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HPLC analysis of bacterial alarmone nucleotide (p)ppGpp and its toxic analogue ppApp

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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. Varik V*, **Oliveira SRA***, Tenson T, Hauryliuk V. 2017. HPLC-Based Quantification of Bacterial Housekeeping Nucleotides and Alarmone Messengers ppGpp and pppGpp. *Scientific Reports* 7 (1): 11022.

II. Kudrin P*, Varik V*, **Oliveira SRA**, Beljantseva J, Del Peso Santos T, Dzhygyr I, Rejman D, Cava F, Tenson T, Hauryliuk V. 2017. Sub-inhibitory concentrations of bacteriostatic antibiotics induce *relA*-dependent and *relA*-independent tolerance to β-lactams. *Antimicrobial Agents and Chemotherapy.* 61: e02173–16.

III. Jimmy S*, Saha CK*, Kurata T*, Stavropoulos C, **Oliveira SRA**, Koh A, Cepauskas A, Takada H, Rejman D, Tenson T, Strahl H, Garcia-Pino A, Hauryliuk V, Atkinson GC. 2020. A widespread toxin-antitoxin system exploiting growth control via alarmone signaling. *Proceedings of the National Academy of Sciences of the United States of America.* 117 (19) 10500–10510.

* Designates shared first authorship.

Author contributions:
In paper I, I have developed the HPLC-based method for analyses of bacterial nucleotide pools. In papers I–III, I have performed HPLC analyses of bacterial nucleotide pools. In paper II, I have performed bacterial time-kill kinetic assays and growth assays.

LIST OF ABBREVIATIONS

ACP	Acyl-carrier protein
ACT	Aspartokinase, Chorismate mutase, TyrA domain
ATP	Adenosine triphosphate
ASF	A-site Finger rRNA structural element
Cam	Chloramphenicol
cAMP	Cyclic adenosine monophosphate
CC	Conserved cysteins domain
c-di-AMP	Cyclic diadenosine monophosphate
c-di-GMP	Cyclic diguanosine monophosphate
CTD	C-terminal domain region
CTP	Cytidine triphosphate
DNA	Deoxyribonucleic acid
EF-G	Elongation factor G
EF-Tu	Elongation factor thermo unstable
GDP	Guanosine diphosphate
GltX	Glutamate-tRNA ligase
GMK	Guanosine monophosphate kinase
GTP	Guanosine triphosphate
HD	Hydrolysis domain
HPLC	High Performance Chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
IPRP	Ion pair reverse phase
ROS	Reactive Oxygen Species
Mup	Mupirocin
mRNA	Messenger RNA
MS	Magic Spot
ppApp	5'-diphosphate 3'-diphosphate
pppApp	5'-triphosphate 3'-diphosphate
(p)ppGpp	Guanosine(penta)tetraphosphate
PVC	*Planctomycetes, Verrucomicrobia, Chlamydiae*
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNAP	RNA polymerase
RRM	RNA Recognition Motif domain
RSH	RelA/SpoT Homolog
SAH	Small Alarmone Hydrolase
SAS	Small Alarmone Synthetase
SAX	Strong anion exchange chromatography
SYNTH	Synthesis domain
SRL	Sarcin-Ricin Loop

tRNA	Transfer RNA
UTP	Uridine triphosphate
TA	Toxin-antitoxin
Tet	Tetracycline
Thio	Thiostrepton
TGS	Threonyl-tRNA synthetase, GTPase, SpoT
TLC	Thin Layer Chromatography
TRIM	Trimethoprim
WT	Wild-type
ZFD	Zinc-finger domain

INTRODUCTION

Bacteria through evolution developed numerous adaptation mechanisms that made them survive in harsh environmental conditions. Therefore, to protect themselves from environmental challenges bacteria evolved complex molecular networks that leads to suitable physiological responses by acquiring resistance to antibiotics, forming biofilms or by entering in a dormant state. These adaptation mechanisms depend on enzymatic activity of specific proteins that sense and respond to stress. The responses of these stresses are mediated by synthesis and degradation of signaling molecules that can regulate transcription and protein activities. The PhD work comprise the study of stringent response that is one of the most widely spread adaptive mechanism in bacteria. This mechanism is orchestrated by RelA SpoT Homologue (RSH) enzymes that produce and degrade a highly charged alarmone nucleotide called guanosine(penta)tetraphosphate ((p)ppGpp), comprising guanosine pentaphosphate (pppGpp) and tetraphosphate (ppGpp), collectively referred as (p)ppGpp. The (p)ppGpp-mediated signaling is one of the master regulators of bacterial physiology and plays an important role in bacterial virulence, and tolerance to antibiotics. In order to quantify the varying levels of (p)ppGpp and housekeeping nucleotides in different stress conditions as well as during normal bacterial growth, we developed a HPLC-based quantification method. Using *Escherichia coli* and *Bacillus subtilis* as the two representatives of Gram-negative and Gram-positive bacteria, I studied the effects of antibiotic treatment on the cellular levels of ppGpp, (p)ppGpp as well as housekeeping nucleotides such as ATP and GTP. Finally, using the HPLC-based approach, I discovered that a toxic Small Alarmone Synthetase RSH from *Cellulomonas marina,* in addition to coproducing ppGpp alarmone synthesizes a highly toxic ppGpp analogue, ppApp. Together with the recent report by Laub and Whitney labs who described *Pseudomonas aeruginosa* Tas1 – a divergent RSH enzyme that acts as a toxic effector of a secretion system via production of (pp)pApp (Ahmad et al., 2019) this discovery opens up a new direction in studies of RSH enzymes.

REVIEW OF LITERATURE

1. The Stringent Response

1.1 Bacterial stress responses

Bacterial cells face rapidly changing environmental conditions. In order to survive, they must sense and adapt to the harsh conditions. Bacteria have evolved multiple protective and adaptive mechanisms that modulate bacterial physiology in response to stress. The adaptation to the severe environment conditions as starvation of Mg^{2+} (Groisman, Kayser, and Soncini 1997), oxidative stress in form of reactive oxygen species (ROSs) (Gu and Imlay, 2011; Touati, 2000), envelope, heat and nutritional stress (Poole, 2012). A regulatory mechanism called the 'stringent control' (SC) or the 'stringent response' that abrogates the synthesis of stable RNA – i.e. transport RNA (tRNA) and ribosomal RNA (rRNA) – upon amino acid limitation in bacteria (Frederick C. Neidhardt, 1964; Neidhardt, 1966).

1.2 Bacterial signaling nucleotide messengers

Bacterial nucleotide secondary messengers that regulate key molecular targets in response to harsh environmental conditions can be divided into two classes: linear and cyclic (**Figure 1**). One of the most ubiquitous and well-studied bacterial cyclic di-nucleotide is c-di-GMP (cyclic diguanosine monophosphate) (Pesavento and Hengge, 2009) (**Figure 1A**). Several studies showed this signaling nucleotide positively regulates biofilm formation and virulence (Pesavento and Hengge, 2009; Ross et al., 1987; Römling and Simm, 2009). The transition between motility and sessility is also regulated by c-di-GMP signaling in bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and others (Jones et al. 2015; Mobley, Spurbeck, and Tarrien 2012; Li et al. 2017). Another cyclic di-nucleotide is c-di-AMP (cyclic diadenosine monophosphate) (**Figure 1B**). This nucleotide regulates diverse cellular processes in bacteria (mainly in Gram-positive), including sporulation, and regulation of potassium ion channels (Blumenthal and Kaczmarek, 1992). c-di-AMP also signals DNA damage (Römling, 2008), cell wall membrane damage and homeostasis irregularities (Luo and Helmann, 2012). The group of cyclic nucleotides also includes cGMP (**Figure 1C**) and cAMP (**Figure 1D**). In was shown that in *Rhodospirillum centenum* cGMP played a key role in development of cyst cells which are metabolically dormant and are able to survive to environmental stresses such as nutrient starvation (Francis and Corbin, 1999). For many years one of the most studied of the signaling nucleotides regarding nutrient starvation in *E. coli* was cAMP (cyclic diadenosine monophosphate) (Makman and Sutherland, 1965). In response to low concentration of ATP in the cell, the levels of

cAMP increase, promoting catabolism and inhibiting anabolism trough transcriptional regulation of gene expression (Francis and Corbin, 1999).

The key linear messengers are alarmones guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp), collectively referred to as (p)ppGpp (**Figure 1E**). Acting at transcriptional and post-transcriptional levels these nucleotides effectuate an array of physiological changes, named the 'stringent response' (Cashel and Gallant, 1969). These nucleotides are the focus of my thesis.

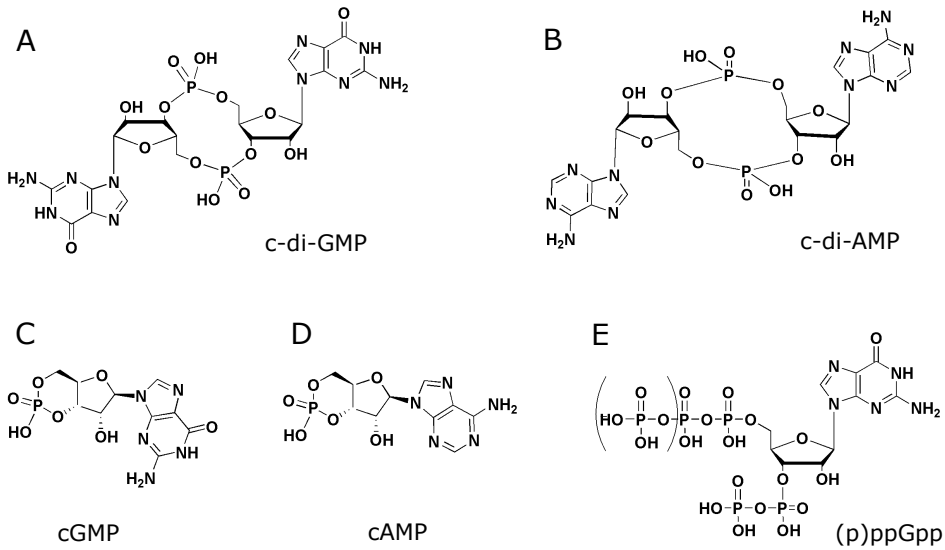

Figure 1. Chemical structures of nucleotides involved in bacterial signaling. (A) c-di-GMP, **(B)** c-di-AMP, **(C)** cGMP**, (D)** cAMP and **(E)** (p)ppGpp.

1.3 The stringent response

In the 60's, Frederick C. Neidhardt observed that during amino acids starvation production of stable RNA (rRNA and tRNA) is rapidly abrogated in *E. coli* (Neidhardt, 1964; Neidhardt, 1966). Later was shown that other nutrient limitations/stress conditions such as starvation of phosphorus (Spira et al., 1995), iron (Vinella et al., 2005), carbon (Flärdh et al., 1994) also result in throttling of stable RNA production.

Cashel and Gallant detected the formation of two new compounds when the bacterial cultures were subjected to amino acid limitation (Cashel and Gallant, 1969). Following metabolic labelling with $^{32}PO_4$, the nucleotide fraction of amino acid-starved stringent *E. coli* was resolved in a thin layer chromatography (TLC), and two unusual spots were observed between GTP and the origin: 'magic spot I' (MSI) and 'magic spot II' (MSII). One year later it was

later shown that MSI is ppGpp and MSII is pppGpp (Cashel and Kalbacher, 1970). In the following years, accumulation of (p)ppGpp was documented upon other stress conditions as fatty acid limitation (Battesti and Bouveret, 2009) and heat shock (Gallant, *et al.*, 1977). In addition to inhibiting production of stable RNA, the stringent response also inhibits synthesis of phospholipids (Yoshihiro Sokawa, Nakao, and Kaziro, 1968; Merlie and Pizer, 1973; Golden and Powell, 1972), nucleotides (J Gallant, Irr, and Cashel, 1971), polyamides (Hölttä, Jänne, and Pispa, 1974) and carbohydrates (Sokawa, Nakao-Sato, and Kaziro 1970), abrogates phosphate incorporation (Irr and Gallant, 1969), membrane transport (Hochstadt-Ozer, 1972) and increases the rate of proteolysis (Sussman and Gilvarg, 1969).

1.4 Synthesis and degradation of (p)ppGpp

Early microbiological studies of the stringent response have discovered that the product of *E. coli relA* gene – protein RelA, or the stringent factor – is active in wild-type but not in so-called 'relaxed' strains, i.e. strains unable to execute the stringent response, and represses the production of stable RNA upon the amino acid limitation (Alföldi, Stent, and Clowes 1962; Gunther and Sydney 1961). Later it was shown that RelA enzymatically synthesizes the (p)ppGpp alarmone, the mediator of the stringent response (Cashel and Gallant, 1969). When the bacterial cells are starved of amino acids the enzyme RelA uses GTP or GDP in combination with ATP (serves as a donor of the pyrophosphate group) to produce pppGpp and ppGpp, respectively (**Figure 2**).

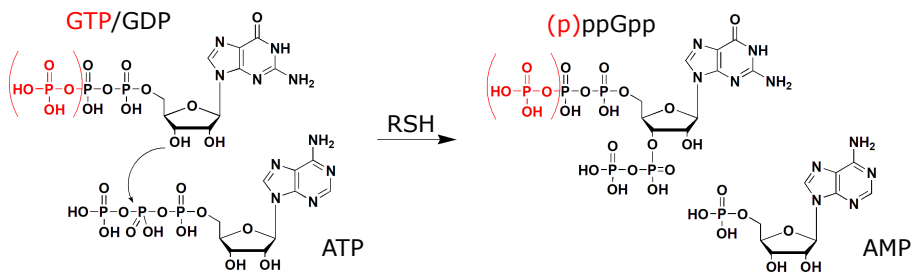

Figure 2. (p)ppGpp synthesis by RSH. RSH utilize ATP and GTP/GDP as substrates to synthesize (p)ppGpp.

The second gene playing a role in *E. coli* stringent response is *spoT*. It encodes SpoT – a bifunctional enzyme, i.e. able to both synthetize and hydrolase (p)ppGpp. SpoT has a predominantly hydrolase activity (Murray and Bremer, 1996; Laffler and Gallant, 1974a), degrading pppGpp to GTP and ppGpp to GDP (Hauryliuk et al., 2015). Production of (p)ppGpp by SpoT enzyme is

triggered by starvation of phosphorus (Spira et al., 1995), fatty acid (Battesti and Bouveret, 2009) and iron (Vinella et al., 2005). The production of (p)ppGpp in basal levels is in μM range while when the cells are starved of amino acids it goes up to mM range (Varik et al., 2017). This means that different levels of (p)ppGpp are important for the survival when bacteria are exposed to harsh conditions.

Additionally, many bacterial species encode small alarmone synthetases (SAS) and encode small alarmone hydrolases SAH, which have just synthase or hydrolase single domain (Jimmy et al., 2020). For example, in Firmicute bacterium *B. subtilis* – the most well-studied model Gram-positive bacterium – a single multidomain 'long' RSH Rel is accompanied two SAS enzymes, RelP (YwaC) and RelQ (YjbM) (**Figure 8**) (Nanamiya et al., 2008).

In addition to RSH enzymes, there are other enzymes involved in ppGpp production. Specifically, pentaphosphate phosphatase, GppA, catalyzes degradation of pppGpp made by RSHs to ppGpp (Keasling, Bertsch, and Kornberg, 1993). In general, bacteria have much higher levels of ppGpp than pppGpp (Varik et al., 2017).

1.5 Regulation of *E. coli* transcription by (p)ppGpp: direct regulation of RNAP

The (p)ppGpp has an important role in metabolism and physiology, the alarmone is capable to regulate hundreds of genes. The transcription regulation mediated by (p)ppGpp can be done in two ways: indirectly through variation of GTP levels in the cell (see below, section **1.8**) or through direct interaction with RNAP (RNA polymerase). Identification of the (p)ppGpp binding site of RNAP was an extremely challenging experimental task. Initially, Artsimovitch and colleagues reported that (p)ppGpp binding site was present in RNAP of *Thermus thermophilus* (Artsimovitch et al., 2004). However, the biological implication of this finding was refuted by Vrentas and colleagues (Vrentas et al., 2008).

In *E. coli* (p)ppGpp directly binds to RNAP (**Figure 3**) and regulates its activity positively or negatively depending on the target promoter sequences (Gourse et al., 2018). In case of *E. coli,* (p)ppGpp binds to two different sites in the RNAP. It was reported that the binding of (p)ppGpp was at the interface of β′ and ω of RNAP subunits, referred as site 1 (Ross et al., 2013). The site 2 is a ligand binding site that is formed by RNAP and the transcription factor, DksA, and the secondary channel interface helices of β′ (Ross et al., 2016) (**Figure 3**). Both biding sites are more than 60 Å apart on the surface of RNAP, and each is around 30 Å from the active site (Ross et al., 2016). While both ppGpp and pppGpp bind to the same sites of RNAP, pppGpp is a somewhat less potent regulator (Mechold et al., 2013).

