pathogens (van der Biezen et al., 2000), reaction on physiological changes (Braeken et al., 2006) and even photosynthesis (Takahashi et al., 2004). Putative control of photosynthesis by (p)ppGpp is based on combined actions of RSH2/3 that maintain high (p)ppGpp levels during the light phase and RSH1 that degrades it during the night (Takahashi et al., 2004). CRSH may as well respond to change of Ca ion levels in response to changes in light intensity (Sai and Johnson, 2002) triggering (p)ppGpp synthesis in response to increased Ca (Masuda, 2012). Plants tend to form symbiotic relationships with such organisms as fungi and bacteria. *Rhizobium etli* and *Sinorhizobium meliloti* are two bacteria species known to form symbiosis with plant roots. They are also known to survive stress conditions with the help of bifunctional RSH enzymes (Braeken et al., 2008; Calderon-Flores et al., 2005; Wei et al., 2004). More of that, in these bacteria the symbiosis with plants depends on (p)ppGpp as it modulates nodulation and nitrogen fixation – processes that are crucial for the establishment of bacteria-plant symbiosis (Calderon-Flores et al., 2005; Moris et al., 2005).

4.8. The feedback between RSH proteins and their product, (p)ppGpp

A remarkable role is assigned to (p)ppGpp in the theory of "intra-domain crosstalk" in RSH proteins. Reaching high intracellular levels (p)ppGpp can possibly bind to CTD of RSH, supporting the inhibitory interaction of CTD with SYNTH domain and giving the permission for HD domain to act (Jain et al., 2007). On the contrary, we recently discovered that at relatively low concentrations (p)ppGpp is able to speed up its own production through allosteric regulation of RelA activity (Shyp et al., 2012). Allosteric binding of pppGpp was also observed in the X-ray structure of *B. subtilis* SAS RelQ (Steinchen et al., 2015).

5. Stringent response and bacterial pathogenicity

5.1. The role of (p)ppGpp in bacterial virulence

As stringent response mechanism is common amongst bacteria, the pathogenic representatives of the kingdom use it in their favor. The first indirect evidence of possible (p)ppGpp role in bacterial virulence appeared when decreased biofilm formation was observed in *E. coli relA spoT* double mutants (Balzer and McLean, 2002). The reports on direct regulation of virulence genes by (p)ppGpp in pathogenic bacteria followed (Godfrey et al., 2002; Lemos et al., 2004; Nakanishi et al., 2006). Thus in enterohemorragic (EHEC) (Nakanishi et al., 2006) and uropathogenic (UPEC) (Aberg et al., 2006, 2008; Kau et al., 2005) *E. coli* (p)ppGpp controls the expression of the genes necessary for host cell invasion and biofilm formation. Similar purposes are being achieved with the help of (p)ppGpp in many other pathogens including *P. aeruginosa* (Shrout et al., 2006), *S. typhimurium* (Thompson et al., 2006), *L. monocytogenes* (Taylor et al., 2002) etc. The stirrers of plague and cholera, *Yersinia pestis* and *V. cholerae* respectively, rely on (p)ppGpp in mediating the activity of virulence regulator proteins (Haralalka et al., 2003; Silva and Benitez, 2006; Sun et al., 2009). In *P. aeruginosa* ppGpp0 strain the *rpoS* levels and virulence are heavily reduced (Erickson et al., 2004) while (p)ppGpp deficient *Salmonella* strain is weakly infectious and shows strong attenuation (Pizarro-Cerda and Tedin, 2004; Thompson et al., 2006).

Besides controlling the expression of virulence genes and biofilm formation, (p)ppGpp also plays its canonical role – promotes the survival of the cell in harsh conditions. In the case of pathogens it can be exposure to acid or aerobic shock during host uptake like with *Helicobacter pylori* (Mouery et al., 2006; Wells and Gaynor, 2006), sanitizer exposure and antibiotic treatment like with *E. faecalis* (Abranches et al., 2009; Yan et al., 2009) or cold of refrigerated food like with *L. monocytogenes* (Liu et al., 2006; Okada et al., 2002a). The survival in host macrophages and avoidance of degradative lysosomes are as well dependent on activated stringent response (Dalebroux et al., 2010) as was shown on the examples of *Campylobacter jejuni* (Gaynor et al., 2005; Stintzl et al., 2005), *Francisella tularensis* (Charity et al., 2009) and *Brucella* species (Dozot et al., 2006). Sporulation is also a method of survival for bacteria and the differentiation into spores is often controlled by (p)ppGpp. A good example is anthrax agent *Bacillus anthracis* where formation of spores occurs depending on Rel protein activity and whose spores can easily survive through decades (van Schaik et al., 2007).

5.2. (p)ppGpp is important for bacterial persistence

Bacterial infections are treated with antibiotics. Sometimes a small population remains alive in an isogenic bacterial culture exposed to certain antibiotic. After the removal of the drug the survived bacteria resume growth. Those bacteria are

not resistant and can be killed if exposed to the drug again. And also during the second treatment a small population survives. That ability of bacteria to survive the drug treatment is termed "persistence" and is of high scientific importance due to causing the recurrent bacterial infections (**Fig. 8**) (Kaldalu et al., 2016). While studying persistence, the question if it has specific genetic traits occurred. The first gene proven to be involved in persistence was called *hipA* where "hip" stands for "high persister" (Korch et al., 2003; Lewis, 2010; Maisonneuve and Gerdes, 2014; Moyed and Bertrand, 1983). Overexpression of HipA leads to suppression of the cell growth on all the levels and increase in antibiotic tolerance (Lewis, 2010; Maisonneuve and Gerdes, 2014). *hipB* is situated upstream of *hipA* and *hipB* gene product binds HipA protein thus inhibiting its activity during normal growth conditions. Together genes *hipB* and *hipA* (*hipBA*) represent a toxin-antitoxin locus (TA) (Lewis, 2010; Maisonneuve and Gerdes, 2014). Other TA loci like *relEB* or *mazEF* are also implemented in persistence suggesting a strong link between the persistence phenomenon and TA modules (Gerdes and Maisonneuve, 2012).

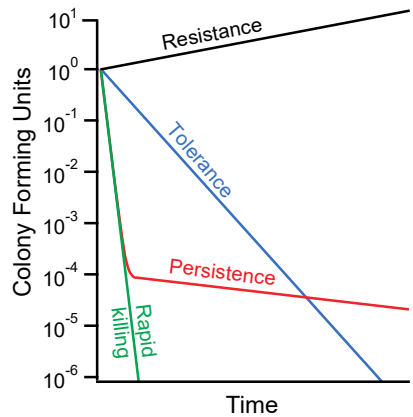

Figure 8. Antibiotic killing kinetics of bacterial cultures showing a rapidly sterilized culture, tolerance and resistance. The slower killing phase or plateau is defined as persisters. Adapted from (Kaldalu et al., 2016).