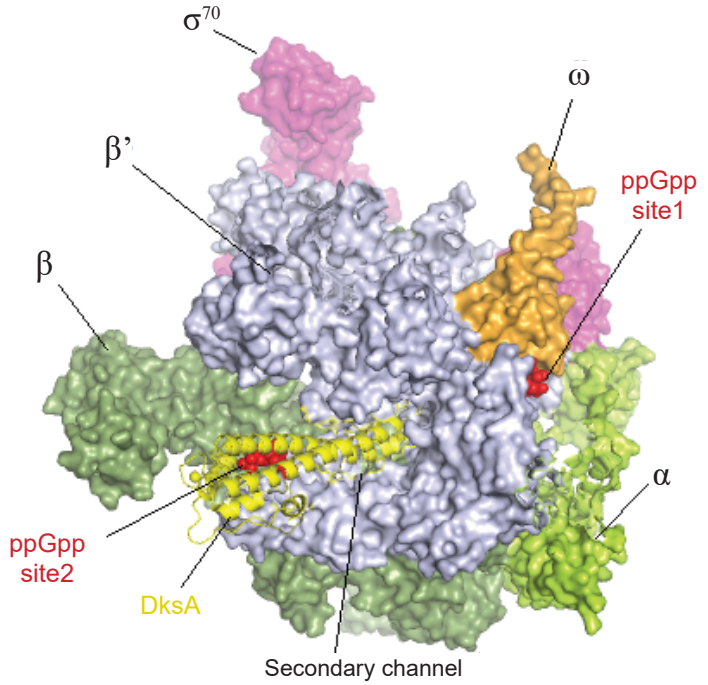

Figure 3. Structure of *E. coli* RNA polymerase in complex with DksA and ppGpp. DksA bound in the secondary channel (yellow), the two ppGpp molecules (red). Adapted from (Ross et al., 2016), PDB accession code 5VSW.

A regulatory protein DksA is crucial for regulation of transcription by (p)ppGpp. Negative and positive regulation of RNAP mediated transcription by (p)ppGpp in presence of DksA have been described (Sanchez-Vazquez et al., 2019). DksA by itself can inhibit transcription to some extent, but when (p)ppGpp binds to the site 2 of the RNAP the DksA inhibitory effect increases ~ 20-fold *in vitro* (Paul, Berkmen, and Gourse, 2005; Paul et al., 2004). (p)ppGpp alone at site 1 and DksA together with (p)ppGpp at site 2 affect the transcription after the initial binding of RNAP to the promoter (Paul et al., 2004). Studies on *rrnB* P1 promoter showed that (p)ppGpp) and/or DksA associates quickly with RNAP, but forms an unstable open complex that inhibits directly the transcription of certain promoters of rRNA genes (Zuo, Wang, and Seitz, 2013). In other hand, DksA/(p)ppGpp increase the isomerization rate of activated amino acids biosynthesis promoters. Nevertheless, this open complex (once formed) are more stable and unresponsive to the inhibitory effects of DksA/ppGpp (Paul, Berkmen, and Gourse, 2005). Sequence motifs associated with promoters are up

or down regulated by (p)ppGpp and DksA were identified, allowing bioinformatic prediction of the regulatory mode (Sanchez-Vazquez et al., 2019).

1.6 Regulation of *E. coli* transcription by (p)ppGpp: regulation via sigma factors

Specific sigma factors are protein factors that promote transcription initiation of specific gene groups in response to environmental signals (Magnusson et al., 2005). (p)ppGpp plays an important role in activation of transcription in the presence of sigma factors (Potrykus and Cashel, 2008). (p)ppGpp modulates of the affinity to RNAP of σ^{70} – housekeeping factor that transcribe the most of the genes in growing bacterial cells – which, in turn, allows other sigma factors to bind to the core of RNAP (Magnusson, et al., 2005; Jishage et al., 2002; Szalewska-Palasz et al., 2007). When acting together with DksA-(p)ppGpp it inhibits strong promoters, this results in increased availability of RNAP core (Wade et al. 2007), which, in turn, indirectly promotes the transcription initiation from promotors that are dependent on alternative sigma factors σ^{S}, σ^{H} and σ^{N} (Maitra, Shulgina, and Hernandez, 2005) and σ^{E} (Costanzo and Ades 2006). σ^{S} is the gene product of *rpoS*, that is the primary regulator of the stationary phase, and strictly regulated by (p)ppGpp (Hengge-Aronis 2002). In starvation conditions σ^{S} is the most common sigma factor to replace σ^{70} (Hengge-Aronis, 2002).

1.7 Non-transcriptional regulation by (p)ppGpp

While the molecular function of (p)ppGpp is intimately connected to control of transcription (Cashel and Gallant, 1969), a wealth of studies has established that in addition to transcription, (p)ppGpp directly controls many other cellular processes, such as replication and translation. Upon amino acid starvation, DNA replication in *E. coli* is inhibited at *oriC* due to the lack of the replication initiation protein, DnaA (Wegrzyn, 1999). Conversely, *B. subtilis* DNA primase (DnaG) is directly inhibited by (p)ppGpp (Potrykus and Cashel, 2008). The alarmone (p)ppGpp was shown to interact with ribosome assembly factor Obg (Buglino et al., 2002). This factor interacts with several regulators (RsbT, RsbW, RsbX) that are involved in the stress activation of σ^{B}, the global regulator of a general stress regulon in *B. subtilis* (Scott and Haldenwang, 1999). Transcription is directly inhibited by binding of (p)ppGpp to target enzymes in *E. coli*. Similarly ppGpp inhibits protein synthesis trough inhibition of the translational GTPase's such as translation initiation factor 2 (IF2) (Mitkevich et al., 2010). It also binds to inducible lysine decarboxylase (LdcI) thus inhibiting stress acid response (Kanjee, Ogata, and Houry, 2012).

1.8 Regulation of *B. subtilis* physiology by (p)ppGpp

In Firmicute bacterium *B. subtilis* (p)ppGpp does not directly bind to and regulate the RNAP activity (Krásný and Gourse, 2004) – likely do to the lack of so-called MAR motif in the ω-subunit of RNAP – but rather regulates by modulation of the GTP pool (Inaoka Why and Ochi, 2002). This regulates the expression of genes that are controlled by promoters that are sensitive to the concentration of initiating nucleotides, such as GTP and ATP (Krásný and Gourse, 2004).

The effects of the stringent response on the NTP pool are mediated by two mechanisms. First, when (p)ppGpp is produced by Rel, the enzyme efficiently consumes GTP (Inaoka and Ochi, 2002; Inaoka et al., 2003). Second, (p)ppGpp inhibits enzymes involved in production of GTP, further depleting the GTP pool (Inaoka and Ochi, 2002; Inaoka et al., 2003) (Lopez, et al., 1981). The severe drop of GTP levels is mediated by the inhibition of IMP dehydrogenase (GuaB), and enzyme that converts IMP into the GMP precursor, xanthine monophosphate (XMP) (Lopez et al., 1981). (p)ppGpp also inhibits Hypoxanthine phosphoribosyl transferase (HprT), the enzyme that synthesizes GMP from of guanine and xanthine (Liu et al., 2015). The alarmone also inhibits GMP kinase (Gmk) that catalyzes conversion of GMP to GDP (Liu et al. 2015). By acting on multiple targets, (p)ppGpp induces a dramatic reduction of the GTP levels.

In Firmicutes an additional regulatory mechanism operates via GTP-biding transcriptional repressor CodY which acts as a sensor of GTP/GDP ratios and branched chain amino acid (BCAA) concentration. To act as a transcriptional repressor, CodY requires GTP and BCAA (Kriel et al., 2012). When GTP-bound, CodY represses the BCAA biosynthesis genes (Liu et al., 2015). Reduction in GTP and BCAA levels inactivates CodY, which leads to an upregulation of amino acid biosynthesis (Liu et al., 2015). Additionally, CodY regulates expression of genes involved in adaptation to stress or sporulation (Geiger and Wolz 2014).

1.9 Domain organization and evolutionary history of 'long' RSH enzymes Rel, RelA and SpoT

RSH enzymes are divided onto 'long' multi-domain RSH – RelA, SpoT, Rel – and small single-domain RSH: small alarmone synthetase (SAS) or small alarmone hydrolase (SAH) (Jimmy et al., 2020). The N-terminal domain region (NTD) of long RSHs consists of (p)ppGpp synthetase domain (SYNTH) and (p)ppGpp hydrolyze domain (HD). In RelA the HD domain is enzymatically inactive, but it is active in SpoT and Rel. The C-terminal domain region (CTD) contains TGS (Threonyl-tRNA synthetase, GTPase, SpoT), helical domain, zinc-finger domain (ZFD), and ACT (Aspartokinase, Chorismate mutase, TyrA) domains. ACT (also known as RNA recognition motif RRM) mediated the interaction of RelA with A-site Finger (ASF) (Brown et al., 2016) (**Figure 4**).

ASF is an element of 23S rRNA involved in translocation step of protein synthesis (Komoda et al., 2006).Short RSH's lack most of these regulatory domains, and consist of only SYNTH or HD domain (Atkinson et al., 2011).

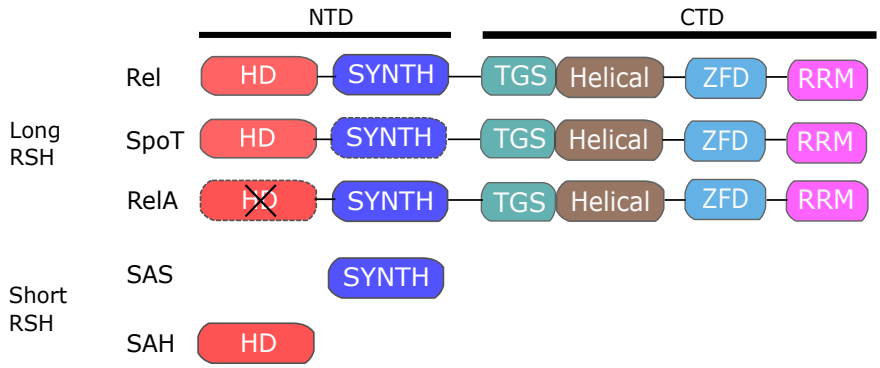

Figure 4 Domain organization of the long RSHs SpoT and Rel and short RSH SAS and SAH. Long RSH enzymes hold six domains: (p)ppGpp hydrolysis domain (HD), (p)ppGpp synthesis domain (SYNTH), TGS (Threonyl-tRNA synthetase, GTPase, SpoT), Helical, and RNA recognition motif (RRM) (Brown et al., 2016). The cross means that the HD domain is not active and the dashed lines show the weak domains. Short RSH enzymes, SAS has the SYNTH domain and SAH has the HD domain (Atkinson et al., 2011).

Recently, Brown and colleagues used cryo-electron microscopy to solve the structure of *E. coli* RelA bound to 'starved' ribosome (Brown et al., 2016). The structure showed that when bounded to the ribosome, RelA assumes an extended conformation which wraps around the uncharged tRNA in the A-site (**Figure 5**). The ZFD and RRM are located next to the anticodon arm of the tRNA. The catalytic N-terminal domain region and TGS domain of RelA stay in the surface of the ribosome, at the acceptor end of the A-site tRNA. In this conformation RelA prevents the accommodation of uncharged tRNA to the peptidyl transferase center (Brown et al., 2016). In previous studies it was reported that RelA binds the ribosomal protein uL11 (Agirrezabala et al., 2013) and uL11 is also crucial for the RelA activation (Parker et al., 1976). However, Brown and colleagues did not observe direct interactions between RelA and uL11 (**Figure 5**) (Brown et al., 2016).

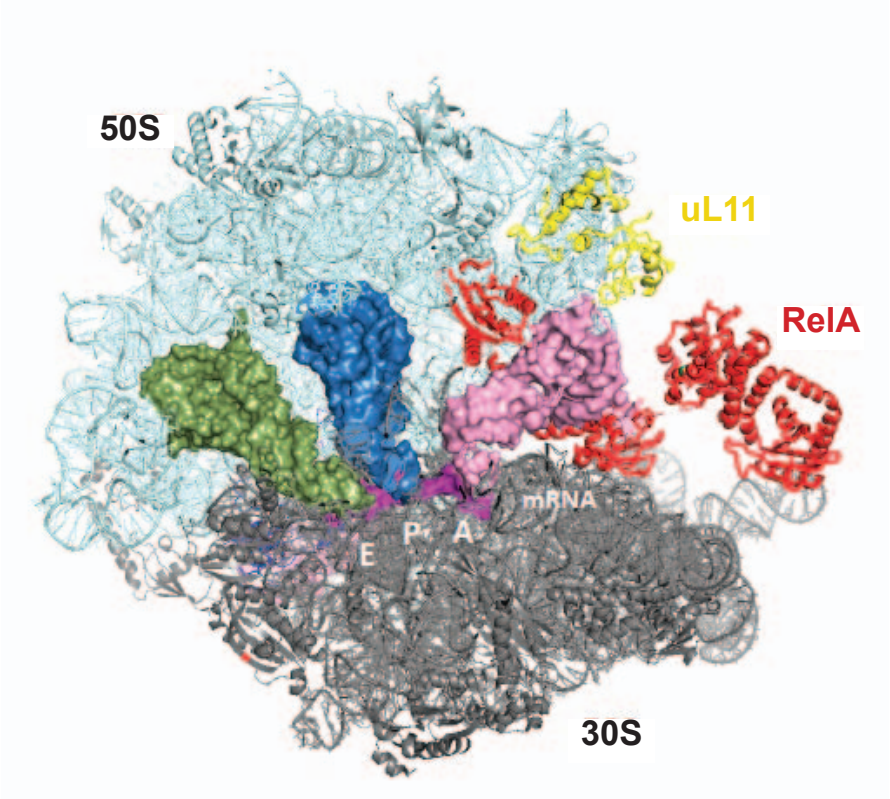

Figure 5. Structure of RelA bound to the ribosome. RelA (red) is wrapped around A-site tRNA (light pink). 50S ribosomal subunit (cyan), 30S (grey). E-site (green) and P-site (blue), mRNA (pink) and uL11 (yellow). PDB accession code is 5IQR. Adapted from (Brown et al., 2016).

The divergence of bifunctional RSH's enzymes, RelA and SpoT can be explained by the gene duplication/horizontal gene transfer (Mittenhuber, 2001). Gram-negative β– and γ–proteobacteria, similarly to *E. coli*, encode the pair of RelA and SpoT enzymes (Mittenhuber, 2001; Atkinson et al., 2011) (**Figure 6**). A big part of bacterial species encode a single bifunctional ribosome-associated Rel factor, e.g. *Bacillus subtilis* (Wendrich and Marahiel, 1997), *Streptococcus equismilis* (Mechold et al., 1996) and *Mycobacterium tuberculosis* (Avarbock et al., 1999). The RHS enzymes are missing in one bacterial group: planctomycetes, verrucomicrobia and, chalmydiales (Atkinson et al. 2011), comprising the so-called PVC superphylum (Santarella-Mellwig et al., 2010; Wagner and Horn, 2006). The reason for RSHs missing in PVC is unclear. Other species that do not encode any RSH are some intracellular endosymbionts (Atkinson et al., 2011). The distribution of SAS and SAH is more diverse comparing to the long RSH's (Jimmy et al., 2020). Usually SAS's are found in proteobacteria, firmicutes, archaea and actinobacteria (Atkinson et al., 2011). A SAH, Mesh1, was found in Animalia, such as humans or *Drosophila melanogaster* (Sun et al., 2010).

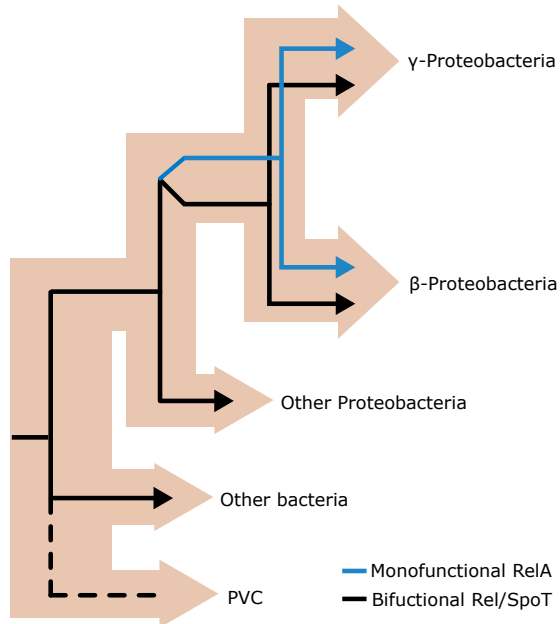

Figure 6. Distribution of long RSH's in bacteria. Bifunctional enzymes Rel/SpoT in black and monofunctional enzyme RelA in blue. Adapted from (Atkinson et al., 2011).

1.10 Regulation of ribosome-associated multi-domain RSH Rel and RelA

RelA's (p)ppGpp synthetic activity is strongly induced when bacteria experience shortage of one (or more) amino acids, which leads to accumulation of uncharged (i.e. not aminoacylated) tRNA, which could constitute up to 80% of the total tRNA in the cell upon acute starvation (Yegian, Stent, and Martin, 1966). Haseltine and Block experimentally validated the hypothesis proposed by Cashel and Gallant (Cashel and Gallant, 1969) by demonstrating that *E. coli* RelA is activated by cognate deacylated tRNA in the ribosomal A-site (Haseltine and Block, 1973). Still, almost half a century after this discovery, the exact molecular mechanism by which long ribosome-associated RSH Rel and RelA sense amino acid starvation is still unresolved.

Through biochemical investigations of *E. coli* RelA, Wendrich and colleagues proposed the 'hopping' model of RelA regulation (Wendrich et al., 2002). According to this model, RelA binds to 'starved' ribosome containing deacylated tRNA in the acceptor A-site, which prompts one act of (p)ppGpp synthesis resulting in dissociation of RelA from the ribosome. Upon release, RelA 'hops' to the next blocked ribosome and synthesis of (p)ppGpp is repeated. A similar 'extending hopping' model was proposed years later by English and colleagues who used single molecule tracking of fluorescently labelled RelA in live *E. coli* cells (English et al., 2011). According to this

model, once activated, RelA spends prolonged time off the ribosome, synthesizing numerous (p)ppGpp molecules. It was recently shown that RelA interacts with uncharged tRNA in rapidly growing cells without being activated (Winther, Roghanian, and Gerdes, 2018). RelA seems to bind to tRNA before being at the A-site. The same study indeed also reported that amino acid starvation leads to a strong increase of interaction with uncharged tRNA and rRNA and a associated activation of (p)ppGpp synthesis activity (Winther, Roghanian, and Gerdes, 2018). Through biochemical and microbiological studies of *B. subtilis* Rel, Takada and colleagues proposed a regulation by starved ribosomal complexes (**Figure 7**). In contrast with previous report, this model shows that Rel interacts with uncharged tRNA on the ribosome. TGS and Helical domains, turn into a "open" conformation, and associates to the vacant A-site of a starved ribosome leading to a specific recognition of uncharged tRNA by Rel. This strong interaction with uncharged tRNA increases the stability of Rel enzyme with starved ribosomes, which leads to a full activation of (p)ppGpp synthesis. After, the complex falls off the ribosome, which makes the protein not active because tRNA inhibits the hydrolysis activity of Rel. When the uncharged tRNA dissociates from Rel, the enzyme turns into a "closed" conformation, where the TGS and Helical domains are hidden and the hydrolysis domain is active (Takada et al., 2020).

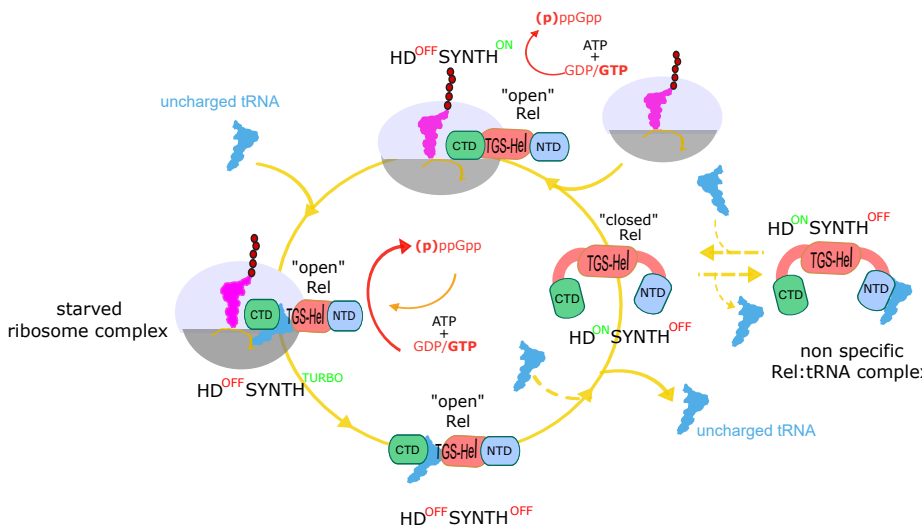

Figure 7. Model of Rel regulation by 'starved' ribosomal complexes. Off the ribosome Rel is in a 'closed' conformation, with SYNTH activity repressed HD activity induced. In this conformation, the factor cannot specifically bind tRNA, inspects the CCA end and be activated for (p)ppGpp synthesis. Binding to vacant A-site 'opens up' Rel, and in this conformation it can recruit the tRNA, resulting in suppression of HD and full activation of SYNTH. Adapted from (Takada et al., 2020).

1.11 Regulation of multi-domain *E. coli* RSH SpoT

Soon after the discovery of *relA*, another *E. coli* gene encoding a protein involved in (p)ppGpp metabolism – *spoT* (Laffler and Gallant, 1974b). Biochemical analysis of SpoT has revealed that this enzyme is able to degrade (p)ppGpp with (GTP) GDP and pyrophosphate as products (Laffler and Gallant, 1974b; Xiao et al., 1991). SpoT has a weak synthetase activity which is triggered by different starvation conditions such as phosphates (Spira et al. 1995), iron (Vinella et al., 2005), carbon (Flärdh et al., 1994) and fatty acids (Battesti and Bouveret, 2009). SpoT is also activated by osmotic and heat shock (Gallant, *et al.*, 1977). The hydrolysis function of SpoT is crucial to maintain the levels of (p)ppGpp during steady-growth conditions in presence of RelA. The HD activity of SpoT requires Mn^{2+} ions (Raué and Cashel, 1975). Since high (p)ppGpp levels stop the cell growth, therefore, disruption of the *spoT* gene in *E. coli* is lethal unless *relA* is also disrupted (Xiao et al., 1991). All attempts to purify full-length SpoT have been so far unsuccessful, postponing a better understanding of the molecular details of these regulatory mechanisms.

It is still unclear if SpoT is ribosome-associated or not. It has been suggested that this enzyme does not bind the ribosome, being a cytosolic protein (Gentry and Cashel, 1995). Another report suggested that SpoT binds to ribosomal 50S subunit (Jiang et al., 2007). Several cellular proteins were suggested to bind to and regulate SpoT. First a 50S ribosomal subunit assembly factor Obg (also known as ObgE and CgtA) that was suggested to control its activity under nutrient rich conditions repressing the synthetic activity of SpoT (Persky et al., 2009). Second, acyl carrier protein (ACP), a central cofactor in fatty-acid starvation, was suggested to activate SpoT's SYNTH activation and inhibit the HD activity by binding to TGS domain (Jiang et al., 2007).

1.12 Regulation of single-domain RSH: SAS and SAH

Many bacterial species in addition to long multi-domain RSH's encode short, single-domain and monofunctional RSH, SAS and SAH. SAS contain an individual SYNTH domain and SAH contains only HD domain, both lacking the CTD domain region altogether (Atkinson, et al., 2011). SAS were described in *Streptococcus mutans* (Lemos et al., 2007), *B. subtilis* (Nanamiya et al., 2008), *Enterococcus faecalis* (Gaca et al., 2015) and *Vibrio cholerae* (Das et al., 2009). The most well-studied SAS representatives are RelP and RelQ (Lemos et al., 2007) and RelV (Das et al., 2009). Just like RelA, synthetic activity of RelQ is positively regulated by (p)ppGpp (Steinchen et al., 2015) (**Figure 8**).

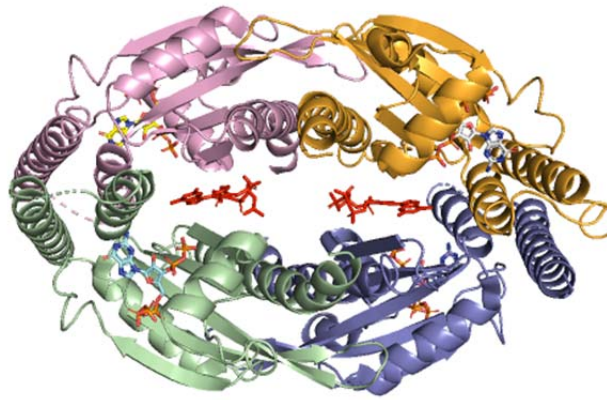

Figure 8. Tetrameric structure of *B. subtilis* RelQ with two allosteric pppGpp molecules (in red). PDB accession code 5DED, adapted from (Steinchen et al., 2015).

Interestingly, Animalia kingdom has SAH called Mesh1, despite these organisms lacking (p)ppGpp synthetases (Sun et al., 2010). It was recently discovered that Mesh1 is a cytosolic NADPH phosphatase (Ding et al., 2020). This contributes for the effectiveness of ferroptosis, a type of programmed cell death that is trigged by oxidative stress dependent on iron and characterized by the accumulation of peroxidation products (Dixon et al., 2012), through the degradation of its central metabolite NADPH (Ding et al., 2020).

2. (p)ppApp as a novel signaling nucleotide

2.1 Synthesis of (p)ppApp by RSH

Adenosine 5'-diphosphate 3'-diphosphate, ppApp, and adenosine 5'-triphosphate 3'-diphosphate, pppApp, commonly referred to as (p)ppApp (**Figure 9**) were first shown to be produced by an excretable SAS RSH enzyme of *Streptomyces morookaensi* (Oki et al., 1975). This enzyme could produce not only (p)ppApp but also pApp and (p)ppGpp (Oki et al., 1975). Detection of (p)ppApp was also reported in sporulating cultures of *B. subtilis* (Rhaese, Grade, and Dichtelmuller, 1976). In the 70's Rhaese and colleagues reported (p)ppApp production by ribosome-associated factors (Rhaese and Groscurth, 1979). More recently, Sobala and colleagues have shown the NTD-only fragment of the Rel enzyme from *Methylobacterium extorquens* could inefficiently synthesize pppApp as well as ppGpp in the presence of unphysiologically high – mM range – levels of Co^{2+} (Sobala et al., 2019). The physiological relevance of this biochemical observation is unclear. The authors also suggested that *E. coli* could produce ppApp, though the result was based solely on TLC assays and the identity of the observed spots was never confirmed by other methods

(Sobala et al., 2019). In the last couple of years, the effects of (p)ppApp on RNAP were characterized, and it was shown that in *E. coli* (p)ppApp binds near to the catalytic center at a shallow cavity of the β' subunit (Bruhn-Olszewska et al., 2018). Additionally, it was observed that (p)ppApp strongly activates the *rrnB* P1 transcription by RNAP, and the authors suggested that (p)ppApp increases the stability of *rrnB* P1 RNAP open complexes. In presence of (p)ppApp DksA inhibits the transcription activation (Bruhn-Olszewska et al., 2018). However, these biochemical papers so far failed to provide a conclusive evidence of (p)ppApp playing a *bona fide* biological role in *E. coli*.

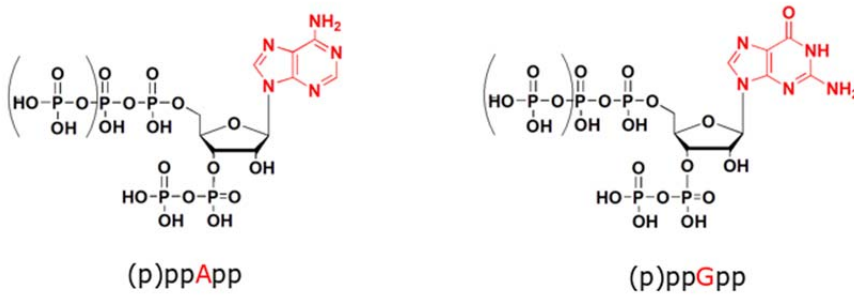

Figure 9. Molecular structure of (p)ppApp and (p)ppGpp. Adenosine and Guanosine bases are shown in red.

2.2 (p)ppApp as a toxic effector

In 2019 Ahmad and colleagues have discovered that *P. aeruginosa* type VI secretion system (T6SS) effector Tas1 is a divergent RSH enzyme which instead of (p)ppGpp produces (pp)pApp, i.e. pApp, ppApp and pppApp (Ahmad et al., 2019). The authors have shown that Tas1-mediated (pp)pApp formation results in depletion of ATP and ADP. Delivery of Tas1 into competitor cells drives the rapid accumulation of (p)ppApp, depletion of ATP, and widespread dysregulation of essential metabolic pathways, resulting in target cell death (Ahmad et al., 2019). These two effects compromise the activity of numerous essential pathways in the cell such as glycolysis, the pentose-phosphate pathway, amino acid biosynthesis (Ahmad et al., 2019). As in case of (p)ppGpp (Wang et al., 2019), the accumulation of (p)ppApp leads to the inhibition of purine biosynthesis by blocking PurF activity (Ahmad et al., 2019). In 2017 Dedrick and colleagues reported for the first time that toxicity can be a function of some SASs (Dedrick et al., 2017). Their bioinformatic studies found a SAS encoded by mycobacterial Cluster N bacteriophage Phrann, gp29, that is a homologue of RSH proteins. The study suggested that gp29 is toxic to *Mycobacterium smegmatis* (Dedrick et al., 2017). The toxicity is neutralized by the expression of its neighboring gene, gp30 (Dedrick et al., 2017). While it is possible that gp29 and Tas1, is producing ppApp, the molecular mechanism of this SAS has not yet been validated directly.

3. Persister cells: possible roles of (p)ppGpp and toxin-antitoxin cells

3.1 Regulation of bacterial virulence by (p)ppGpp

Numerous studies have implicated (p)ppGpp-mediated signaling in regulation of bacterial virulence. (p)ppGpp is a key regulator of expression of genes involved in virulence, invasion and survival during infection in several bacterial species such as *Streptococcus pneumoniae* (Kazmierczak et al., 2009)*, M. tuberculosis* (Stallings et al., 2009; Dahl et al., 2003)*, Vibrio cholerae* (Silva and Benitez, 2006). Loss of ppGpp results in defects in biofilm formation in *P. aeruginosa* (Shrout et al., 2006)*, L. monocytogenes* (Taylor et al., 2002). Deletion of *relA* and *spoT* genes results in a significant decrease of *Burkholderia pseudomallei* virulence in mouse infection model (Müller et al., 2012). In *P. aeruginosa* loss of (p)ppGpp results in decreased *rpoS* levels and virulence (Shrout et al., 2006).

(p)ppGpp also promotes cell survival under stress condition during infection. (p)ppGpp is required for survival of *H. pylori* upon acid and aerobic shock (Mouery et al., 2006). It is important for *E. faecalis* exposed to antibiotic challenge (Abranches et al., 2009). Finally, the stringent response is also involved in sporulation, the ultimate bacterial survival strategy. Deletion of the *rel* gene in *Bacillus anthracis* reduced the sporulation efficiency 10 000-fold (Schaik, Prigent, and Fouet, 2007).

3.2 Persister cell formation and (p)ppGpp

In 1944 Joseph Bigger discovered that antibiotic penicillin does not sterilize bacterial cultures: a small population surviving bacteria, called persisters cells, while not able to grow in the presence of the penicillin, can resume growth after antibiotic is removed (Bigger, 1944). When a culture started with these surviving cells – he called them 'persisters' – is challenged by antibiotic, the majority of the new cells die, suggesting that persisters are not genetically modified resistant bacteria. The molecular mechanism underlying formation of persisters is unclear, but toxin-antitoxin systems, and (p)ppGpp-mediated signaling were implicated (Kaldalu, Hauryliuk, and Tenson, 2016). Below I discuss these two aspects of bacterial persistence in more detail.

3.3 Toxin-antitoxin systems

Toxin-antitoxin (TA) systems are bicistronic operons composed of a gene encoding a toxin gene that inhibits the cell growth and a gene encoding an antitoxin that protects the cell against the toxin (Unterholzner, Poppenberger, and Rozhon, 2013; Page and Peti, 2016). Four main types of TA loci have been identified **(Figure 10).** TA loci type I and III encode small RNA's that neutralize protein toxins at translational and post-translational levels, respec-

tively (Blower, Salmond, and Luisi, 2011). Type II toxins are neutralized by the protein antitoxins that directly bind and inhibit the toxin protein (Kenn Gerdes, Christensen, and Løbner-Olesen, 2005). In the case of type IV TA system the antitoxin inhibits the toxin by affecting its molecular target rather than via direct interaction between the toxin and antitoxin proteins (Brown and Shaw, 2003).

TA systems are widely distributed in bacterial genomes and have been studied for several decades. However, their biological function still not fully understood. Three biological functions have been suggested: post-segregational killing (Gerdes, Rasmussen, and Molin, 1986), abortive infection (Rhizobium, 2013) and formation of persister cells (Harms, Maisonneuve, and Gerdes, 2016). TA models were initially described on bacterial plasmids, type II and type III TA loci are usually related mobile elements and inclined to horizontal gene transfer (Gerdes, Rasmussen, and Molin, 1986; Ogura and Hiraga, 1983). On the other hand, type I TA loci are usually associated to vertical gene transfer. Most recently, was reported new families of type I TA system. All types of TA systems are found on bacterial chromosomes (Blower et al., 2012; Goeders et al., 2016). Though, the chromosomal TA system differ among bacteria and between close related organisms (Pandey and Gerdes, 2005; Fozo et al., 2010; Coray et al., 2017; Leplae et al., 2011; Goeders et al., 2016).

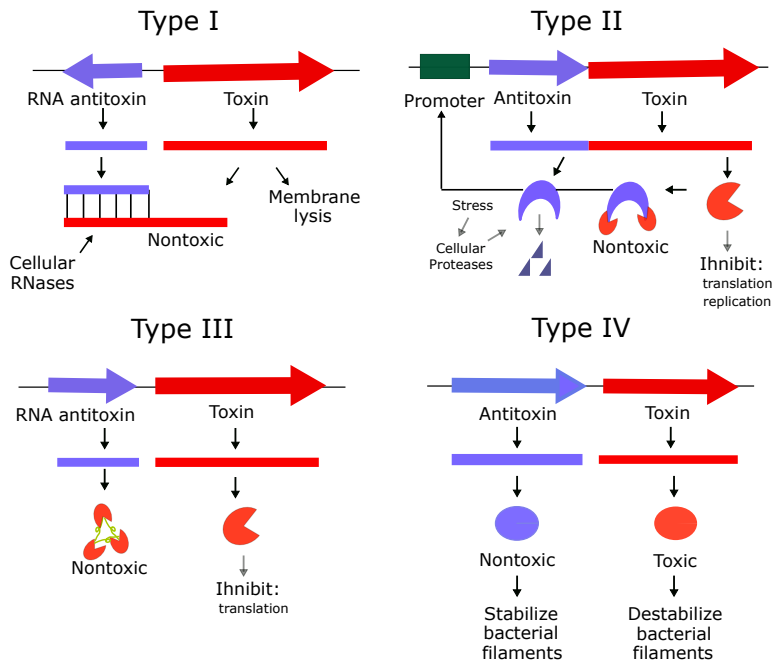

Figure 10. Four types of toxin-antitoxin systems. Type I, II, III and IV toxin-antitoxin systems are shown. Toxins are in red and antitoxins in blue. Adapted from (Page and Peti, 2016).