The third player, (p)ppGpp, stepped on the scene when the function of *hipA* gene product was discovered. HipA turned out to be a kinase able to inhibit the activity of Glu-tRNA synthetase (Germain et al., 2013; Kaspy et al., 2013). Accordingly, overproduction of HipA leads to increase in deacylated tRNA levels followed by the activation of stringent response (Germain et al., 2013; Kaspy et al., 2013). Increased (p)ppGpp levels are, on the other hand, beneficial for the translation of toxins from TA loci (Aizenman et al., 1996; Dahl et al., 2003; Gerdes et al., 2005; Hazan et al., 2004) as (p)ppGpp regulates the activity of Lon protease (Maisonneuve et al., 2013) which, in turn, is able to degrade

antitoxins (Gerdes and Maisonneuve, 2012; Maisonneuve et al., 2011). Therefore, a possible positive feedback loop exists between the activation of stringent response and toxin-antitoxin activity resulting in elevated drug tolerance (Hauryliuk et al., 2015).

The importance of stringent response for persistence is also supported in numerous studies dedicated to bacteria other than *E. coli*. For example, in *M. tuberculosis*, where stringent response is activated through the carbon source limitation (Sureka et al., 2007), Rel protein was *in vivo* shown to be of absolute necessity for chronic persistence (Dahl et al., 2003; Primm et al., 2000). Another famous pathogen, *S. typhimurium*, produces considerably fewer persisters in macrophages when lacking either s*poT*, *relA* or *lon* genes (Helaine et al., 2014).

Bacterial persistence is relatively poorly understood phenomenon and the exact mechanisms that allow persistent bacteria to tolerate high antibiotic concentrations remain elusive (Kaldalu et al., 2016). Nevertheless, for certain antibiotics the direct or indirect but crucial influence on stringent response has been documented.

5.3. Drug resistance acquired through the stringent response

Considering how the physiology of the bacterial cell is being reprogrammed upon the activation of stringent response, there can be a huge variety of pathways to the drug tolerance. The absolute majority of these pathways is dependent on the indirect effect of rising in (p)ppGpp levels.

One of the first antibiotics, penicillin, belongs to the family of β-lactam antibiotics and literally made a revolution in medicine upon the discovery. Nowadays there are dozens of β-lactam antibiotics but their target is still the same – the cell wall synthesis. During the stringent response the synthesis of peptidoglycan, the main component of the cell wall, is inhibited, consequently, the target of β-lactam antibiotics is absent (Hesketh et al., 2007). Rodionov and Ishiguro studying the effects of β-lactam amdinocillin showed that overexpression of cell division protein FtsZ triggered by (p)ppGpp promotes cell division without additional synthesis of peptidoglycan therefore diminishing the effect of the antibiotic (Rodionov and Ishiguro, 1995). Suchlike solution is also applied against the glycopeptide antibiotic vancomycin that as well targets cell wall synthesis (Abranches et al., 2009). Another strategy takes into advantage the fact that (p)ppGpp binds to RNAP; polypeptide antibiotic microcin J25 (Solbiati et al., 1999) acts through binding into RNAP secondary channel and blocking the access for NTP substrates (Mukhopadhyay et al., 2004) – (p)ppGpp binding site on RNAP is located in a close proximity and with the assistance of DksA (p)ppGpp can neutralize the inhibitory effect of microcin J25 (Pomares et al., 2008). Other RNAP-targeting antibiotics like rifamycin, streptolydigin, myxopyronin and ripostatin are counteracted by (p)ppGpp in a more or less similar manner (Mukhopadhyay et al., 2008).

6. Inhibition of bacterial stringent response

6.1. Antibiotics targeting protein synthesis as modulators of the SR

Shortly after the discovery of the stringent response the attempts to modulate the mechanism of SR with drugs began (**Table 1**). Considering that (p)ppGpp production in *E. coli* is activated upon starvation (Cashel and Gallant, 1969), a shortage in amino acids source can lead towards the activation of the stringent response. Therefore, the drugs that can ensure or mimic the shortage in nutrients can possible be the inducer for the stringent response. Accordingly, serine hydroxamate (SHX), that inhibits the activity of Ser-tRNA synthetase, has shown itself as a very prominent SR inducer. Antibiotic mupirocin or pseudomonic acid works in the same manner, though it limits access to isoleucine through inhibiting Ile-tRNA synthetase activity (Hughes and Mellows, 1978). Another antibiotic, trimethoprim, is also able to elicit (p)ppGpp production (Khan and Yamazaki, 1972; Lund and Kjeldgaard, 1972a) via the very strategy of mimicking amino acid deprivation (Roche et al., 1976). Trimethoprim is known DNA synthesis inhibitor that inhibits the activity of the enzyme dihydrofolate reductase resulting in a drop of tetrahydrofolate (THF) production (Gleckman et al., 1981). THF is crucial for thymidine synthesis but is also very important for the production of such amino acids as Met and Gly and lack of it leads to the starvation for methionine and glycine.

The measurements of cellular nucleotide pools, including (p)ppGpp, show the strong decrease in (p)ppGpp level upon exposure to certain antibiotics. For example, rifampicin binds RNAP and blocks transcription. As a result, no mRNA is synthesized, consequently decreasing the level of translation. Following the same logic, slow translation means less protein and more aminoacilated tRNAs what acts as a signal for RSH enzymes to turn off the stringent response and start (p)ppGpp degradation (Wong and Nazar, 1970).

The studies on several translational antibiotics are controversial; translocation inhibitor Fus (fusidic acid, blocks EF-G on the ribosome) and Cam (chloramphenicol, binds into the ribosomal P-site and prevents peptide bond formation) were shown to have moderate effect on stringent response (Lund and Kjeldgaard, 1972a). In the presence of these antibiotics (p)ppGpp is being degraded or not produced at all if pre-treated with them while RNA synthesis is being restored (Lund and Kjeldgaard, 1972a). In another study, however, the results are opposite with none of the antibiotics able to affect the stringent response (Kaplan et al., 1973). Anyways, possible inhibition by either Cam or Fus seems to be indirect – the same mechanism as with rifampicin comes into play – arrested translation causes drop in amino acid consumption subsequently increasing the rate of tRNA aminoacylation (Kaplan et al., 1973; Kurland and Maaloe, 1962; Midgley and Gray, 1971). The data on the other putative stringent response inhibitor, tetracycline, are more consistent. Tet-initiated inhibition

of translation is based on the blockage of the ribosomal A-site by the antibiotic (Nguyen et al., 2014). Deacylated tRNA cannot bind into the A-site therefore eliminating the possibility for the stringent response to be activated. Tet was confirmed to be very effective against the SR in bacteria (Kaplan et al., 1973; Lund and Kjeldgaard, 1972a) and also in chloroplasts of the plants (Kasai et al., 2004).

6.2. Direct inhibition of RelA by thiostrepton

The best-known representative of thiopeptide antibiotics, thiostrepton or Ts, inhibits translation by preventing the correct positioning of translational GTPases IF2 (Brandi et al., 2004) and EF-G (Modolell et al., 1971; Thompson et al., 1988; Walter et al., 2012) on the ribosome during initiation and elongation steps respectively. Thiostrepton binds into the cleft between r-protein L11 and helices H43 and H44 of 23S rRNA subunit making four contacts with the 50S subunit: two with Pro22 and Pro26 of L11 and two with 23S rRNA residues A1095 and A1067 (Harms et al., 2008). Although RelA and Ts binding sites on the ribosome (Brown et al., 2016; Harms et al., 2008) do not seem to overlap, the fact that RelA-bound A/T-tRNA makes a stacking interaction with A1067 of 23S rRNA (Brown et al., 2016) and L11 r-protein was shown to be of importance for the activation of the SR (Yang and Ishiguro, 2001b) leaves a possibility that the conformational changes upon Ts binding can prevent the activation of the stringent response. As a proof, decades ago it was discovered that thiostrepton completely inhibits (p)ppGpp formation in vitro (Haseltine et al., 1972c; Knutsson Jenvert and Holmberg Schiavone, 2005; Sy, 1974) but only in a ribosome-dependent manner (Sy et al., 1973). Being the most specific antibiotic towards the SR so far discovered, Ts has one major minus – it is insoluble in water and has no effect on *E. coli in vivo* due to the lack of cellular uptake (Pestka, 1970). But having the base for the further development, thiostrepton can certainly be improved in terms of inhibiting the SR. One of the ways is to make it more soluble, either by chemically modifying or using surfactants to help the drug get into the cell.

Table 1. Antibacterial agents known to have an effect on the stringent response.

Antibacterial agent	Type	Main target	Effect on the SR	Mechanism of action	References and comments
Serine Hydroxamate (SHX)	Serine analog	Ser-tRNA synthetase; translation	Activation	Mimics amino acid depletion	
Mupirocin (pseudomonic acid)	Monoxycarbolic acid, antibiotic	Ile-tRNA synthetase; translation	Activation	Mimics amino acid depletion	(Hughes and Mellows, 1978)
Trimethoprim	Pyrimidine analog, antibiotic	Dihydrofolate reductase; replication	Activation	Mimics amino acid depletion	(Khan and Yamazaki, 1972)
Rifampicin	Bacteriocidal antibiotic	RNA polymerase; transcription	Inhibition	Increases tRNA aminoacylation level	(Wong and Nazar, 1970)
Fusidic acid	Bacteriostatic antibiotic	EF-G; translation	Inhibition	Increases tRNA aminoacylation level	(Kaplan et al., 1973; Lund and Kjeldgaard, 1972b)
Chloram phenicol	Mostly bacteriostatic antibiotic	Ribosome, peptidyl-transferase center; translation	Inhibition	Increases tRNA aminoacylation level	(Kaplan et al., 1973; Lund and Kjeldgaard, 1972b)
Tetracycline	Tetracycline, polyketide antibiotic	Ribosomal A-site, prevents tRNA ac-commodation; translation	Inhibition	Prevents the SR activation by blocking A-site tRNA binding	(Kaplan et al., 1973; Kasai et al., 2004)
Thiostrepton	Thiopeptide antibiotic	Ribosome, blocks correct EF-G positioning; translation	Inhibition	Ribosome-dependent	(Knutsson Jenvert and Holmberg Schiavone, 2005)
Relacin	(p)ppGpp analog	RSH proteins; stringent response	Inhibition	Direct binding to Rel enzymes	(Wexselblatt et al., 2012); questioned (Andresen et al., 2016a)
Peptide 1018	Anti-biofilm peptide	(p)ppGpp; stringent response	Inhibition	Binds (p)ppGpp and marks it for degradation	(de la Fuente-Nunez et al., 2014); refuted (Andresen et al., 2016a)

6.3. New generation of the SR inhibitors

While one of the approaches to inhibit the stringent response includes the re-assessment and upgrade of the classical antibiotics, another suggests discovering new molecules that can be effective against RSH proteins. Some efforts along this way have already been taken, resulting in a number of (p)ppGpp-based chemical compounds (Wexselblatt et al., 2010). Several (p)ppGpp-analogues, especially Relacin, were shown to inhibit Rel activity almost completely, however only in millimolar range (Wexselblatt et al., 2012). The specificity of Rel activity inhibition by Relacin was also questioned (Andresen et al., 2016b). Nevertheless, the approach of making RSH inhibitors based on (p)ppGpp structure looks promising.

A completely different strategy to inhibit the SR was recently presented with a discovery of antibiofilm peptide 1018 by the group of R. Hancock (de la Fuente-Nunez et al., 2014). According to the authors, the peptide eradicates mature biofilms and prevents the formation of new ones by blocking the signaling pathway involving (p)ppGpp (de la Fuente-Nunez et al., 2014). Subsequently, the authors claim that 1018 peptide specifically binds (p)ppGpp and initiates its degradation (de la Fuente-Nunez et al., 2014). In recent studies, however, the specificity of the SR inhibition by 1018 peptide was put under question (Andresen et al., 2016a; Andresen et al., 2016b).

AIM OF THE STUDY

The general aim of the study is to characterize the relationships between *E. coli* RelA, its product (p)ppGpp and its activators 70S ribosome and deacylated tRNA by studying the inhibitory effects of different antibacterial agents on the SR.

Specific objectives of the study:

- To reveal the effect of product on (p)ppGpp synthetases *E. coli* stringent factor RelA and *E. faecalis* SAS RelQ during the early steps of the SR activation (Papers I and II)

- To evaluate the possibilities of developing novel antibacterial agents based on a (p)ppGpp scaffold (Paper III)

- To test the effect of existing translational antibiotics on the stringent response and reveal the mechanisms of their action if inhibitory effect is present (Paper IV)

- To characterize the effects of combined antibiotic treatment onto the stringent response and bacterial persistence in living bacterial cultures (Paper IV)

RESULTS AND DISCUSSION

1. The activities of the *E. coli* stringent response factor RelA and *E. faecalis* small alarmone synthetase RelQ are regulated by their product, (p)ppGpp (Papers I, II)

1.1. ppGpp positively controls the activity of *E. coli* RelA

The activation of the stringent response is not a spontaneous process and needs certain circumstances and factors to get started. As it was previously discussed, lack of nutritional sources leads to increase in the levels of deacylated tRNAs which, in turn, when bound to the 70S ribosome represents the major condition for the activation of the SR. The change in alarmone molecule (p)ppGpp levels is what regulates the SR and, therefore, measuring (p)ppGpp synthetic activity can reflect the possibility for the SR induction. In *in vitro* (p)ppGpp synthesis system consistent of 70S, mRNA, deacylated tRNA and substrates for (p)ppGpp production the stalled ribosomal complex indeed fulfills the conditions for the induction of (p)ppGpp synthesis by activating RelA, however the kinetics of (p)ppGpp production does not seem to be linear, representing sigmoid-like curve with a pronounced lag-effect (Payoe and Fahlman, 2011). One of the possible explanation for the occurrence of the lag-effect can be the lack of crucial components for the rapid activation of the (p)ppGpp synthesis in the experimental system used.

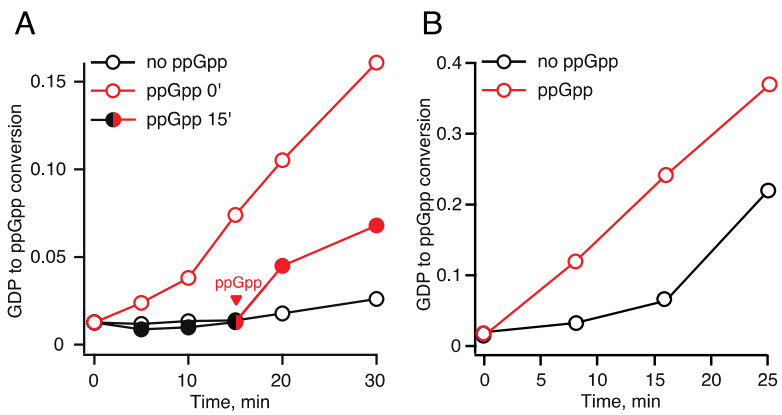

Figure 9. Synthetic activities of *E. coli* RelA (A) and *E. faecalis* RelQ (B) in the presence (red empty circles) and absence (black empty circles) of 100 μM ppGpp. Axis OY=1 means all the substrate (GDP) has been converted into ppGpp. Filled circles on a panel A represent an assay with ppGpp addition at certain timepoint, black before the addition of 100 μM ppGpp, red after it.