3.4 Possible role of (p)ppGpp and toxin-antitoxin systems in persistance

The *E. coli hip* (high persistence) mutant *hipA7* was the first identified mutant producing highly elevated persister levels, and it was a focus of research striving to understand the molecular mechanisms of persistence. Bicistronic *hip* locus consists of two protein-encoding genes *hipA* and *hipB* (*hipBA*). *hipBA* belongs to type II TA system where toxins are inhibited by the cognate antitoxin (Harms et al., 2018). HipB antitoxin neutralizes HipA toxin through sequestering it into an inactive complex; consequently, expression of HipA is highly toxic to *hipB*-deficient strain (Black, Irwin, and Moyed, 1994; Black et al., 1991). The toxic protein HipA is a serine/threonine kinase that phosphorylates the glutamine tRNA synthetase, GltX. This leads to inhibition of tRNA aminoacylation and abrogates protein synthesis (Germain et al., 2013; Kaspy et al., 2013). The *hipA7* mutant retains its toxic activity via phosphorylation of GltX, but the complex between the mutant HipA and HipB is weaker, and some of the toxin is released (Schumacher et al., 2009). This affects the growth and increases the persistence. However, deletion of the *hipBA* didn't affect the formation persisters, suggesting that the wild-type system is not the key to bacterial persistence (Keren et al., 2004; Luidalepp et al., 2011).

The type II mRNA endonuclease TA systems of *E. coli* suggested to mediate persister formation in response to stochastic accumulation of (p)ppGpp (Maisonneuve et al., 2013). Maisonneuve and colleagues proposed that an increase of (p)ppGpp accumulation inhibits exopolyphosphatase (Ppx) that leads to polyphosphate (polyP) accumulation and antitoxin degradation by Lon protease, activated by polyP (Kuroda et al., 2001; Maisonneuve, Castro-Camargo, and Gerdes, 2013). However, it was soon questioned since the results were not reproducible when verified in deletion strains and were explained by bacteriophage infection and activation of prophages, among other experimental problems (Harms, Maisonneuve, and Gerdes, 2016; Goormaghtigh et al. 2018). Moreover, the effects of Lon protease as well as polyP synthesis were not reproducible (Ramisetty et al., 2016; Shan et al., 2017). Taking together all these studies, the connection of (p)ppGpp to persisters cell formation is not clear and further research is needed to clarify the topic.

4. (p)ppGpp-mediated signaling as a therapeutic target

4.1 Inhibition of (p)ppGpp accumulation by antibiotics targeting protein synthesis

Since (p)ppGpp-mediated signaling plays a role in bacterial virulence, antibiotic tolerance and biofilm formation, it was recently targeted for development of novel anti-infectives. Treatment with translation inhibitors – such as transpeptidation inhibitor chloramphenicol or fusidic acid which locks GDP-bound elongation factor EF-G on the ribosome and prevents the translocation – abrogates (p)ppGpp accumulation in the cell (Lund and Kjeldgaard, 1972). Similar effects were reporter for tetracycline which inhibits protein synthesis by blocking the delivery of charged tRNA to the A-site of the ribosome. The cyclic peptide thiostrepton is also a strong inhibitor of translation by blocking productive recruitment of translational GTPases IF2 (Brandi et al., 2004) and the elongation factors EF-Tu (Modolell et al., 1971) and EF-G (Walter et al., 2012) to the ribosome. Collectively, these results demonstrate that all antibiotics that inhibit protein synthesis indirectly abrogate (p)ppGpp production by ribosome-associated long RSH enzymes Rel or RelA.

4.2 Dedicated stringent response inhibitors

Several research groups recently attempted to develop new molecules that can efficiently and specifically inhibit (p)ppGpp-mediated signaling (Wexselblatt et al,. 2012; 2010; de la Fuente-Núñez et al., 2014). One of the compounds that was proposed to directly inhibit RSH enzymes is a (p)ppGpp analogue Relacin. This analogue of ppGpp, when tested in the test tube, inhibits the activity of Rel RSH (Wexselblatt et al., 2012). When added to cultures, it affects sporulation and biofilm formation (Wexselblatt et al., 2012). However, the efficiency specificity of Rel inhibition by Relacin was questioned in the follow-up studies (Andresen et al., 2016b).

Another approach for inhibition (p)ppGpp-mediated signaling is exemplified by charged anti-biofilm peptide 1018 (de la Fuente-Núñez et al., 2014). It was proposed that peptide 1018 binds directly to (p)ppGpp and induces the degradation of the alarmone by an unknown mechanism (de la Fuente-Núñez et al., 2014). However, this model was later questioned (Andresen, Tenson, and Hauryliuk, 2016a), and it was suggested that rather than specifically targeting (p)ppGpp, the compound acts as a general antibacterial.

5. Quantification of bacterial nucleotide pools

Concentrations of housekeeping (such as ATP, GDP, etc.) and signaling (ppGpp, c-di-AMP etc.) nucleotides are the key parameters of bacterial metabolism. Separating and quantifying nucleotides is challenging. There are several challenges on quantifying specific nucleotides because one has to extract compounds from a complex biological mixture. Different approaches are used to harvest the cells, different chromatography columns, and different compounds are used to release the nucleotides from the cell. The nucleotides quantification is technically challenging for various reasons. First, because of turnover of the nucleotides is quite fast, as ATP with a half-life of around one-tenth of a second (Walsh and Koshland, 1984; Holms, Hamilton, and Robertson, 1972). ppGpp is more stable with a half-life of 30 to 200 seconds (Gallant, Margason, and Finch, 1972; Fiil et al., 1977; Harshman and Yamazaki, 1971) and (p)ppGpp has a half-life of around 10 seconds (Fiil et al., 1977). Second, some nucleotides are not stable during the sample process either due to enzymatic activity or due to the intrinsic chemical instability. Third, achieving good resolution of the full spectrum of nucleotide species is challenging due the complexity of the cellular nucleotide pools; both identification and quantification can be a challenge. The method can be divided in three steps: acquisition, extraction and quantification of the nucleotides. Currently, the most commonly used analytical techniques used for the analysis of nucleotide pool are TLC, HPLC and HPLC coupled to mass-spectrometry, HPLC-MS.

5.1 Sample acquisition for nucleotide analysis

The sample acquisition can be done by separating cells from culture medium or by sampling whole culture broth. The cells either can be separated from the medium by filtration or centrifugation. Nevertheless, if the acquisition of the sample is relatively slow – and invasive, affecting the metabolic status of the cell – which is a problem for the quantification of rapidly metabolizing nucleotides (Bennett et al., 2009; Buckstein, He, and Rubin, 2008). Centrifugation is poorly suited for analysis of bacterial nucleotides since it causes dramatic changes in the nucleotide levels, with highly phosphorylated species such as ATP converted to less phosphorylated species, such as ADP (Payne and Ames, 1982; Buckstein, He, and Rubin, 2008). Rapid vacuum filtration of the bacterial culture through nitrocellulose filters followed by fast snap-freezing the sample with liquid nitrogen overcome this issue (Payne and Ames, 1982). Challenges in sample acquisition can also be overcome by using with whole-culture broth sampling, followed by quenching by snap-freezing the sample with liquid nitrogen (Chassagnole et al., 2002; Dominguez et al., 1998). However, this last approach can have several disadvantages since it results in more diluted metabolites, nucleotides in intracellular or extracellular material cannot be distinguished, and components of growth media can interfere with the following analysis steps.

5.2 Sample extraction for nucleotide analysis

The extraction step can be mechanical (Meyer, Liebeke, and Lalk, 2010) or by using sonication (Lundquist and Olivera, 1971). The most popular choice is chemical, using with hot or cold solutes. Several options were tested for hot nucleotides extraction such as alkaline, chloroform, water, buffer solutions and ethanol (Meyer, Liebeke, and Lalk, 2010). One should be careful while choosing the exact protocol since signaling nucleotides, especially cyclic species, are unstable in alkaline conditions or the lysis of the bacterial cell is not efficient (Markham and Smith, 1952) which leads to (p)ppGpp losses (Cashel and Kalbacher, 1970). At higher temperatures chemical and enzymatic degradation are more likely to degrade the nucleotide pools. Thus, cold extraction is a more reliable approach. However, nucleotides still can be unstable at low temperatures. Use of strong acids TCA and PCA do not extract ppGpp efficiently, for that reason they are not the best options for our experiments (Cashel, 1969). The cold formic acid was very commonly used and we used in our first experiments when using whole culture acquisition experiments. It was also reported that formic acid induces ppGpp to ppGp degradation (Lagosky and Chang, 1978). Lastly, the acids are removed by freeze-drying using lyophilizator.

5.3 Thin Layer Chromatography

The thin-layer chromatography (TLC) is widely used to separate complex mixtures. This analytical technique was invented in 1905 in Tartu, Estonia, by Russian botanist Mikhail Semenovich Tswet (Михаил Семёнович Цвет) (Tswett, 1905).When the sample is applied on a plate covered with thin layer of polyethylenimine and cellulose (this the name, TLC), and one side of the plate is inserted in the solvent, as KH_2PO_4, the liquid mobile phase is drawn up the plate via capillary action. Through differential strength of the interaction with the sorbent, different species are resolved. In the case of nucleotides, the stationary phase is usually made of cellulose.

This technique was the first approach used for separation and detection of ppGpp and pppGpp (Cashel and Gallant, 1969). To assist the detection of nucleotides, bacterial cultures are metabolically labelled by $^{32}PO_4$, and it is essential that the cultures are grown for at least two generations in the presence of the label to ensure the uniform labelling of all the nucleotide species (Cashel, 1994). The classical protocol developed by Michael Cashel relies on nucleotide extraction with formic acid followed by TLC on polyethyleneimine cellulose using phosphates buffer (Cashel, 1994). With this method safety procedures are very important to avoid exposure to radioactive isotope. TLC is a fast, relatively reproducible and sensitive technique. It is highly versatile and relatively cheap procedure. The main drawback of TLC is its relatively low resolving power: TLC achieves about 5 000 theoretical plates (efficiency parameter used in chromatography) while HPLC achieves 10 000 to 20 000 theoretical plates (Bernard Fried, 1999).

5.4 High Performance Liquid Chromatography, HPLC

High performance liquid chromatography (HPLC) is a powerful technique for separation, identification and quantification of components in liquid phase. In the 1940's HPLC was applied to identify and quantify housekeeping bacterial nucleotides (such as NTP's, NDP's and NMP's) from a complex mixture material using anion exchange chromatography (Cohn, 1949). This technique was further developed during the 1980's when 10 µm 4.6 × 250 mm SAX (Partisil) columns with irregular silica particles became a standard for nucleotide analysis (Ochi, Kandala, and Freese, 1981; Payne and Ames, 1982). As well IPRP is being extensively used for nucleotide analysis (Buckstein, He, and Rubin, 2008; Au, Su, and Wientjes, 1989; Payne and Ames, 1982). To detect nucleotides the machine has to have an UV detector. HPLC offers a rapid and automated and highly precise method to separate and quantify compounds from a complex biological mixture. A gradient solvent can be applied and it is highly reproducible.

5.5 HPLC coupled to mass-spectrometry, HPLC-MS

TLC and HPLC methods achieve a very good separation of bacterial nucleotides according to analyte charge and size. However, identification of the compounds relies on the comparison of the eluted peaks with external standards. This is not always reliable since the compounds need to be baseline-separated, and co-elution of compounds that absorb at the same wavelength can lead to mis-identification. To overcome this limitation, ion-pair liquid chromatography was paired with mass spectrometry (MS) (Qin and Wang ,2018; Seifar et al., 2013). The disadvantage is that the MS signal deteriorates when ion pair reagents are used, rendering the MS spectra exceedingly complex (Holčapek et al., 2004). Recently ion chromatography coupled electrospray ionization high-resolution mass spectrometry (IC-ESI-HRMS) using isotope dilution mass spectrometry (IDMS) was applied for quantification of ppGpp and pppGpp (Patacq, Chaudet, and Létisse, 2018). However, this paper demonstrated only quantification of these two nucleotides, not the whole pool of bacterial nucleotides. In 2019 Zborníková and colleagues demonstrated the feasibility of quantification the whole bacterial nucleotide pool using hydrophilic Interaction Liquid Chromatography (HILIC) coupled with mass-spectrometry (Zborníková et al., 2019).

AIMS OF THE STUDY

The aim of this study is to develop and apply a reliable HPLC-based approach for quantification of bacterial nucleotide pools, with a special focus on alarmones (p)ppGpp and (p)ppApp.

The specific objectives of this study were:

- To develop a HPLC-based method to quantify bacterial housekeeping nucleotide pools with a focus on the second-messenger alarmones ppGpp and pppGpp (Paper I)
- To validate the HPLC-based method applied to studies of acute stringent response and quantify the nucleotide pool dynamics throughout the bacterial growth curve (Paper I)
- To describe the effects of translational antibiotics on bacterial nucleotide pools, with a special focus on ppGpp (Paper II)
- To characterize the dynamics of the nucleotide pools upon expression of *C. marina* FaRel toxSAS TA toxin and uncover the molecular basis of FaRel-mediated growth inhibition (Paper III)

RESULTS AND DISCUSSION

I. HPLC-based quantification of bacterial nucleotides (Paper I)

To investigate the fluctuation of housekeeping nucleotides concentration in bacteria we defined a HPLC-UV method for nucleotide quantification that is able of quantification of bacterial nucleotide pools including adenosine, guanosine and pyrimidine species. Nucleotide quantification methods are divided into three steps: acquisition, extraction and quantification (**Figure 11**).

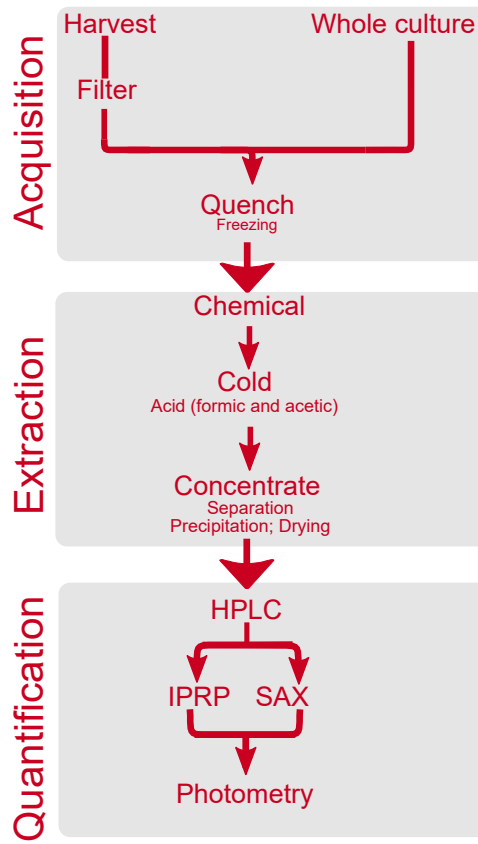

Figure 11. Nucleotide quantification workflow tested in this work. The workflow can be subdivided into three steps: sample acquisition, nucleotide extraction, and quantification. Sample acquisition can be accomplished using either cell harvesting or whole culture sampling. During extraction, the nucleotide content is released from the cells chemically, with cold formic or acetic acid. The quantification was done using high performance liquid chromatography with an UV detector. The column used were Kinetix C18 2.6 µm 4.6 × 150 mm and 5 µm 4.6 × 150 mm Strong anion exchange (SAX). Adapted from (Varik et al., 2017).

Centrifugation was earlier reported to perturb the composition of bacterial nucleotide pools (Lundin and Thore, 1975; Leps and Ensign, 1979; Buckstein, He, and Rubin, 2008). We tested its effect and also concluded that centrifugation has a pronounced effect and should be avoided. Another option for sample collection is rapid filtration which is a common procedure for harvesting cells (Payne and Ames, 1982; Ochi, Kandala, and Freese, 1981). We commonly filtered 10–40 ml of bacterial culture through a 0.45 μm membrane filter using a vacuum pump, and the filters were immediately transferred into 1.5 ml tubes that contained ice-cold acid. After, the tube containing the filter and acid were snap-frozen in liquid nitrogen and stored at −80 °C. The extraction was performed with the samples thawed on ice for around 30 minutes with occasionally vortexing. The filter was removed and the sample again freeze-dried in liquid nitrogen. This approach to collect samples shortens the method and the loss of monophosphates and ADP is way less compared with the whole culture procedure. To concentrate the nucleotides we used a freeze-drying.

The application of anion exchange chromatography for separation of nucleotide pools is well-documented (Payne and Ames, 1982; Ochi, Kandala, and Freese, 1981). We used a 5 μm 4.6 × 150 mm Strong anion exchange (SAX) column with spherical porous particles. We adopted two modes of elution: isocratic or gradient elution with ammonium phosphate buffer. To detect pppGpp and ppGpp (**Figure 12B**), we have used the isocratic elution at pH 3.4. However, the resolution of the rest of the nucleotide standards is not as robust (**Figure 12A**). The separation of the nucleotide standards in isocratic mode is not possible (**Figure 12A**). However, the nucleotide standard resolution is improved when gradient elution is applied (**Figure 13A** and **13B**). We noticed that the retention time of the SAX column decrease as the columns ages, requiring consistent adjustments of the gradient and/or buffer strength.

SAX is a more reliable approach for detection of pppGpp and ppGpp (**Figure 12B**), but not well-suited for analysis of other nucleotide species (**Figure 13A**). To detect and quantify housekeeping nucleotides, we used IPRP chromatography that is also widely used (Huang, Zhang, and Chen, 2003; Cserjan-Puschmann et al., 1999; Buckstein, He, and Rubin, 2008; Au, Su, and Wientjes, 1989; Payne and Ames, 1982). In comparison to SAX, IPRP approach has several advantages. First, IPRP-HPLC has higher sensitivity and the peaks are better resolved (**Figure 13C**). Second, the retention times are significantly more stable. Third, this approach doesn't require high-salt buffers. However, we were not successful to implementing this protocol to quantify (p)ppGpp. In conclusion, IPRP is a reliable method for detection and quantification of bacterial housekeeping nucleotides, with an exception of GMP which co-elutes with IMP (**Figure 13D**).