The regulation of enzyme activity by its product is a widely distributed phenomenon; in our case we observed that upon the addition of ppGpp to our *in vitro* system the lag-effect has been removed suggesting ppGpp enhances the activity of RelA (**Fig. 9A**). Considering this, the lag-effect can be explained by progressive stimulation of RelA activity by *in situ* produced ppGpp. The further investigation of the phenomenon of RelA activation by its product led to the conclusion that the existence of a positive feedback loop between RelA and ppGpp is not exclusive amongst RSHs. When studying the small alarmone synthetase of *E. faecalis*, RelQ, *in vitro*, we observed similar elevation in enzyme's activity upon addition of either ppGpp (**Fig. 9B**) or pppGpp. Guanosine pentaphosphate activation of *B. subtilis* SAS1 was also reported previously (Steinchen et al., 2015).

The positive feedback loop between (p)ppGpp synthetases and their product ensures accelerated response towards stress stimuli. Sped up activation of total cellular population of RelA enzymes allows to increase the adaptation that is extremely crucial for cell survival.

2. ppGpp and molecules structurally related to it can negatively affect the activity of *E. coli* stringent response factor RelA (Paper III)

2.1. ppGpp inhibits RelA activity at higher concentrations

When knowing that ppGpp can positively regulate the activity of RelA the logical next step would be to check to what extent ppGpp is able to activate RelA. To our surprise, when titrating ppGpp on RelA *in vitro*, the activity of the enzyme starts to drop down with ppGpp higher than ~150 μM and it is almost completely gone at the 1 mM concentration of ppGpp (**Fig. 10A**). One possible explanation for this inhibition could be that in an *in vitro* system saturated with ppGpp the alarmone molecule starts to compete with native substrates GDP and GTP for the enzyme's active center. Considering that ppGpp can possibly bind into both RelA's active center and allosteric binding site the molecule can be chemically modified into RelA inhibitor that is likely to block the activation of stringent response.

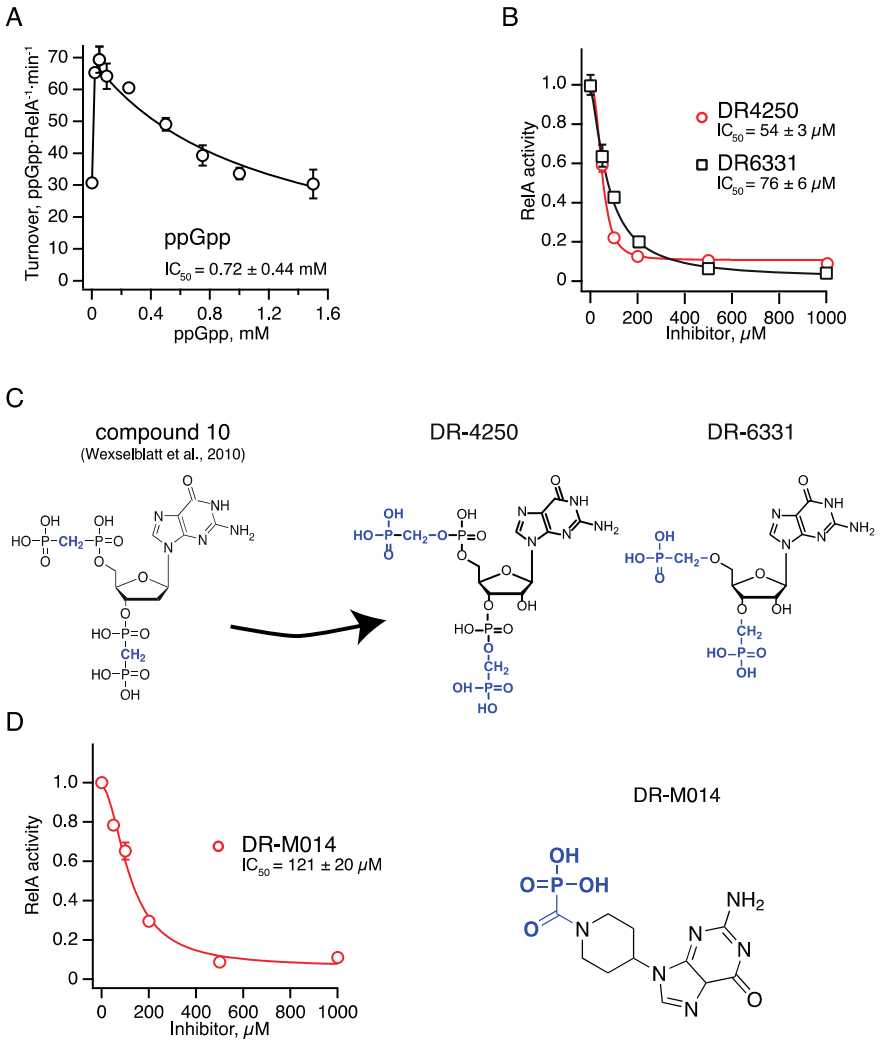

Figure 10. ppGpp and its derivatives negatively affect the synthetic activity of RelA. RelA activity is tested in the presence of 0.5 μM of 70S ribosomes. A) ppGpp activates RelA at low and turns into an inhibitor at higher concentrations. B) ppGpp-analogues DR-4250 (red circles), DR-6331 (black squares) and D) piperidine phosphonate DR-M014 efficiently inhibit ppGpp synthesis of RelA, activated by 70S ribosome. RelA activity without an inhibitor equals 1 and RelA activity in the presence of the inhibitor is normalized to that. C) The structures of compound 10 from (Wexselblatt et al., 2010), one of the first inhibitors with ppGpp-like structure, and compounds DR-4250 and DR-6331 used in this study. Error bars represent standard deviation of the turnover estimated by linear regression. Each experiment was performed at least three times.

2.2. Modifying ppGpp into better RelA inhibitor

Considering that RelA is of great importance for the stringent response activation and, therefore, is a very prominent target for the development of new antibacterial agents, one could possibly to try to use ppGpp as a platform for the development of anti-RelA drug. There are, however, certain problems to overcome on the pathway of making ppGpp into efficient RelA inhibitor: a) ppGpp is considerably hydrophilic which may cause problems with cellular uptake i.e. delivery of the molecule to its target; b) ppGpp is degradation-prone – enzymes like SpoT or intracellular chemical conditions can easily degrade it back to GDP; c) the flexibility of pyrophosphate moieties of ppGpp should not be constrained as it is of importance for correct allosteric binding (Steinchen et al., 2015). First attempts in the direction of modifying ppGpp into an inhibitor were undertaken by an Israeli research group and resulted in the development of Relacin (Wexselblatt et al., 2010; Wexselblatt et al., 2012). Despite of all modifications (Relacin is less hydrophilic and chemically protected from degradation) it is still not very effective against RSH enzymes with IC_{50} around 0.8 mM. Therefore it is not suitable for drug development pipeline.

To follow up in the development of new ppGpp-based effector molecules we tested a library of 69 nucleotide analogues on their ability to inhibit the activity of *E. coli* stringent response factor RelA *in vitro*. The library was produced in collaboration with a group of chemists led by Dominik Rejman from Czech Academy of Sciences. Structurally the library of tested compounds can be divided into groups with the first group representing compounds strongly related to ppGpp. The "true" ppGpp analogues differ from ppGpp only in minor aspects leaving the overall structure intact.

As a starting point we used previously described compound 10 (**Fig. 10C**) (Wexselblatt et al., 2010) where the oxygen atoms connecting the phosphate groups have been replaced with methylene bridges to improve the chemical stability of the compound. As the given compound showed considerably weak activity against RelA our efforts were directed towards improving the inhibitory effect without losing chemical stability. Modifying the nucleobase did not give much in this case while changing the pyrophosphate moieties of ppGpp, on the contrary, resulted in much more efficient compounds DR-4250 (IC_{50} = 54 ± 3 μM) and its derivative with reduced phosphate groups and, therefore, the net charge, DR-6331 (IC_{50} = 76 ± 6 μM) (**Fig. 10B**).

Most of the remaining compounds in the library were divided into additional groups according to the modifications in the sugar cycle moving from ribose towards acyclic compounds. The vast majority of the compounds, unfortunately, were either poor inhibitors for RelA activity or had no effect at all. The only partial success was achieved with the compound DR-M014. DR-M014 belongs to the group of piperidine phosphonates and, while most of the group representatives in the library were hardly effective against RelA, DR-M014 has IC_{50} of 121 ± 20 μM (**Fig. 10D**). Compared to DR-4250 it has simpler structure and of smaller size with, however, smaller inhibitory effect. As most promising com-

pounds both DR-4250 and DR-M014 were further investigated to reveal the mechanism of their action. During the first stage all the compounds were tested in a simplistic *in vitro* system where only minimal requirements for RelA activity were fulfilled (**Fig. 10B,D**). In a following experiment RelA was activated to its maximum by starved ribosomal complex (**Fig. 11**) and the effects of the most promising compounds were analyzed. Both DR-4250 and DR-M014 showed relatively the same IC_{50}'s as obtained previously, 41 ± 7 μM and 155 ± 14 μM respectively (**Fig. 11**). The independence of the inhibitory effect on the presence of the major activator of (p)ppGpp synthesis by RelA, the starved ribosomal complex, suggests both compounds could potentially be effective *in vivo*.

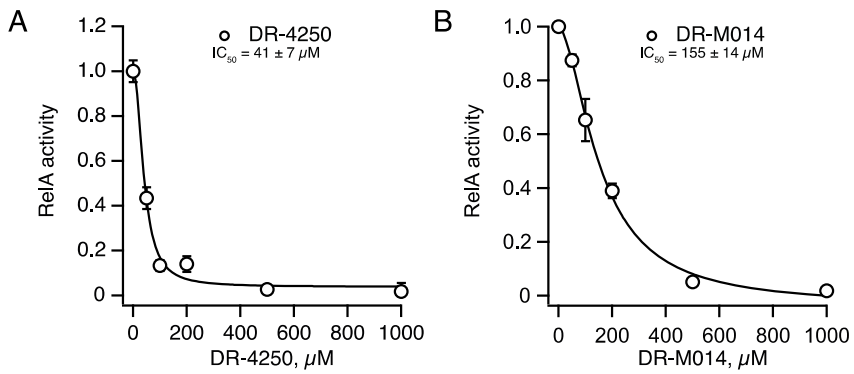

Figure 11. ppGpp synthesis of RelA, activated with starved ribosomal complex, is inhibited by ppGpp derivaives A) DR-4250 and B) DR-M014. RelA activity without an inhibitor equals 1 and RelA activity in the presence of the inhibitor is normalized to that. Error bars represent standard deviation of the turnover estimated by linear regression. Each experiment was performed at least three times.

2.3. RelA inhibition by naturally occurring nucleotides and 6-thio-ppGpp

Bacteria have many other signal molecules than ppGpp. Some of them, like cAMP, c-di-GMP etc., share structural similarities with the substrates for (p)ppGpp synthesis and theoretically can compete with the substrates for RelA's active center and with ppGpp for the allosteric binding site. We tested a set of suchlike molecules in our system though with a negative result. Lastly, we also tested two more nucleotides that are structurally very close to ppGpp. One of them is naturally occurring nucleotide with unknown function, ppApp (Oki et al., 1976) and the other is ppGpp derivative 6-thio-ppGpp used for mapping ppGpp binding sites on RNAP (Ross et al., 2013). One can assume that by being structurally close to ppGpp, ppApp and 6-thio-ppGpp should

activate RelA in a manner ppGpp itself does, however this is not the case as both nucleotides turned out to be potent RelA activity inhibitors with IC_{50}'s of ~20 μM for both of them (**Fig. 12**).

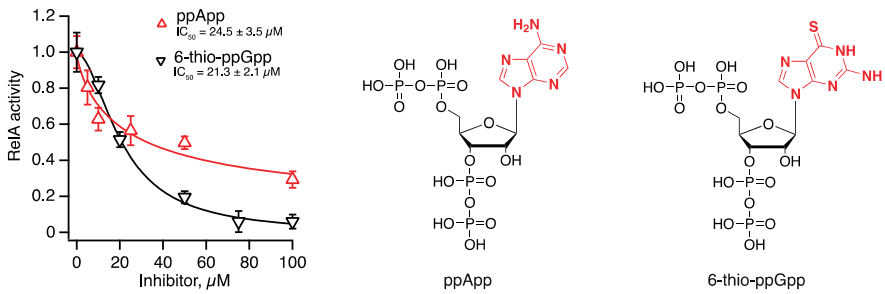

Figure 12. Substitution of the base in ppGpp for adenine (red triangles) or 6-thio-guanine (black triangles) increases the inhibitory effect dramatically. RelA activity is tested in the presence of 0.5 μM of 70S ribosomes. RelA activity without an inhibitor equals 1 and RelA activity in the presence of the inhibitor is normalized to that. Error bars represent standard deviation of the turnover estimated by linear regression. Each experiment was performed at least three times.

In a current state the strategy of modifying ppGpp scaffold to make a ppGpp-synthesis inhibitor looks promising but definitely needs improvement. We demonstrated that ppGpp-derivatives can inhibit RelA's activity *in vitro* but the problem of making the putative inhibitor suitable for clinical trials remained unsolved. We tested the compounds for their *in vivo* effect in *E. coli* live culture with no inhibition of (p)ppGpp production observed. The most obvious reason could be that the compound could not reach its target i.e. enter the bacterial cell. One of the possible solutions could be chemically modifying the most promising compounds into prodrugs to overcome the possible problem of cellular uptake. In the way they are now, the compounds of the library described here can be beneficial in structural studies by helping to stabilize RelA on the ribosome.