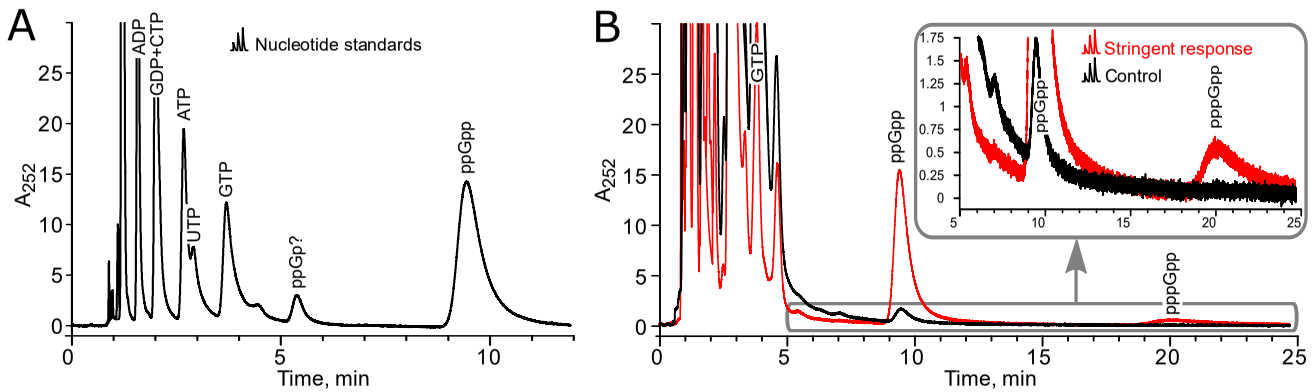

Figure 12. Isocratic elution in SAX-HPLC. (A) Nucleotide standards were run on isocratic SAX-HPLC using absorbance at 252 nm. **(B)** The stringent response was induced with 150 µg/ ml of mupirocin in E. coli cells. Samples were collected both before (black) and 5 min after induction (red). The column used was a 5 µm SphereClone column 4.6 × 150 mm that was run with a buffer containing 0.36 M NH_4H2PO_4 pH 3.4, 2.5% acetonitrile at 26 °C at a flow rate of 1.5 ml/min. Since the 'ppGp?' peak was never validated by the addition of a spike-in standard, it is marked with a question mark. Adapted from (Varik et al., 2017).

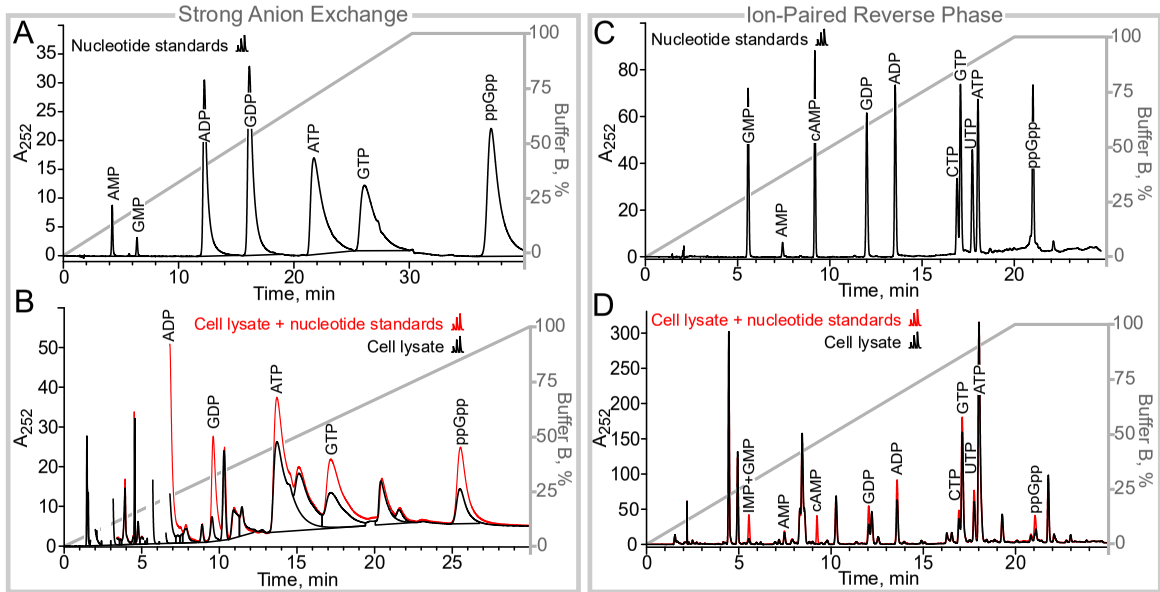

Figure 13. Gradient elution in SAX and IPRP-HPLC. (A) Nucleotide standard were resolved in a SAX-HPLC run using gradient elution followed by tracking absorbance at 252 nm. The degradation of di-, tri- and tetraphosphates leads to the appearance of AMP and GMP in the standard. **(B)** Nucleotides extracted from an *E. coli* were resolved with SAX-HPLC using gradient elution both without (black) and with (red) a spiked-in 2 nmol nucleotide standard used to validate identity of the peaks. **(C)** 0.5 nmol of nucleotide standard (GMP, cAMP, GDP, ADP, CTP, GTP, UTP, ATP and ppGpp) were resolved in an IPRP-HPLC run using gradient elution. **(D)** Nucleotides extracted from *E. coli* were resolved using IPRP with the aid of a spiked-in 0.25 nmol standard (red) used to validate the identity of the peaks. IMP and GMP were not resolved and co-migrate as one peak. SAX-HPLC: A 5 µm Spherisorb 4.6 × 150 mm column was run at 1 ml/min and 26°C. Buffer A: 0.05 M $NH_4H_2PO_4$, pH 3.4. Buffer B: 0.5 $NH_4H_2PO_4$, pH 3.4. IPRP: Kinetix C18 2.6 µm 4.6 × 150 mm, 0.8 ml/ min, 26°C. Buffer A: 5 mM Bu_4NOH, 30 mM KH_2PO_4 pH 6.0. Buffer B: 100% acetonitrile. Adapted from (Varik et al., 2017).

II. Nucleotide pools in *E. coli* throughout the growth curve and during acute stringent response (Paper I)

With our HPLC-UV method we could follow the changes in nucleotide levels in *E. coli* throughout the growth curve (**Figure 14A**). The experiments were done using fast vacuum filtration, followed by acid extraction and freeze-drying. To quantify the nucleotide pools – GMP unresolved and co-migrating with IMP; GDP and GTP; AMP, ADP and ATP; CTP; UTP – we used C18 IPRP-HPLC column. To resolve ppGpp and pppGpp we used a SAX column in isocratic mode. Through the growth curve the most pronounce nucleotide is GTP, 60-80% of the total pool. When ppGpp accumulated, the levels of GTP decreased down to 54%. The explanation is that in *E. coli* ppGpp inhibits IMP dehydrogenase, thus down-regulating the production of guanosine nucleotides. Adenosines are very stable throughout the growth curve. The triphosphates are stable with the exception of GTP, see above (**Figure 14A**).

We also analyzed the nucleotide pools of *E. coli* during acute amino acids starvation. To induce the stringent response the bacterial cells were treated with mupirocin (pseudomonic acid), inhibitor of isoleucine aminoacyl-tRNA synthetase. Soon after the stringent response was induced, we observed a dramatic increase in the levels of ppGpp (**Figure 14B**). 4 minutes post-treatment, ppGpp becomes the most abundant nucleotide in the pool (60% total). GTP levels drop from 73% to 25%, reciprocating the accumulation of (p)ppGpp. pppGpp, which is undetectable in untreated cultures, increases to about 8% and becomes more abundant than GDP, which drops down to 4% of the guanosine pool. Conversely, the adenosine pools are constant and the triphosphate pool changes in a similar way when there is the transition from exponential to stationary phase. GTP is the exception that goes from 28% to 16% of the total NTP pool, see above, AEC, adenylate energy charge, a key physiological parameter showing how energized and viable the cells are (Atkinson 1968), is stable at approximately 0.9, showing the viability of the bacterial cells.

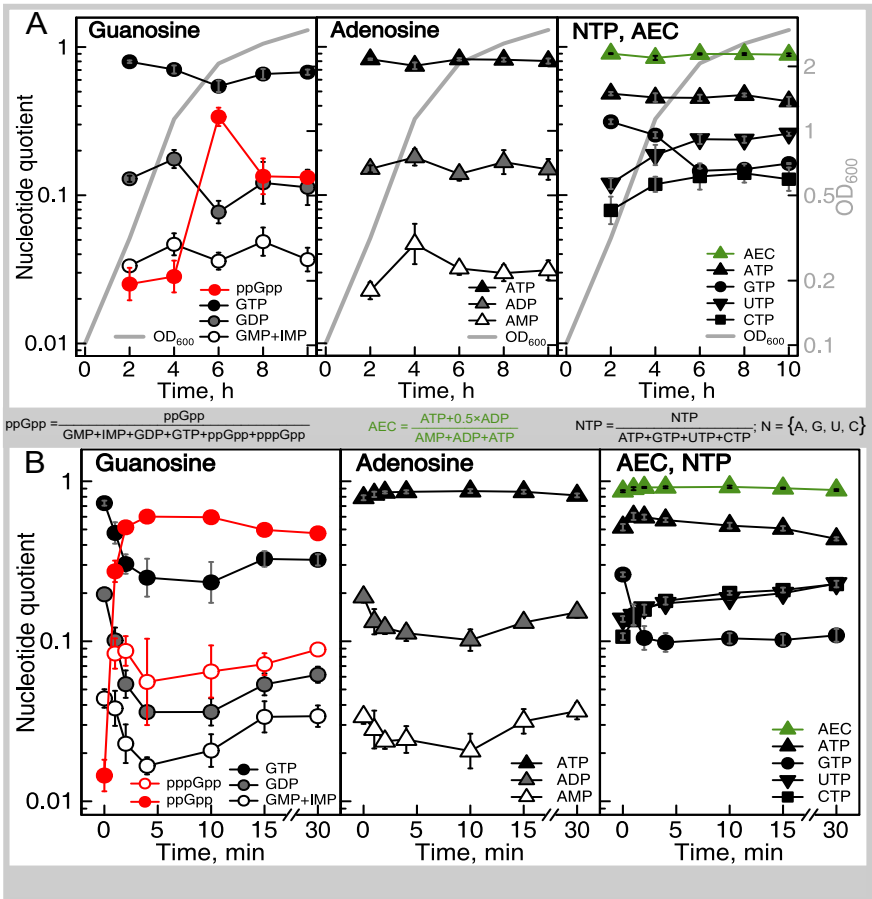

Figure 14. Intracellular nucleotide measurements of *E. coli*. (A) Nucleotides measurements of *E. coli* through the growth curve show that the nucleotides pool is very stable with the exception of ppGpp. **(B)** Kinetics of nucleotide upon induced stringent response. The stringent response was induced with 150 µg/ml of mupirocin, added when cells reached OD_{600} 0.5. In both cases *E. coli* cultures grown in MOPS 0.4% glucose at 37 °C with vigorous aeration are expressed as ratios of guanosine, adenosine, and NTP pools as indicated in the insert. Cells were harvested by filtration and nucleotides extracted with acetic acid. ppGpp and pppGpp were measured using isocratic SAX and the remaining nucleotide species were quantified using gradient IPRP. Error bars indicate the standard error of the mean of biological replicates. AEC refers to the adenylate energy charge defined as per (Atkinson, 1968). Adapted from (Varik et al., 2017).

III. Translation inhibitors block RelA-mediated stringent response (Paper II)

The (p)ppGpp-mediated signaling is a promising target for new antibacterial agents since this signaling system plays important role in bacterial antibiotic tolerance, such as beta-lactams (Rodionov et al., 1995), and it is lacking in in eukaryotes. Antibiotics that target translation – chloramphenicol, tetracycline and thiostrepton – have been shown to inhibit the accumulation of (p)ppGpp (Kaplan, Atherly, and Barrett, 1973; Jenvert and Schiavone, 2005) suggesting that these antibiotics could abrogate (p)ppGpp accumulation, have a knock-on effect by sensitizing bacteria to cell-wall targeting beta-lactams. We characterized the effect of translation inhibitors on the intracellular levels of ppGpp, GTP, GDP and ATP nucleotide pools using our method of quantification of nucleotides by HPLC-based approach. Here we revisit the classic translation inhibitors in *E. coli* (**Figure 15**) and *B. subtilis* (**Figure 16**) upon amino acid starvation.

We tested several antibiotics that target protein synthesis (thiostrepton, chloramphenicol, and tetracycline), as well as antibiotic trimethoprim used as a control. Trimethoprim blocks the production of tetrahydrofolate by dihydrofolate reductase, following in the inhibition of glycine, methionine, dTTP, and purine biosynthesis. Antibiotic mupirocin was used to pretreat exponentially growing bacterial cultures to induce the accumulation of (p)ppGpp. These results in accumulation of uncharged tRNA leading to activation of RelA or Rel and effectuating. Afterwards, the antibiotic of interest was added at sub-inhibitory concentrations. The same experiments were done for both organisms with the exception of thiostrepton since *E. coli* is insensitive to this antibiotic due to a lack of uptake. Therefore, the experiments with thiostrepton are done only in *B. subtilis* (**Figure 16A**). In the case of both organisms all the translation inhibitors tested inhibited the ppGpp accumulation (**Figure 15 and 16 A, C and D**). In case of trimethoprim, the inhibition of growth does not result in the decrease of ppGpp levels (**Figure 15B and 16B**). The effects of translation inhibitors are three-fold. First, thiostrepton and tetracycline directly inhibit activation of Rel/RelA by starved ribosomal complexes. Second, it was proposed that all translation inhibitors abrogate consumption of amino acids, which results in charging up of tRNA's indirectly inhibiting RelA activation. In the absence of deacylated tRNA, Rel and RelA are not activated. Finally, inhibition of translation results in abrogation of production of RSH enzymes. This is especially important in case of *E. coli* SpoT which has a protein functional lifespan of 40 seconds or less (Murray and Bremer 1996) and its synthetic activity is rapidly lost upon inhibition of protein production.

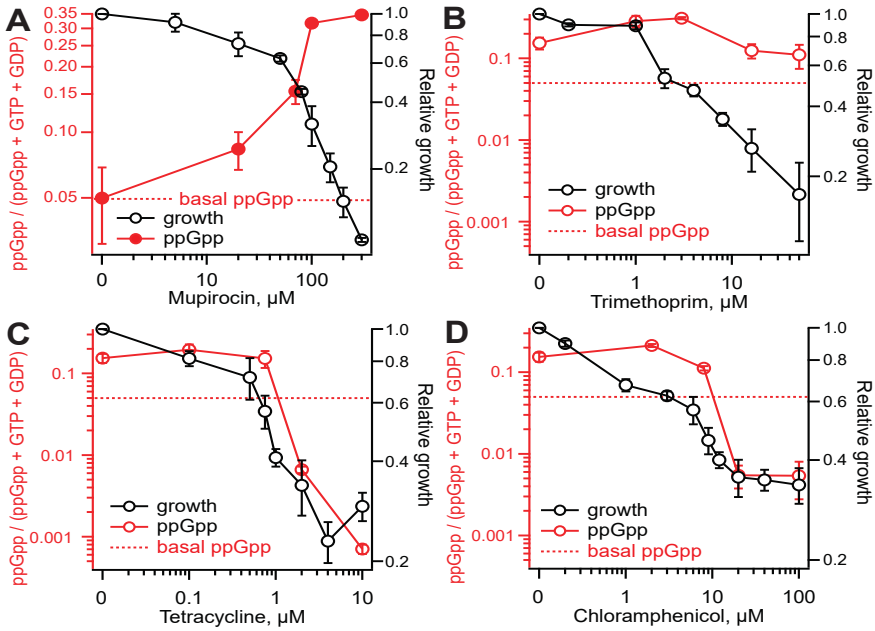

Figure 15. Concurrent inhibition of *E. coli* growth and ppGpp production by antibiotics targeting translation. (A) The stringent response was induced by the addition of increasing concentrations of mupirocin followed by 30 min of incubation and HPLC analysis (**B** to **D**). *E. coli* cultures were treated for 30 min with different concentrations of **(B)** trimethoprim, **(C)** tetracycline or **(D)** chloramphenicol combined with 70 μM of mupirocin. Samples were collected, and nucleotide levels were determined by HPLC. We used BW25113 *E. coli* strain grown at 37°C in MOPS medium supplemented with 0.4% glucose and a full set of 20 amino acids at 25 μg/ml. Growth inhibition was calculated as an increase in the OD_{600} after1h of antibiotic treatment compared to the untreated control. The ppGpp levels are calculated as a ppGpp fraction of a combined GTP, GTP, and ppGpp nucleotide pool; the dashed red trace indicates the level in unstressed cells. Error bars indicate the standard errors of the mean. Adapted from (Kudrin et al. 2017).