3. Revising the effect of classical antibiotics (Paper IV)

3.1. Thiostrepton specifically inhibits RelA activation by deacylated A-site tRNA

With the progress in uncovering the mechanism of the stringent response the dependence of the stringent factor RelA on the ribosome and its functions became obvious. Consequently the information concerning RelA positioning on the ribosome allows to suggest ways to brake the interaction between the enzyme and the organelle and therefore inhibit (p)ppGpp synthesis. Certain translational antibiotics (**Table 1**) have previously been shown to inhibit RelA activity with, however, no data about the possible mechanism of action (Kaplan et al., 1973; Knutsson Jenvert and Holmberg Schiavone, 2005). Here we re-examined the effect of classical translational antibiotics in the context of latest structural data.

According to structural data (Brown et al., 2016; Harms et al., 2008), the antibiotic thiostrepton can possibly block correct positioning of RelA on the ribosome the same way as it does with EF-G. Nevertheless, thiostrepton has very low solubility in non-organic solvents and that becomes an issue when applying it in bacterial systems. Common organic solvents like DMSO (dimethyl sulfoxide) and TFE (2,2,2-trifluorethanol) at the concentration of 3% (v/v) keep thiostrepton poorly in solution while the addition of 0.1% (w/v) non-ionic surfactant Pluronic F-127 helps keeping thiostrepton soluble up to the concentration of 15 μM that we confirmed using DLS (dynamic light scattering) analysis. At the same time, Pluronic F-127 does not affect either ppGpp synthesis by RelA or GTPase activity of EF-G. Therefore, in all the following experiments Pluronic F-127 was added at the concentration of 0.1% (w/v).

To test the effect of thiostrepton on RelA activity we titrated it in *in vitro* systems with either weakly active RelA (70S ribosome activation) or fully active RelA (activated by 70S loaded with mRNA/polyU and deacylated tRNAs). As a positive control we reproduced thiostrepton effect on the GTPase activity of EF-G (Walter et al., 2012) with WT and as a negative control with A1067U mutant 70S ribosomes. When assaying thiostrepton against RelA we demonstrated that the antibiotic has absolutely no effect on RelA activity in the simplified *in vitro* system with just 70S ribosome and substrates (**Fig. 13A**), however, with the addition of mRNA/polyU and tRNAs the picture changes dramatically with thiostrepton totally nullifying the activation by additional factors already at the concentration of 0.5 μM (**Fig. 13B,D**). At the same time the activation by deacylated A-site tRNA in the case of A1067U mutant 70S remains untouched (**Fig. 13B,C**). The residue 1067A of 23S rRNA is one of the four major contacts of 70S ribosome and thiostrepton (Harms et al., 2008) and inability of antibiotc to inhibit RelA activity on mutant ribosomes underlines the importance of 23S rRNA position 1067A for RelA-thiostrepton relationship.

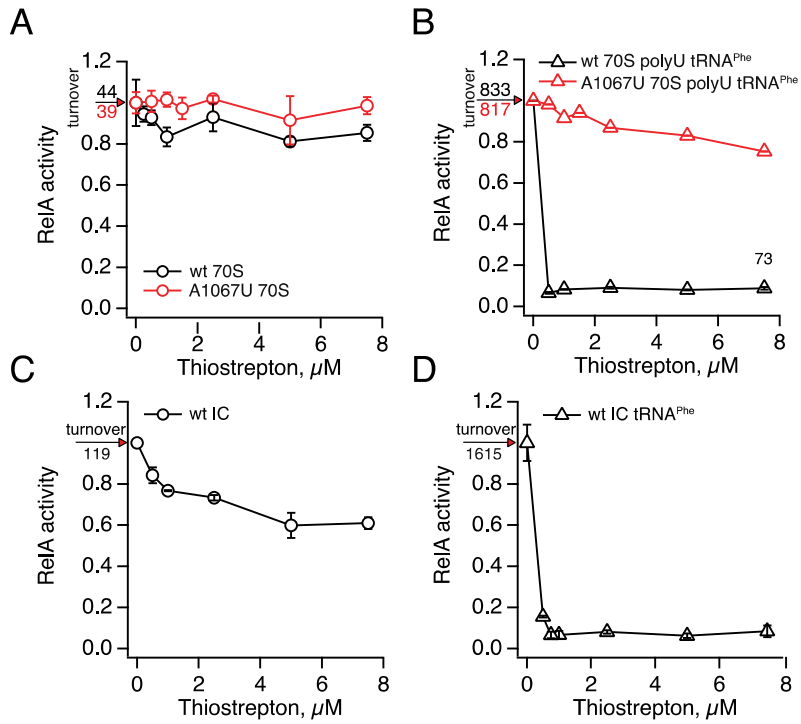

Figure 13. Thiostrepton prevents RelA activation by deacylated A-site tRNA. Thiostrepton titrations were performed in *in vitro* systems with either 70S ribosome only (A), starved ribomal complex (B), ribosomal initiation complex (C) or ribosomal initiation complex with the addition of deacylated A-site tRNA (D). Turnovers represent ppGpp synthesis activity per Rela per min. Enzymatic activities were normalized to that of the corresponding system in the absence of thiostrepton and uninhibited turnover values corresponding to 1.0 activity are provided on individual panels. Error bars represent standard deviation of the turnover estimated by linear regression. Each experiment was performed at least three times.

Apparently, the encounter of RelA with deacylated tRNA in the ribosomal A-site leads to the conformational switch in the enzyme with thiostrepton making this switch impossible. One can speculate on the contribution of ribosomal L11 protein into this conformational change. Structural studies suggest the movement of L11's NTD upon binding of thiostrepton (Harms et al., 2008) and at the same time L11 is implemented in activation of RelA (Yang and Ishiguro, 2001b). Recent structural studies on RelA and 70S interactions, however, reported no contacts between RelA and L11 (Arenz et al., 2016; Brown et al., 2016; Loveland et al., 2016). According to these studies RelA wraps around A-site tRNA promoting it into A/T-like conformation (**Fig. 7**) allowing establish the stacking interaction between residues C56 of tRNA and A1067 of 23S rRNA. In the absence of A-site tRNA RelA has no indirect contacts with A1067

and therefore, thiostrepton, that directly interacts with A1067 has no effect on RelA activity. The protection from inhibiton of RelA activity by thiostrepton granted by mutation in residue 1067 of 23S rRNA can be interpreted by the inability of thiostrepton to correctly bind onto the ribosome (Thompson et al., 1988). However it is still not clear why the very mutation does not affect RelA activity inspite of the possible occurrence of impairment in stacking interaction between C56 of A-site tRNA and 1067U of 23S rRNA upon RelA binding to the ribosome.

3.2. Tetracycline and chloramphenicol do not directly inhibit RelA activity

Apart from thiostrepton two other translational antibiotics were shown to be effective (p)ppGpp synthesis inhibitors; these are tetracycline and chloramphenicol (Kasai et al., 2004; Lund and Kjeldgaard, 1972b). To their advantage over thiostrepton both antibiotics are water-soluble. Both antibiotics were assayed against RelA and, while chloramphenicol, that binds into ribosomal P-site and inhibits peptide bond formation, was unable to cause any direct effect on RelA activity (**Fig. 14B**), tetracycline weakly inhibited ppGpp biosynthesis in both systems it was tested (**Fig. 14A**).