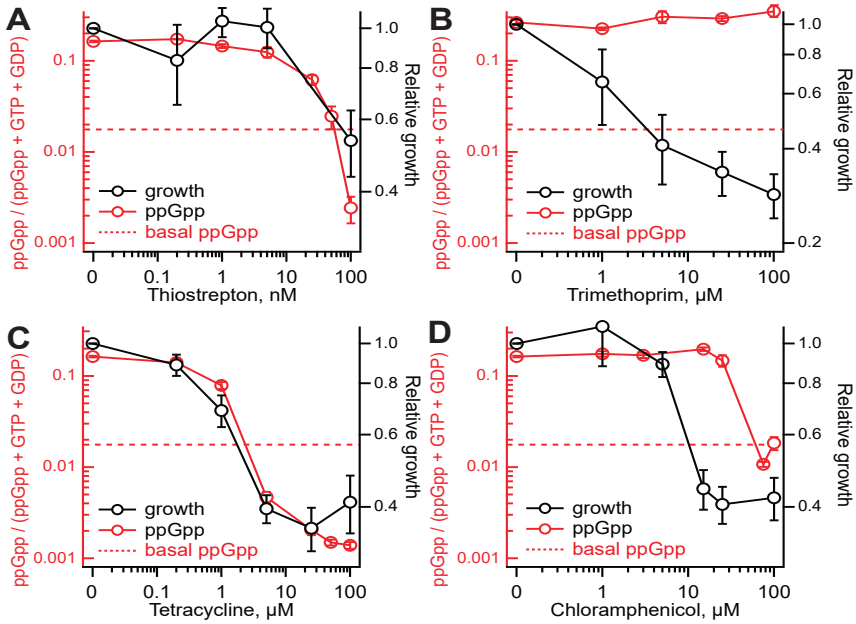

Figure 16. *B. subtilis* cells starved for isoleucine and treated with translation inhibitors. (A) The stringent response was induced with 70 nM of mupirocin, followed by the addition of increasing concentrations of thiostrepton, **(B)** trimethoprim, **(C)** tetracycline, **(D)** chloramphenicol. After 30 minutes of addition of antibiotics the samples were collected. We used BSB1 *B. subtilis* strain grown at 37°C in MOPS medium supplemented with 0.4% glucose and a full set of 20 amino acids at 25 μg/ml. Growth inhibition was calculated as an increase in the OD_{600} after1h of antibiotic treatment compared to the untreated control. The ppGpp levels are calculated as a ppGpp fraction of a combined GTP, GTP, and ppGpp nucleotide pool; the dashed red trace indicates the level in unstressed cells. Error bars indicate the standard errors of the mean. Adapted from (Kudrin et al. 2017).

IV. FaRel toxicity is mediated by accumulation of ppGpp and ppApp alarmones (Paper III)

Analysis of SAS gene neighborhood conservation across a panel of bacterial species was used to identify the SAS genes that are a part of conserved operon; this, in turn, indicates a functional association of several genes (Jimmy et al., 2020). It was discovered that some subfamilies of SAS can be encoded in conserved and overlapping two-gene operons resembling toxin-antitoxin (TA) loci. Since high levels of (p)ppGpp are toxic, it was hypothesized that these TA-like SAS could act as toxic effectors. Using growth assays, several toxic SAS's were discovered, toxSAS – faRel, phRel2, and phRel – were validated as TA effectors (**Figure 17**). The antitoxin ATfaRel from *Cellulomonas marina* is an

SAH, suggesting it acts trough degradation of alarmone produced by toxSAS; the rest of the antitoxins were suggested to act via formation of an inactive, non-toxic complex with the toxSAS (Jimmy et al., 2020). Finally, FaRel was shown to produce ppApp, which causes, similarly to the case of Tas1, depletion of ATP and GTP pools.

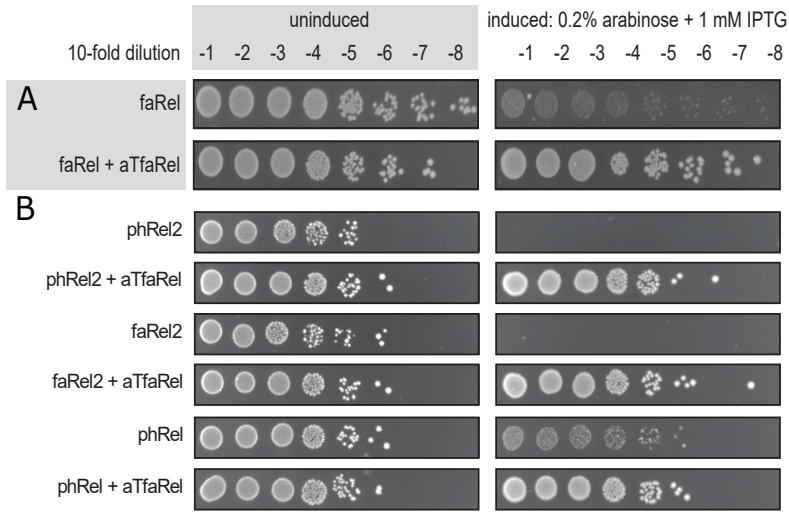

Figure 17. *C. marina* ATfaRel SAH universally counteracts all identified toxSASs. (A) *C. marina* aTfaRel neutralizes the cognate toxin. **(B)** *C. marina* aTfaRel neutralizes all identified toxSAS toxins. aT stands for antitoxin. Adapted from (Jimmy et al., 2020).

To understand better the effects of *C. marina* faRel toxSAS on the intracellular nucleotide pools, I applied the HPLC-based approach to quantify the nucleotide pools upon expression of either FaRel (toxin) alone **(Figure 18A-C)**, co-expressed with ATfaRel (anti-toxin) **(Figure 18D-F)** or ATfaRel by itself **(Figure 18G-I)**. When FaRel was expressed, I observed a dramatic decrease of ATP and GTP pools **(Figure 18A-B)**. ppGpp accumulated after five minutes after induction of FaRel, but it dropped five minutes later **(Figure 18B)**. I noticed a very pronounced peak on IPRP upon FaRel induction. The hypothesis was that FaRel is able to synthesise ppApp, similarly to a *S. morookaensis* SAS (Oki et al. 1975) and Tas1 toxic effector (Ahmad et al. 2019). Using pure ppApp nucleotide as a spike-in standard, I validated that the new distinct peak was, indeed, ppApp. The accumulation of ppApp after five minutes and kept a high level of production, with ppApp becoming the dominant adenosine species; simultaneously GTP and ATP levels dropped **(Figure 18A and 19B)**. When the aTfaRel in combination with faRel ppGpp and ppApp are not accumulate and the levels of GTP and ATP do not decrease as much. The induction of atfRel alone shows the nucleotide pool stable and no accumulation of either ppGpp or ppApp (**Figure 18D-F**). Collectively, my results demons-

trated that HPLC approach is capable of detecting novel alarmones such as ppApp, thus explaining the mechanisms of toxicity of FaRel SAS.

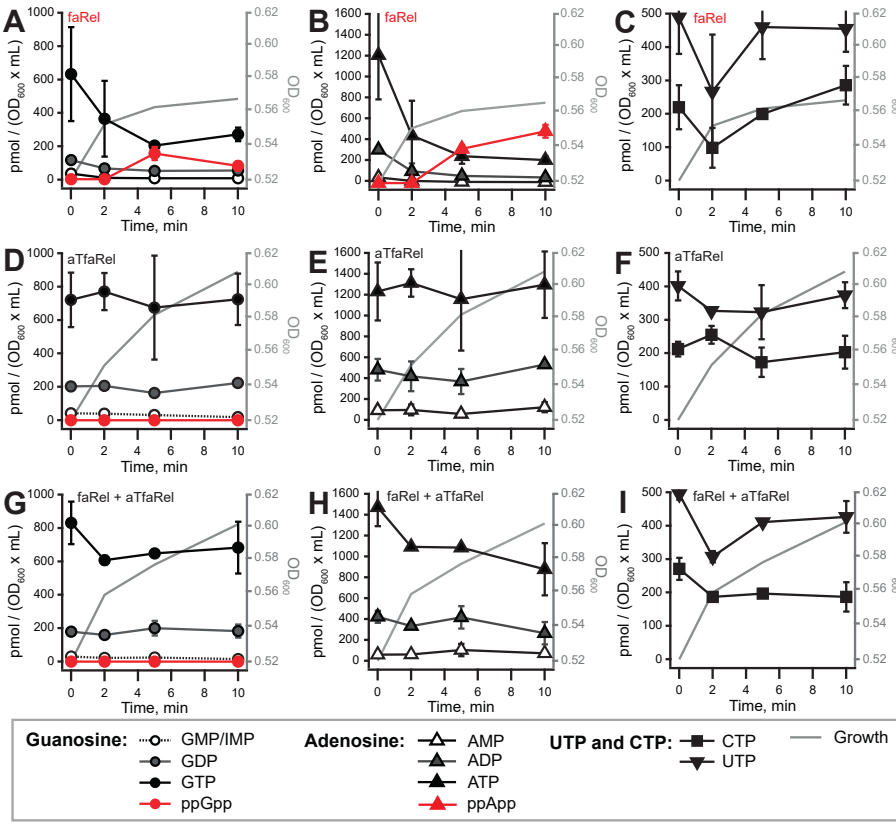

Figure 18. Measurements of nucleotide pools in *E. coli* BW25113 expressing (A-C) *C. marina* faRel, (D-F) *C. marina* aTfaRel and (G-I) the combination of *C. marina* faRel and aTfaRel. Cell cultures were grown in defined minimal MOPS medium supplemented with 0.5% glycerol at 37 °C with vigorous aeration. The expression of *C. marina* faRel was induced with 0.2% L-arabinose at the OD_{600} 0.5 (**A-C**, **G-I**). The expression of *C. marina* aTfaRel was induced by 1 mM IPTG at the zero time point (**D-F**, **G-I**). Intracellular nucleotides are expressed in pmol per OD600 • mL of bacterial culture. Error bars indicate the standard error of the arithmetic mean of three biological replicates. Adapted from (Jimmy et al., 2020).

The hypothesis was that FaRel is able to synthesise (p)ppApp, similarly to a *S. morookaensis* SAS (Oki et al., 1975) and Tas1 toxic effector (Ahmad et al., 2019). Using pure ppApp nucleotide as a spike-in standard, I validated that the new distinct peak was, indeed, ppApp (**Figure 19**). The accumulation of ppApp after five minutes and kept a high level of production, with ppApp becoming

the dominant adenosine species; simultaneously GTP and ATP levels dropped **(Figure 18A** and **19B)**. When the aTfaRel is in combination with faRel then ppGpp and ppApp do not accumulate and the levels of GTP and ATP do not decrease as much. The induction of atfRel alone shows the nucleotide pool stable and no accumulation of either ppGpp or ppApp (**Figure 18D-F**). Collectively, my results demonstrated that HPLC approach is capable of detecting novel alarmones such as ppApp, thus explaining the mechanisms of toxicity of FaRel SAS.

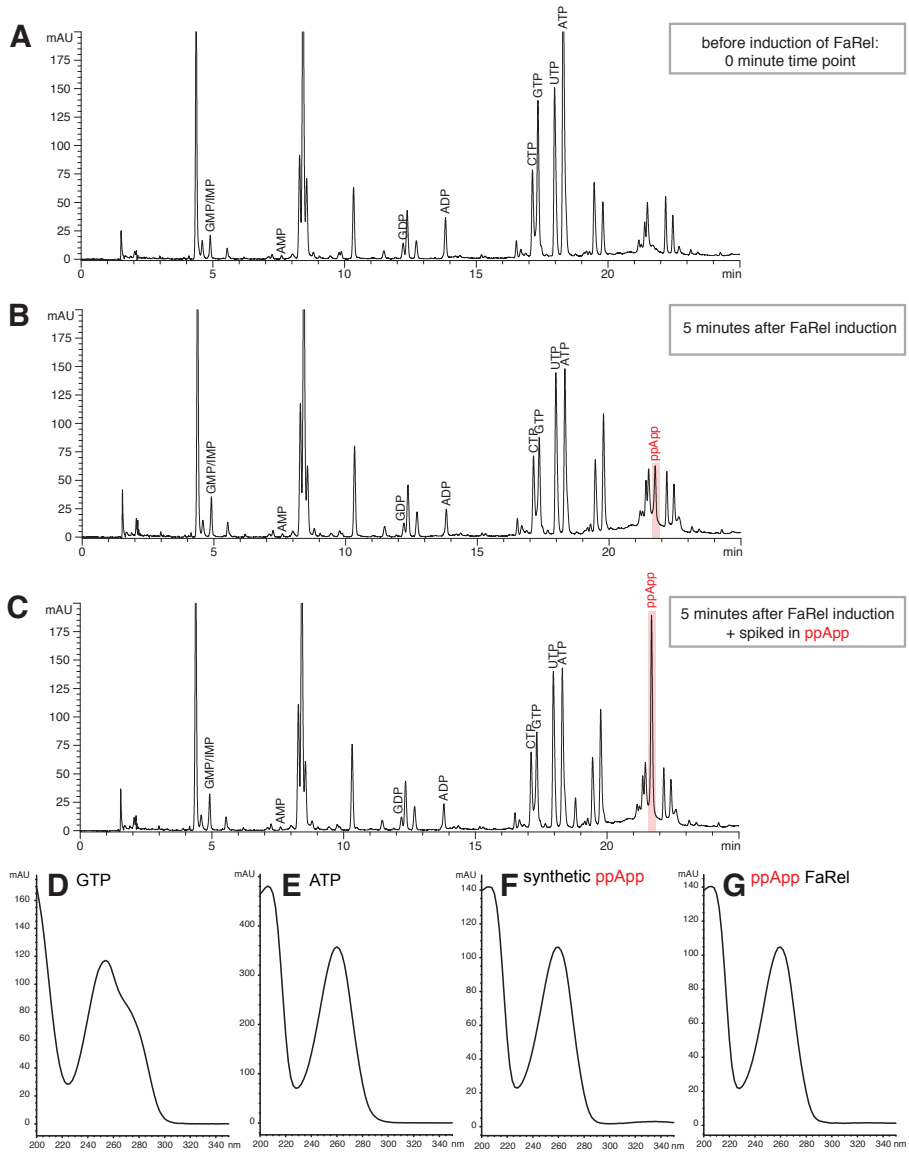

Figure 19. Detection of ppApp accumulation in *E. coli* BW25113 expressing *C. marina* FaRel using Ion-Paired Reverse Phase (IPRP) chromatography. Detection

of ppApp accumulation in E. coli BW25113 expressing C. marina FaRel using Ion-Paired Reverse Phase (IPRP) chromatography. Cell cultures were grown in defined minimal MOPS medium supplemented with 0.5% glycerol at 37 °C with vigorous aeration. The expression of C. marina faRel was induced with 0.2% L-arabinose at the OD600 0.5. Nucleotides (including ppApp) were resolved and quantified on Ion-Paired Reverse Phase (IPRP) at 26 °C using Kinetix C18 2.6 µm 4.6×150 mm column on 5 (0 minutes) – 35% (20 minutes) gradient of buffer B. Buffer A: 5 mM TBA-OH, 30 mM KH_2PO_4 pH 6.0. Buffer B: 100% acetonitrile. (A) Nucleotide pool prior to induction of *C. marina* faRel expression. **(B)** Nucleotide pool after 5 minutes of induction of *C. marina* faRel expression **(C)** same sample as **(B)** but spiked in with 1000 pmol of chemically synthesised ppApp standard. ppGpp was detected and quantified in a separate experiment by SAX-HPLC using Spherisorb 5 µm 4.6×150 mm column with isocratic elution in 0.27 M $NH_4H_2PO_4$ pH 3.4, 2.5% acetonitrile at 26 °C at a flow rate of 1.5 ml/min. **(D-G)** UV-spectra of GTP, ATP and ppApp standards as well as the ppApp peak that accumulates upon expression of FaRel. Adapted from (Jimmy et al., 2020).

CONCLUSIONS

The HPLC-based method for quantification of bacterial nucleotides was successfully implemented using two approaches for harvesting the cells – either using whole culture sampling or rapid filtration – in combination with two complementary methods for nucleotide quantification, SAX and IPRP. Using this method, we show that:

First, the nucleotide pools of *E. coli* are stable across the growth curve, with the exception of ppGpp peaking during the transition to stationary phase and stabilizing at higher stable level at the stationary phase.

Second, tetracycline and chloramphenicol abolish accumulation of ppGpp in *E. coli* cells acutely starved for amino acids by mupirocin treatment. Same falls for *B. subtilis*, in which additionally ppGpp accumulation can be abrogated by thiostrepton.

Third, *C. marina* SAS toxin FaRel produced both ppGpp as well as ppApp. The latter alarmone is the causative agent of the toxic effect of FaRel and its antitoxin can counteract the toxicity of all the ToxSAS tested.

REFERENCES

Abranches, Jacqueline, Alaina R. Martinez, Jessica K. Kajfasz, Violeta Chavez, Danielle A. Garsin, and José A. Lemos. 2009. "The Molecular Alarmone (p)PpGpp Mediates Stress Responses, Vancomycin Tolerance, and Virulence in Enterococcus Faecalis." *Journal of Bacteriology* 191 (7): 2248–56. https://doi.org/10.1128/JB.01726-08.

Agirrezabala, Xabier, Israel S Fernández, Ann C Kelley, David Gil Cartón, Venki Ramakrishnan, and Mikel Valle. 2013. "The Ribosome Triggers the Stringent Response by RelA via a Highly Distorted TRNA." *EMBO Reports* 14 (9): 1–6. https://doi.org/10.1038/embor.2013.106.

Ahmad, Shehryar, Boyuan Wang, Matthew D. Walker, Hiu Ki R. Tran, Peter J. Stogios, Alexei Savchenko, Robert A. Grant, Andrew G. McArthur, Michael T. Laub, and John C. Whitney. 2019. "An Interbacterial Toxin Inhibits Target Cell Growth by Synthesizing (p)PpApp." *Nature* 575 (7784): 674–78. https://doi.org/10.1038/s41586-019-1735-9.

Alföldi, Lajos, Gunther S. Stent, and Royston C. Clowes. 1962. "The Chromosomal Site of the RNA Control (RC) Locus in Escherichia Coli." *Journal of Molecular Biology* 5 (3): 348–55. https://doi.org/10.1016/S0022-2836(62)80077-1.

Andresen, Liis, Tanel Tenson, and Vasili Hauryliuk. 2016. "Cationic Bactericidal Peptide 1018 Does Not Specifically Target the Stringent Response Alarmone (p)PpGpp." *Scientific Reports* 6 (November). https://doi.org/10.1038/srep36549.

Andresen, Liis, Vallo Varik, Yuzuru Tozawa, Steffi Jimmy, Stina Lindberg, Tanel Tenson, and Vasili Hauryliuk. "Auxotrophy-Based High Throughput Screening Assay for the Identification of Bacillus Subtilis Stringent Response Inhibitors." *Scientific Reports* 6 (1): 1–8. https://doi.org/10.1038/srep35824.

Artsimovitch, Irina, Vsevolod Patlan, Shun-ichi Sekine, Marina N Vassylyeva, Takeshi Hosaka, Kozo Ochi, Shigeyuki Yokoyama, and Dmitry G Vassylyev. 2004. "Structural Basis for Transcription Regulation by Alarmone PpGpp." *Cell* 117 (3): 299–310. http://www.ncbi.nlm.nih.gov/pubmed/15109491.

Atkinson, Daniel E. 1968. "The Energy Charge of the Adenylate Pool as a Regulatory Parameter. Interaction with Feedback Modifiers." *Biochemistry* 7 (11): 4030–34. https://doi.org/10.1021/bi00851a033.

Atkinson, Gemma C, Tanel Tenson, and Vasili Hauryliuk. 2011. "The RelA/SpoT Homolog (RSH) Superfamily: Distribution and Functional Evolution of PpGpp Synthetases and Hydrolases across the Tree of Life." *PloS One* 6 (8): e23479. https://doi.org/10.1371/journal.pone.0023479.

Au, J. L.S., M. H. Su, and M. G. Wientjes. 1989. "Extraction of Intracellular Nucleosides and Nucleotides with Acetonitrile." *Clinical Chemistry* 35 (1): 48–51. https://doi.org/10.1093/clinchem/35.1.48.

Avarbock, David, Jerome Salem, Lin Sheng Li, Zhi Mei Wang, and Harvey Rubin. 1999. "Cloning and Characterization of a Bifunctional RelA/SpoT Homologue from Mycobacterium Tuberculosis." *Gene* 233 (1–2): 261–69. https://doi.org/10.1016/S0378-1119(99)00114-6.

Battesti, Aurelia, and Emmanuelle Bouveret. 2009. "Bacteria Possessing Two RelA/SpoT-like Proteins Have Evolved a Specific Stringent Response Involving The Acyl Carrier Protein-SpoT Interaction." *Journal of Bacteriology* 191 (2): 616–24. https://doi.org/10.1128/JB.01195-08.

Bennett, Bryson D., Elizabeth H. Kimball, Melissa Gao, Robin Osterhout, Stephen J. Van Dien, and Joshua D. Rabinowitz. 2009. “Absolute Metabolite Concentrations and Implied Enzyme Active Site Occupancy in Escherichia Coli.” *Nature Chemical Biology* 5 (8): 593–99. https://doi.org/10.1038/nchembio.186.

Bernard Fried, Bernard Sherma. 1999. “Thin-Layer Chromatography, Revised And Expanded.” CRC Press. 1999. https://books.google.se/books?id=gkLIqBT7VUIC&pg=PA5&lpg=PA5&dq=,+HPLC,+theoretical+plates+are+around+5000+on+TLC+and+in+on+HPLC+10+000+to+20+000+theoretical+plates&source=bl&ots=RVY6du-XXi&sig=ACfU3U1XPuVv1-ZcEuIMyy1vx3wBiEE9SA&hl=pt-PT&sa=X&ved=2ahUKEwi.

Bigger, JosephW. 1944. “TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION.” *The Lancet* 244 (6320): 497–500. https://doi.org/10.1016/S0140-6736(00)74210-3.

Black, D S, B Irwin, and H S Moyed. 1994. “Autoregulation of Hip, an Operon That Affects Lethality Due to Inhibition of Peptidoglycan or DNA Synthesis.” *Journal of Bacteriology* 176 (13): 4081–91.

Black, D S, A J Kelly, M J Mardis, and H S Moyed. 1991. “Structure and Organization of Hip, an Operon That Affects Lethality Due to Inhibition of Peptidoglycan or DNA Synthesis.” *Journal of Bacteriology* 173 (18): 5732–39.

Blower, Tim R., George P.C. Salmond, and Ben F. Luisi. 2011. “Balancing at Survival’s Edge: The Structure and Adaptive Benefits of Prokaryotic Toxin-Antitoxin Partners.” *Current Opinion in Structural Biology*. https://doi.org/10.1016/ j.sbi.2010.10.009.

Blower, Tim R., Francesca L. Short, Feng Rao, Kenji Mizuguchi, Xue Y. Pei, Peter C. Fineran, Ben F. Luisi, and George P.C. Salmond. 2012. “Identification and Classification of Bacterial Type III Toxin-Antitoxin Systems Encoded in Chromosomal and Plasmid Genomes.” *Nucleic Acids Research* 40 (13): 6158–73. https://doi.org/10.1093/nar/gks231.

Blumenthal, E. M., and L. K. Kaczmarek. 1992. “Modulation by CAMP of a Slowly Activating Potassium Channel Expressed in Xenopus Oocytes.” *Journal of Neuroscience* 12 (1): 290–96. https://doi.org/10.1523/jneurosci.12-01-00290.1992.

Brandi, Letizia, Stefano Marzi, Attilio Fabbretti, Carola Fleischer, Walter E. Hill, Claudio O. Gualerzi, and J. Stephen Lodmell. 2004. “The Translation Initiation Functions of IF2: Targets for Thiostrepton Inhibition.” *Journal of Molecular Biology* 335 (4): 881–94. https://doi.org/10.1016/j.jmb.2003.10.067.

Brown, Alan, Israel S. Fernández, Yuliya Gordiyenko, and V. Ramakrishnan. 2016. “Ribosome-Dependent Activation of Stringent Control.” *Nature* 534 (7606): 277–80. https://doi.org/10.1038/nature17675.

Brown, Jason M., and Karen Joy Shaw. 2003. “A Novel Family of Escherichia Coli Toxin-Antitoxin Gene Pairs.” *Journal of Bacteriology* 185 (22): 6600–6608. https://doi.org/10.1128/JB.185.22.6600-6608.2003.

Bruhn-Olszewska, Bożena, Vadim Molodtsov, Michał Sobala, Maciej Dylewski, Katsuhiko S. Murakami, Michael Cashel, and Katarzyna Potrykus. 2018. “Structure-Function Comparisons of (p)PpApp vs (p)PpGpp for Escherichia Coli RNA Polymerase Binding Sites and for RrnB P1 Promoter Regulatory Responses in Vitro.” *Biochimica et Biophysica Acta – Gene Regulatory Mechanisms* 1861 (8): 731–42. https://doi.org/10.1016/j.bbagrm.2018.07.005.

Buckstein, Michael H., Jian He, and Harvey Rubin. 2008. “Characterization of Nucleotide Pools as a Function of Physiological State in Escherichia Coli.” *Journal of Bacteriology* 190 (2): 718–26. https://doi.org/10.1128/JB.01020-07.

Buglino, John, Vincent Shen, Payam Hakimian, and Christopher D. Lima. 2002. “Structural and Biochemical Analysis of the Obg GTP Binding Protein.” *Structure* 10 (11): 1581–92. https://doi.org/10.1016/S0969-2126(02)00882-1.

Cashel, M. 1969. “The Control of Ribonucleic Acid Synthesis in Escherichia Coli. IV. Relevance of Unusual Phosphorylated Compounds from Amino Acid-Starved Stringent Strains.” *Journal of Biological Chemistry* 244 (12): 3133–41. https://pubmed.ncbi.nlm.nih.gov/4893338/.

Cashel, M, and B Kalbacher. 1970. “The Control of Ribonucleic Acid Synthesis in Escherichia.” *The Journal of Biological Chemistry* 245 (9): 2309–18.

Cashel, Mike. 1994. “Detection of (p)PpGpp Accumulation Patterns in Echeria Coli Mutants.”

Chashel, M, and J Gallant. 1969. “Two Compounds Implicated in the Function of the RC Gene of Escherichia Coli.” *Nature Publishing Group* 221: 838–841.

Chassagnole, Christophe, Naruemol Noisommit-Rizzi, Joachim W. Schmid, Klaus Mauch, and Matthias Reuss. 2002. “Dynamic Modeling of the Central Carbon Metabolism OfEscherichia Coli.” *Biotechnology and Bioengineering* 79 (1): 53–73. https://doi.org/10.1002/bit.10288.

Cohn, W E. 1949. “The Separation of Purine and Pyrimidine Bases and of Nucleotides by Ion Exchange.” *Science (New York, N.Y.)* 109 (2833): 377–78. https://doi.org/10.1126/science.109.2833.377.

Coray, Dorien S., Nicole E. Wheeler, Jack A. Heinemann, and Paul P. Gardner. 2017. “Why so Narrow: Distribution of Anti-Sense Regulated, Type I Toxin-Antitoxin Systems Compared with Type II and Type III Systems.” *RNA Biology*. Taylor and Francis Inc. https://doi.org/10.1080/15476286.2016.1272747.

Costanzo, Alessandra, and Sarah E. Ades. 2006. “Growth Phase-Dependent Regulation of the Extracytoplasmic Stress Factor, ΣE, by Guanosine 3′,5′-Bispyrophosphate (PpGpp).” *Journal of Bacteriology* 188 (13): 4627–34. https://doi.org/10.1128/JB.01981-05.

Cserjan-Puschmann, M., W. Kramer, E. Duerrschmid, G. Striedner, and K. Bayer. 1999. “Metabolic Approaches for the Optimisation of Recombinant Fermentation Processes.” *Applied Microbiology and Biotechnology* 53 (1): 43–50. https://doi.org/10.1007/s002530051612.

Dahl, John L., Carl N. Kraus, Helena I.M. Boshoff, Bernard Doan, Korrie Foley, David Avarbock, Gilla Kaplan, Valerie Mizrahi, Harvey Rubin, and Clifton E. Barry. 2003. “The Role of RelMtb-Mediated Adaptation to Stationary Phase in Long-Term Persistence of Mycobacterium Tuberculosis in Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (17): 10026–31. https://doi.org/10.1073/pnas.1631248100.

Das, Bhabatosh, Ritesh Ranjan Pal, Satyabrata Bag, and Rupak K. Bhadra. 2009. “Stringent Response in Vibrio Cholerae: Genetic Analysis of SpoT Gene Function and Identification of a Novel (p)PpGpp Synthetase Gene.” *Molecular Microbiology* 72 (2): 380–98. https://doi.org/10.1111/j.1365-2958.2009.06653.x.

Dedrick, Rebekah M., Deborah Jacobs-Sera, Carlos A. Guerrero Bustamante, Rebecca A. Garlena, Travis N. Mavrich, Welkin H. Pope, Juan C. Cervantes Reyes, et al. 2017. “Prophage-Mediated Defence against Viral Attack and Viral Counter-

Defence." *Nature Microbiology* 2 (3): 16251. https://doi.org/10.1038/nmicrobiol.2016.251.

Ding, Chien Kuang Cornelia, Joshua Rose, Tianai Sun, Jianli Wu, Po Han Chen, Chao Chieh Lin, Wen Hsuan Yang, et al. 2020. "MESH1 Is a Cytosolic NADPH Phosphatase That Regulates Ferroptosis." *Nature Metabolism* 2 (3): 270–77. https://doi.org/10.1038/s42255-020-0181-1.

Dixon, Scott J., Kathryn M. Lemberg, Michael R. Lamprecht, Rachid Skouta, Eleina M. Zaitsev, Caroline E. Gleason, Darpan N. Patel, et al. 2012. "Ferroptosis: An Iron-Dependent Form of Nonapoptotic Cell Death." *Cell* 149 (5): 1060–72. https://doi.org/10.1016/j.cell.2012.03.042.

Dominguez, Hélène, Catherine Rollin, Armel Guyonvarch, Jean Luc Guerquin-Kern, Muriel Cocaign-Bousquet, and Nicholas D. Lindley. 1998. "Carbon-Flux Distribution in the Central Metabolic Pathways of Corynebacterium Glutamicum during Growth on Fructose." *European Journal of Biochemistry* 254 (1): 96–102. https://doi.org/10.1046/j.1432-1327.1998.2540096.x.

English, Brian P, Vasili Hauryliuk, Arash Sanamrad, Stoyan Tankov, Nynke H Dekker, and Johan Elf. 2011. "Single-Molecule Investigations of the Stringent Response Machinery in Living Bacterial Cells." *Proceedings of the National Academy of Sciences of the United States of America* 108 (31): E365-73. https://doi.org/10.1073/pnas.1102255108.

Fiil, Niels P., Berthe M. Willumsen, J. D. Friesen, and Kaspar von Meyenburg. 1977. "Interaction of Alleles of the RelA, RelC and SpoT Genes in Escherichia Coli: Analysis of the Interconversion of GTP, PpGpp and PppGpp." *MGG Molecular & General Genetics* 150 (1): 87–101. https://doi.org/10.1007/BF02425329.

Flärdh, K, T Axberg, N H Albertson, and S Kjelleberg. 1994. "Stringent Control during Carbon Starvation of Marine Vibrio Sp. Strain S14: Molecular Cloning, Nucleotide Sequence, and Deletion of the RelA Gene." *Journal of Bacteriology* 176 (19): 5949–57. https://doi.org/10.1128/jb.176.19.5949-5957.1994.

Fozo, Elizabeth M., Kira S. Makarova, Svetlana A. Shabalina, Natalya Yutin, Eugene V. Koonin, and Gisela Storz. 2010. "Abundance of Type I Toxin-Antitoxin Systems in Bacteria: Searches for New Candidates and Discovery of Novel Families." *Nucleic Acids Research* 38 (11): 3743–59. https://doi.org/10.1093/nar/gkq054.

Francis, Sharron H., and Jackie D. Corbin. 1999. "Cyclic Nucleotide-Dependent Protein Kinases: Intracellular Receptors for CAMP and CGMP Action." *Critical Reviews in Clinical Laboratory Sciences*. https://doi.org/10.1080/10408369991239213.

Gaca, Anthony O., Pavel Kudrin, Cristina Colomer-Winter, Jelena Beljantseva, Kuanqing Liu, Brent Anderson, Jue D. Wang, et al. 2015. "From (p)PpGpp to (Pp)PGpp: Characterization of Regulatory Effects of PGpp Synthesized by the Small Alarmone Synthetase of Enterococcus Faecalis." *Journal of Bacteriology* 197 (18): 2908–19. https://doi.org/10.1128/JB.00324-15.

Gallant, J., G. Margason, and B. Finch. 1972. "On the Turnover of PpGpp in Escherichia Coli." *Journal of Biological Chemistry* 247 (19): 6055–58.

Gallant, J, J Irr, and M Cashel. 1971. "The Mechanism of Amino Acid Control of Guanylate and Adenylate Biosynthesis." *The Journal of Biological Chemistry* 246 (18): 5812–16. http://www.ncbi.nlm.nih.gov/pubmed/4938039.

Gallant, Jonathan, Linda Palmer, and Chia Chu Pao. 1977. "Anomalous Synthesis of PpGpp in Growing Cells." *Cell* 11 (1): 181–85. https://doi.org/10.1016/0092-8674(77)90329-4.

Geiger, Tobias, and Christiane Wolz. 2014. “Intersection of the Stringent Response and the CodY Regulon in Low GC Gram-Positive Bacteria.” *International Journal of Medical Microbiology : IJMM*, December. https://doi.org/10.1016/j.ijmm.2013.11.013.

Gentry, D. R., and M. Cashel. 1995. “Cellular Localization of the Escherichia Coli SpoT Protein.” *Journal of Bacteriology* 177 (13): 3890–93. https://doi.org/10.1128/jb.177.13.3890-3893.1995.

Gerdes, K., P. B. Rasmussen, and S. Molin. 1986. “Unique Type of Plasmid Maintenance Function: Postsegregational Killing of Plasmid-Free Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 83 (10): 3116–20. https://doi.org/10.1073/pnas.83.10.3116.

Gerdes, Kenn, Susanne K. Christensen, and Anders Løbner-Olesen. 2005. “Prokaryotic Toxin-Antitoxin Stress Response Loci.” *Nature Reviews Microbiology*. https://doi.org/10.1038/nrmicro1147.

Germain, Elsa, Daniel Castro-Roa, Nikolay Zenkin, and Kenn Gerdes. 2013. “Molecular Mechanism of Bacterial Persistence by HipA.” *Molecular Cell* 52 (2): 248–54. https://doi.org/10.1016/j.molcel.2013.08.045.

Goeders, Nathalie, Ray Chai, Bihe Chen, Andrew Day, and George Salmond. 2016. “Structure, Evolution, and Functions of Bacterial Type III Toxin-Antitoxin Systems.” *Toxins* 8 (10): 282. https://doi.org/10.3390/toxins8100282.

Golden, N. G., and G. L. Powell. 1972. “Stringent and Relaxed Control of Phospholipid Metabolism in Escherichia Coli.” *Journal of Biological Chemistry* 247 (20): 6651–58.

Goormaghtigh, Frédéric, Nathan Fraikin, Marta Putrinš, Thibaut Hallaert, Vasili Hauryliuk, Abel Garcia-Pino, Andreas Sjödin, et al. 2018. “Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of Escherichia Coli Type II Persister Cells.” *MBio* 9 (3). https://doi.org/10.1128/mBio.00640-18.

Gourse, Richard L., Albert Y. Chen, Saumya Gopalkrishnan, Patricia Sanchez-Vazquez, Angela Myers, and Wilma Ross. 2018. “Transcriptional Responses to PpGpp and DksA.” *Annual Review of Microbiology* 72 (1): 163–84. https://doi.org/10.1146/annurev-micro-090817-062444.

Groisman, E A, J Kayser, and F C Soncini. 1997. “Regulation of Polymyxin Resistance and Adaptation to Low-Mg2+ Environments.” *Journal of Bacteriology* 179 (22): 7040–45. https://doi.org/10.1128/jb.179.22.7040-7045.1997.