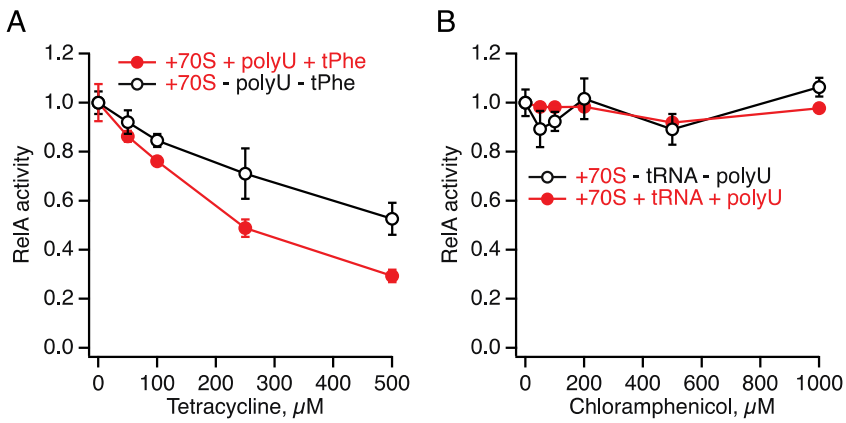

Figure 14. Classical translational antibiotics tetracycline (A) and chloramphenicol (B) cause only little or no effect on RelA activity. RelA activity was assayed in the *in vitro* systems with either 70S ribosomes with vacant A-site (black empty circles) or starved ribosomal complex (filled red circles). RelA activity without an inhibitor equals 1 and RelA activity in the presence of the inhibitor is normalized to that. Error bars represent standard deviation of the turnover estimated by linear regression. Each experiment was performed at least three times.

As tetracycline acts through blocking the accommodation of A-site tRNA one could expect to observe the tRNA-dependent mechanism of RelA activity inhibition in the manner similar to what we described for thiostrepton (**Fig. 13B**). With the addition of deacylated tRNA the inhibitory effect of tetracycline, however, did not change (**Fig. 14A**) suggesting other mechanism of RelA activity inhibition by tetracycline than through the binding of deacylated A-site tRNA.

3.3. Inhibition of translation by antibiotics blocks the stringent response *in vivo*

To support the *in vitro* data we characterized the effect of thiostrepton, tetracycline and chloramphenicol *in vivo*. Thiostrepton was assayed in *B. subtilis* culture (**Fig. 15B**) to solve the cellular uptake problem and two others in *E. coli* culture. First, the stringent response in bacterial cultures was induced through the pre-treatment with mupirocin (**Fig. 15A**) with the following addition of the antibiotic of interest. The effect of antibiotics was studied by measuring the changes in ppGpp levels using HPLC-based (High Performance Liquid Chromatography) approach.

Pre-treatment of bacterial cultures with 70 μM mupirocin caused Ile starvation accompanied by the activation of the stringent response and, therefore, increase in ppGpp levels (**Fig. 15A**). All the antibiotics used here showed pronounced inhibitory effect on stringent response *in vivo* with ppGpp synthesis being abrogated in all the cases (**Fig. 15**). Current experimental system, however, does not provide the information about the specificity of antibiotics towards the respective stringent factors – RelA in **Fig. 15C,D** and Rel in **Fig. 15B**. The stringent response is indeed, repressed, although we cannot confidently confirm the mechanism of such repression. With tetracycline and chloramphenicol that were titrated in *E. coli* culture remains a possibility of their influence on SpoT since both antibiotics were hardly effective against RelA *in vitro*. Thiostrepton, on the other hand, showed high specificity *in vitro* towards RelA but due to the lack of cellular uptake in *E. coli* we could only study its effect in *B. subtilis* that does not have a cell membrane but has the set of RSH enzymes different from that in enterobacteria. Taking into account the range of concentrations for antibiotics used in *in vivo* experimetns and *in vitro* obtained data, of the antibiotics tested thiostrepton represents the highest potential towards being a specific inhibitor of the stringent response. Modifying thiostrepton structure in a way to improve the solubility and therefore overcome cellular uptake problem without losing inhibitory activity looks like a promising strategy for the further studies aiming for the stringent response inhibition.

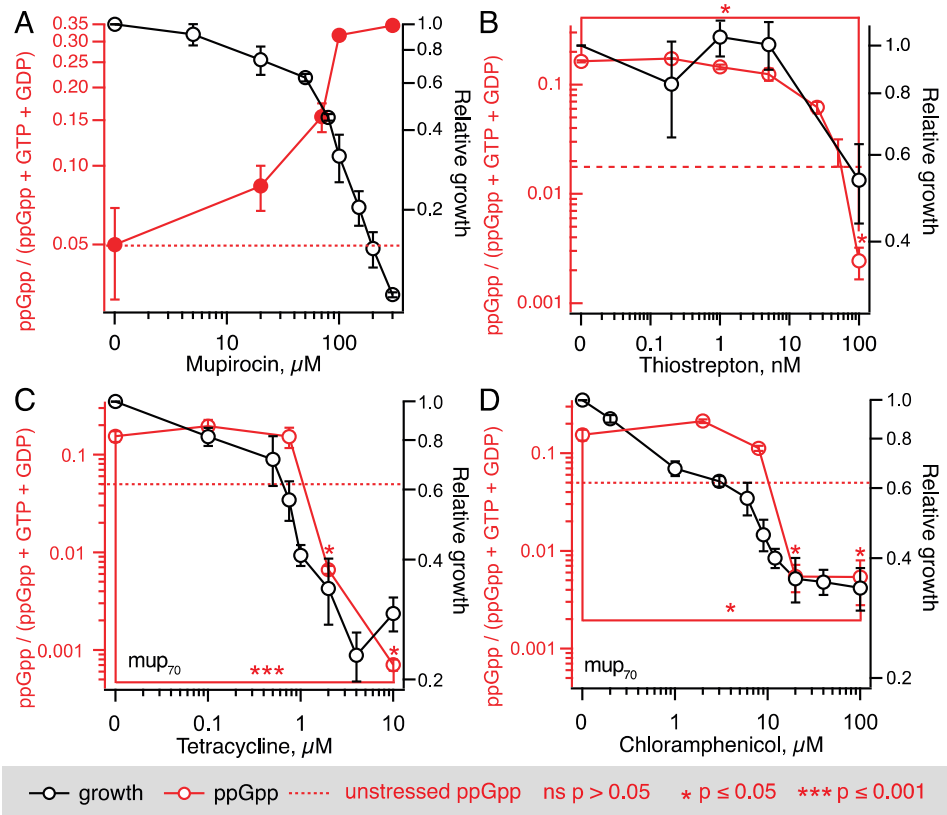

Figure 15. Inhibition of bacterial growth by translational antibiotics is in correlation with a drop in ppGpp levels. Red circles represent changing in ppGpp levels while black circles reflect the bacterial growth. A) The stringent response was induced by the addition of increasing concentrations of mupirocin. B) to D) Cell cultures were treated for 30 min with increasing concentrations of thiostrepton (B), tetracycline (C), or chloramphenicol (D) combined with 70 µM mupirocin (mup70). Experiments were performed with BW25113 *E. coli* wild-type strain (A, C and D) or BSB1 *B. subtilis* wild-type strain (B). The dashed red trace indicates the level in unstressed cells. Error bars indicate the standard errors of the mean (three to five biological replicates). The P values were calculated using a two-tailed Welch's t test either relative to the unstressed ppGpp levels or, where indicated by brackets, within the titration series.

CONCLUSIONS

ppGpp synthesis activity of certain RSH enzymes is allosterically regulated at considerably low concentration of ppGpp in a positive way and, in the case of *E. coli* RelA, negatively at high concentrations of the alarmone molecule.

Compounds DR-4250 and DR-M014 that are based on ppGpp-scaffold specifically inhibit *E. coli* RelA activity *in vitro.*

ppApp and 6-thio-ppGpp specifically inhibit *E. coli* RelA activity *in vitro.*

E. coli RelA activity inhibition by antibiotic thiostrepton is tRNA-dependent. A1067U point mutation in 23S rRNA rescues RelA ppGpp synthetase activity and EF-G GTPase activity from inhibition by thiostrepton.

Treatment with translational antibiotics thiostrepton, tetracycline and chloramphenicol abolishes ppGpp production *in vivo*.

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SUMMARY IN ESTONIAN

Otsides *Escherichia coli* poomisvastuse valgu RelA inhibiitoreid

Keskkonnatingimuste jälgimiseks ja muutustega kohanemiseks on bakteritel mitmeid sensoorseid süsteeme. Seejuures on levinud strateegiaks sekundaarsete signaalmolekulite kasutamine: stressisignaali ilmumisega muutub signaalmolekulide rakusisene kontsentratsioon ning see omakorda reguleerib sihtensüümide aktiivsust. Üks sellistest stressivastuse süsteemidest on poomisvastus *(stringent response)*, mis aktiveeritakse sõltuvalt alarmoonmolekuli (p)ppGpp rakusisesest kontsentratsioonist. (p)ppGpp on võimeline reguleerima mitmete ensüümide aktiivsust, kuid peamiseks märklauaks on RNA polümeraas. (p)ppGpp rakusisest taset kontrollivad *E. coli* RelA-SpoT-ga homoloogsed valgud (RSH), mis kas sünteesivad või hüdrolüüsivad (p)ppGpp-d vastavalt keskkonnatingimustele. Eksperimentaalselt on tõestatud, et poomisvastus kontrollib bakterite virulentsust, persisterite moodustamist, antibiootikumide taluvust ning antibiootikumide tootmist, samuti osaleb bakterite hulgatunnetuses (*quorum sensing*) ja bakterite ellujäämises fagotsütoosi jooksul. Seepärast on poomisvastuse mehhanismide mõistmine väga oluline ja (p)ppGpp rakusisest taset kontrollivate ühendite loomine võiks viia meditsiini ja biotehnoloogia seisukohalt oluliste rakendusteni. Hiljuti avastati, et ppGpp struktuuril põhinev aine Relacin on võimeline inhibeerima RelA aktiivsust kuid tõhusa inhibeerimise jaoks on vaja Relacini kasutada väga kõrges kontsentratsioonis.

Käesoleva töö raames iseloomustasin (p)ppGpp struktuuri põhjal disainitud uudsete keemiliste ainete efekti *E. coli* RelA aktiivsusele. Samuti pakun välja uusi võimalusi RelA inhibiitorite arendamiseks. Peale uute sünteesitud ainete efektide vaatasin üle võimalusi mõjutada poomisvastust juba tuntud translatsiooni inhibiitorite abil ning iseloomustasin thiostreptooni tugevat inhibeerimisvõimet (p)ppGpp sünteesile.

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PUBLICATIONS

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Master Thesis: Intersubunit bridge B3 is important for ribosomal functionality due to its role in subunit association

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Scientific interests:

My research interests are molecular mechanisms of ribosomal protein synthesis and its regulation. My PhD thesis is dedicated to the structure-functional studies of ribosome-associated and ribosome-independent RelA-SpoT Homologue (RSH) enzymes controlling intracellular levels of alarmone nucleotide (p)ppGpp in bacteria; development of selective inhibitors of RSH enzymes.

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Publications:

1. Positive allosteric feedback regulation of the stringent response enzyme RelA by its product.
   Shyp V, Tankov S, Ermakov A, **Kudrin P**, English BP, Ehrenberg M, Tenson T, Elf J, Hauryliuk V. EMBO Rep. 2012 Sep;13(9):835-9. doi: 10.1038/embor.2012.106. Epub 2012 Jul 20.
2. From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of *Enterococcus faecalis*. Gaca AO, **Kudrin P**, Colomer-Winter C, Beljantseva J, Liu K, Anderson B, Wang JD, Rejman D, Potrykus K, Cashel M, Hauryliuk V, Lemos JA. J Bacteriol. 2015 Sep;197(18):2908-19. doi: 10.1128/JB. 00324-15. Epub 2015 Jun 29.
3. Molecular mutagenesis of ppGpp: turning a RelA activator into an inhibitor. Beljantseva J*, **Kudrin P***, Jimmy S, Ehn M, Pohl R, Varik V, Tozawa Y, Shingler V, Tenson T, Rejman D, Hauryliuk V. Sci Rep. 2017 Feb 3;7:41839. doi: 10.1038/srep41839.
4. Subinhibitory Concentrations of Bacteriostatic Antibiotics Induce *relA*-Dependent and *relA*-Independent Tolerance to β-Lactams. **Kudrin P***, Varik V*, Oliveira SR, Beljantseva J, Del Peso Santos T, Dzhygyr I, Rejman D, Cava F, Tenson T, Hauryliuk V. Antimicrob Agents Chemother. 2017 Mar 24;61(4). pii: e02173-16. doi: 10.1128/AAC. 02173-16. Print 2017 Apr
5. Negative allosteric regulation of *Enterococcus faecalis* small alarmone synthetase RelQ by single-stranded RNA. Beljantseva J*, **Kudrin P***, Andresen L, Shingler V, Atkinson GC, Tenson T, Hauryliuk V. Proc Natl Acad Sci U S A. 2017 Apr 4;114(14):3726-3731. doi: 10.1073/ pnas.1617868114.

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Teadustegevus:
Minu uurimisvaldkond on olnud valgubiosünteesi molekulaarsed mehhanismid ja regulatsioon. Doktoritöö on pühendatud ribosoomiga assotsieerunud või iseseisvalt funktsioonerivate RelA-SpoT homoloogsete (RSH) ensüümide struktuur-funktsionaalsetele uuringutele ja võimalustele nende inhibeerimiseks.

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Publikatsioonid

1. Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. Shyp V, Tankov S, Ermakov A, **Kudrin P**, English BP, Ehrenberg M, Tenson T, Elf J, Hauryliuk V. EMBO Rep. 2012 Sep;13(9):835-9. doi: 10.1038/embor.2012.106. Epub 2012 Jul 20.
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3. Molecular mutagenesis of ppGpp: turning a RelA activator into an inhibitor. Beljantseva J*, **Kudrin P***, Jimmy S, Ehn M, Pohl R, Varik V, Tozawa Y, Shingler V, Tenson T, Rejman D, Hauryliuk V. Sci Rep. 2017 Feb 3;7:41839. doi: 10.1038/srep41839.
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