Gu, Mianzhi, and James A. Imlay. 2011. “The SoxRS Response of Escherichia Coli Is Directly Activated by Redox-Cycling Drugs Rather than by Superoxide.” *Molecular Microbiology* 79 (5): 1136–50. https://doi.org/10.1111/j.1365-2958.2010.07520.x.

Gunther, G. S, and B Sydney. 1961. “A Genetic Locus for the Regulation of Ribonucleic Acid Synthesis.” *Proc Natl Acad Sci U S A* 47 (12): 2005–14. https://doi.org/STENT GS, BRENNER S. A genetic locus for the regulation of ribonucleic acid synthesis. Proc Natl Acad Sci U S A. 1961;47(12):2005–2014. doi:10.1073/pnas.47.12.2005.

Harms, Alexander, Ditlev Egeskov Brodersen, Namiko Mitarai, and Kenn Gerdes. 2018. “Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology.” *Molecular Cell*. Cell Press. https://doi.org/10.1016/j.molcel.2018.01.003.

Harms, Alexander, Etienne Maisonneuve, and Kenn Gerdes. 2016. “Mechanisms of Bacterial Persistence during Stress and Antibiotic Exposure.” *Science* 354 (6318). https://doi.org/10.1126/science.aaf4268.

Harshman, R B, and H Yamazaki. 1971. “Formation of PpGpp in a Relaxed and Stringent Strain of Escherichia Coli during Diauxie Lag.” *Biochemistry* 10 (21): 3980–82. https://doi.org/10.1021/bi00797a027.

Haseltine, W. A., and R. Block. 1973. “Synthesis of Guanosine Tetra and Pentaphosphate Requires the Presence of a Codon Specific, Uncharged Transfer Ribonucleic Acid in the Acceptor Site of Ribosomes.” *Proceedings of the National Academy of Sciences of the United States of America* 70 (5): 1564–68. https://doi.org/10.1073/pnas.70.5.1564.

Hauryliuk, Vasili, Gemma C. Atkinson, Katsuhiko S. Murakami, Tanel Tenson, and Kenn Gerdes. 2015. “Recent Functional Insights into the Role of (p)PpGpp in Bacterial Physiology.” *Nature Reviews Microbiology* 13 (5): 298–309. https://doi.org/10.1038/nrmicro3448.

Hengge-Aronis, Regine. 2002. “Signal Transduction and Regulatory Mechanisms Involved in Control of the Sigma(S) (RpoS) Subunit of RNA Polymerase.” *Microbiology and Molecular Biology Reviews : MMBR* 66 (3): 373–95, table of contents. https://doi.org/10.1128/mmbr.66.3.373-395.2002.

Hochstadt-Ozer, J. 1972. “The Regulation of Purine Utilization in Bacteria. IV. Roles of Membrane-Localized and Pericytoplasmic Enzymes in the Mechanism of Purine Nucleoside Transport across Isolated Escherichia Coli Membranes.” *The Journal of Biological Chemistry* 247 (8): 2419–26. http://www.ncbi.nlm.nih.gov/pubmed/4336374.

Holčapek, Michal, K. Volná, P. Jandera, L. Kolářová, K. Lemr, M. Exner, and A. Církva. 2004. “Effects of Ion-Pairing Reagents on the Electrospray Signal Suppression of Sulphonated Dyes and Intermediates.” *Journal of Mass Spectrometry* 39 (1): 43–50. https://doi.org/10.1002/jms.551.

Holms, W. H., I. D. Hamilton, and A. G. Robertson. 1972. “The Rate of Turnover of the Adenosine Triphosphate Pool of Escherichia Coli Growing Aerobically in Simple Defined Media.” *Archiv Für Mikrobiologie* 83 (2): 95–109. https://doi.org/10.1007/BF00425016.

Hölttä, E, J Jänne, and J Pispa. 1974. “The Regulation of Polyamine Synthesis during the Stringent Control in Escherichia Coli.” *Biochemical and Biophysical Research Communications* 59 (3): 1104–11. https://doi.org/10.1016/s0006-291x(74)80092-6.

Huang, Dan, Yazhuo Zhang, and Xiaoguang Chen. 2003. “Analysis of Intracellular Nucleoside Triphosphate Levels in Normal and Tumor Cell Lines by High-Performance Liquid Chromatography.” *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 784 (1): 101–9. https://doi.org/10.1016/S1570-0232(02)00780-8.

Inaoka, Takashi, and Kozo Ochi. 2002. “RelA Protein Is Involved in Induction of Genetic Competence in Certain Bacillus Subtilis Strains by Moderating the Level of Intracellular GTP.” *Journal of Bacteriology* 184 (14): 3923–30. https://doi.org/10.1128/JB.184.14.3923-3930.2002.

Inaoka, Takashi, Kosaku Takahashi, Mayumi Ohnishi-Kameyama, Mitsuru Yoshida, and Kozo Ochi. 2003. “Guanine Nucleotides Guanosine 5′-Diphosphate 3′-Diphosphate and GTP Co-Operatively Regulate the Production of an Antibiotic Bacilysin in Bacillus Subtilis.” *Journal of Biological Chemistry* 278 (4): 2169–76. https://doi.org/10.1074/jbc.M208722200.

Irr, J, and J Gallant. 1969. “The Control of Ribonucleic Acid Synthesis in Escherichia Coli. II. Stringent Control of Energy Metabolism.” *The Journal of Biological Chemistry* 244 (8): 2233–39. http://www.ncbi.nlm.nih.gov/pubmed/4889467.

Jenvert, Rose Marie Knutsson, and Lovisa Holmberg Schiavone. 2005. “Characterization of the TRNA and Ribosome-Dependent PppGpp-Synthesis by Recombinant Stringent Factor from Escherichia Coli.” *FEBS Journal* 272 (3): 685–95. https://doi.org/10.1111/j.1742-4658.2004.04502.x.

Jiang, Mengxi, Susan M. Sullivan, Patrice K. Wout, and Janine R. Maddock. 2007. “G-Protein Control of the Ribosome-Associated Stress Response Protein SpoT.” *Journal of Bacteriology* 189 (17): 6140–47. https://doi.org/10.1128/JB.00315-07.

Jimmy, Steffi, Chayan Kumar Saha, Tatsuaki Kurata, Constantine Stavropoulos, Sofia Raquel Alves Oliveira, Alan Koh, Albinas Cepauskas, et al. 2020. “A Widespread Toxin−antitoxin System Exploiting Growth Control *via* Alarmone Signaling.” *Proceedings of the National Academy of Sciences*, 201916617. https://doi.org/10.1073/pnas.1916617117.

Jimmy, Steffi, Chayan Kumar Saha, Constantine Stavropoulos, Sofia Raquel Alves Oliveira, Tatsuaki Kurata, Alan Koh, Albinas Cepauskas, et al. 2020. “A Widespread Toxin-Antitoxin System Exploiting Growth Control via Alarmone Signalling.” *BioRxiv*, April, 575399. https://doi.org/10.1101/575399.

Jishage, Miki, Kristian Kvint, Victoria Shingler, and Thomas Nyström. 2002. “Regulation of σ Factor Competition by the Alarmone PpGpp.” *Genes and Development* 16 (10): 1260–70. https://doi.org/10.1101/gad.227902.

Jones, Christopher J., Andrew Utada, Kimberly R. Davis, Wiriya Thongsomboon, David Zamorano Sanchez, Vinita Banakar, Lynette Cegelski, Gerard C. L. Wong, and Fitnat H. Yildiz. 2015. “C-Di-GMP Regulates Motile to Sessile Transition by Modulating MshA Pili Biogenesis and Near-Surface Motility Behavior in Vibrio Cholerae.” Edited by Matthew R. Parsek. *PLOS Pathogens* 11 (10): e1005068. https://doi.org/10.1371/journal.ppat.1005068.

Jöres, Lars, and Rolf Wagner. 2003. “Essential Steps in the PpGpp-Dependent Regulation of Bacterial Ribosomal RNA Promoters Can Be Explained by Substrate Competition.” *Journal of Biological Chemistry* 278 (19): 16834–43. https://doi.org/10.1074/jbc.M300196200.

Kaldalu, Niilo, Vasili Hauryliuk, and Tanel Tenson. 2016. “Persisters—as Elusive as Ever.” *Applied Microbiology and Biotechnology*. Springer Verlag. https://doi.org/10.1007/s00253-016-7648-8.

Kanjee, Usheer, Koji Ogata, and Walid a Houry. 2012. “Direct Binding Targets of the Stringent Response Alarmone (p)PpGpp.” *Molecular Microbiology* 85 (6): 1029–43. https://doi.org/10.1111/j.1365-2958.2012.08177.x.

Kaplan, S., A. G. Atherly, and A. Barrett. 1973. “Synthesis of Stable RNA in Stringent Escherichia Coli Cells in the Absence of Charged Transfer RNA.” *Proceedings of the National Academy of Sciences of the United States of America* 70 (3): 689–92. https://doi.org/10.1073/pnas.70.3.689.

Kaspy, Ilana, Eitan Rotem, Noga Weiss, Irine Ronin, Nathalie Q. Balaban, and Gad Glaser. 2013. “HipA-Mediated Antibiotic Persistence via Phosphorylation of the Glutamyl-TRNA-Synthetase.” *Nature Communications* 4 (December): 3001. https://doi.org/10.1038/ncomms4001.

Kazmierczak, Krystyna M., Kyle J. Wayne, Andreas Rechtsteiner, and Malcolm E. Winkler. 2009. “Roles of RelSpn in Stringent Response, Global Regulation and Virulence of Serotype 2 Streptococcus Pneumoniae D39.” *Molecular Microbiology* 72 (3): 590–611. https://doi.org/10.1111/j.1365-2958.2009.06669.x.

Keasling, J D, L Bertsch, and A Kornberg. 1993. “Guanosine Pentaphosphate Phosphohydrolase of Escherichia Coli Is a Long-Chain Exopolyphosphatase.”

Proceedings of the National Academy of Sciences of the United States of America 90 (15): 7029–33. https://doi.org/10.1073/PNAS.90.15.7029.

Keren, I., D. Shah, A. Spoering, N. Kaldalu, and K. Lewis. 2004. "Specialized Persister Cells and the Mechanism of Multidrug Tolerance in Escherichia Coli." *Journal of Bacteriology* 186 (24). https://doi.org/10.1128/JB.186.24.8172-8180.2004.

Komoda, Taeko, Neuza S. Sato, Steven S. Phelps, Naoki Namba, Simpson Joseph, and Tsutomu Suzuki. 2006. "The A-Site Finger in 23 S RRNA Acts as a Functional Attenuator for Translocation." *Journal of Biological Chemistry* 281 (43): 32303–9. https://doi.org/10.1074/jbc.M607058200.

Krásný, Libor, and Richard L Gourse. 2004. "An Alternative Strategy for Bacterial Ribosome Synthesis: Bacillus Subtilis RRNA Transcription Regulation." *The EMBO Journal* 23 (22): 4473–83. https://doi.org/10.1038/sj.emboj.7600423.

Kriel, Allison, Alycia N Bittner, Sok Ho Kim, Kuanqing Liu, Ashley K Tehranchi, Winnie Y Zou, Samantha Rendon, Rui Chen, Benjamin P Tu, and Jue D Wang. 2012. "Direct Regulation of GTP Homeostasis by (p)PpGpp: A Critical Component of Viability and Stress Resistance." *Molecular Cell* 48 (2): 231–41. https://doi.org/ 10.1016/j.molcel.2012.08.009.

Kudrin, Pavel, Vallo Varik, Sofia Raquel Alves Oliveira, Jelena Beljantseva, Teresa Del Peso Santos, Ievgen Dzhygyr, Dominik Rejman, Felipe Cava, Tanel Tenson, and Vasili Hauryliuk. 2017. "Subinhibitory Concentrations of Bacteriostatic Antibiotics Induce RelA-Dependent and RelA-Independent Tolerance to β-Lactams." *Antimicrobial Agents and Chemotherapy* 61 (4). https://doi.org/10.1128/ AAC.02173-16.

Kuroda, A., K Nomura, R Ohtomo, J Kato, T Ikeda, N Takiguchi, H Ohtake, and A Kornberg. 2001. "Role of Inorganic Polyphosphate in Promoting Ribosomal Protein Degradation by the Lon Protease in E. Coli." *Science* 293 (5530): 705–8. https://doi.org/10.1126/science.1061315.

la Fuente-Núñez, César de, Fany Reffuveille, Evan F Haney, Suzana K Straus, and Robert E W Hancock. 2014. "Broad-Spectrum Anti-Biofilm Peptide That Targets a Cellular Stress Response." *PLoS Pathogens* 10 (5): e1004152. https://doi.org/ 10.1371/journal.ppat.1004152.

Laffler, Thomas, and Jonathan Gallant. 1974a. "SpoT, a New Genetic Locus Involved in the Stringent Response in E. Coli." *Cell* 1 (1): 27–30. https://doi.org/ 10.1016/ 0092-8674(74)90151-2.

———. 1974b. "SpoT, a New Genetic Locus Involved in the Stringent Response in E. Coli." *Cell* 1 (1): 27–30. https://doi.org/10.1016/0092-8674(74)90151-2.

Lagosky, Peter A., and F. N. Chang. 1978. "The Extraction of Guanosine 5′-Diphosphate, 3′-Diphosphate (PpGpp) from Escherichia Coli Using Low PH Reagents: A Reevaluation." *Biochemical and Biophysical Research Communi cations* 84 (4): 1016–24. https://doi.org/10.1016/0006-291X(78)91685-6.

Lemos, José A., Vanessa K. Lin, Marcelle M. Nascimento, Jacqueline Abranches, and Robert A. Burne. 2007. "Three Gene Products Govern (p)PpGpp Production by Streptococcus Mutans." *Molecular Microbiology* 65 (6): 1568–81. https://doi.org/ 10.1111/j.1365-2958.2007.05897.x.

Leplae, Raphaël, Damien Geeraerts, Régis Hallez, Julien Guglielmini, Pierre Drze, and Laurence Van Melderen. 2011. "Diversity of Bacterial Type II Toxin-Antitoxin Systems: A Comprehensive Search and Functional Analysis of Novel Families." *Nucleic Acids Research* 39 (13): 5513–25. https://doi.org/10.1093/nar/gkr131.

Leps, W T, and J C Ensign. 1979. “Adenylate Nucleotide Levels and Energy Charge in Arthrobacter Crystallopoietes during Growth and Starvation.” *Archives of Micro biology* 122 (1): 69–76. https://doi.org/10.1007/BF00408048.

Li, Kewei, Guangjian Yang, Alexander B. Debru, Pingping Li, Li Zong, Peizhen Li, Teng Xu, Weihui Wu, Shouguang Jin, and Qiyu Bao. 2017. “SuhB Regulates the Motile-Sessile Switch in Pseudomonas Aeruginosa through the Gac/Rsm Pathway and c-Di-GMP Signaling.” *Frontiers in Microbiology* 8 (JUN). https://doi.org/10.3389/fmicb.2017.01045.

Liu, Kuanqing, Alycia N. Bittner, and Jue D. Wang. 2015. “Diversity in (p)PpGpp Metabolism and Effectors.” *Current Opinion in Microbiology*. Elsevier Ltd. https://doi.org/10.1016/j.mib.2015.01.012.

Liu, Kuanqing, Angela R. Myers, Tippapha Pisithkul, Kathy R. Claas, Kenneth A. Satyshur, Daniel Amador-Noguez, James L. Keck, and Jue D. Wang. 2015. “Molecular Mechanism and Evolution of Guanylate Kinase Regulation by (p)PpGpp.” *Molecular Cell* 57 (4): 735–49. https://doi.org/10.1016/J.MOLCEL. 2014.12.037.

Lopez, J. M., A. Dromerick, and E. Freese. 1981. “Response of Guanosine 5’-Triphosphate Concentration to Nutritional Changes and Its Significance for Bacillus Subtilis Sporulation.” *Journal of Bacteriology* 146 (2): 605–13. https://doi.org/10.1128/jb.146.2.605-613.1981.

Luidalepp, Hannes, Arvi Jõers, Niilo Kaldalu, and Tanel Tenson. 2011. “Age of Inoculum Strongly Influences Persister Frequency and Can Mask Effects of Mutations Implicated in Altered Persistence.” *Journal of Bacteriology* 193 (14): 3598–3605. https://doi.org/10.1128/JB.00085-11.

Lund, Elsebet, and Niels Ole Kjeldgaard. 1972. “Metabolism of Guanosine Tetraphosphate in Escherichia Coli.” *European Journal of Biochemistry* 28 (3): 316–26. https://doi.org/10.1111/j.1432-1033.1972.tb01916.x.

Lundin, Arne, and Anders Thore. 1975. “Comparison of Methods for Extraction of Bacterial Adenine Nucleotides Determined by Firefly Assay.” *Applied Microbiology* 30 (5): 713–21. https://doi.org/10.1128/aem.30.5.713-721.1975.

Lundquist, Ronald, and Baldomero Olivera. 1971. “Pyridine Nucleotide Metabolism in Escherichia Coli.” *The Journal of Biological Chemistry* 246 (4): 1107–16. https://www.jbc.org/content/248/14/5137.

Luo, Yun, and John D. Helmann. 2012. “Analysis of the Role of Bacillus Subtilis σ M in β-Lactam Resistance Reveals an Essential Role for c-Di-AMP in Peptidoglycan Homeostasis.” *Molecular Microbiology* 83 (3): 623–39. https://doi.org/10.1111/j.1365-2958.2011.07953.x.

Magnusson, Lisa U, Anne Farewell, and Thomas Nyström. 2005. “PpGpp: A Global Regulator in Escherichia Coli.” *Trends in Microbiology* 13 (5): 236–42. https://doi.org/10.1016/j.tim.2005.03.008.

Maisonneuve, Etienne, Manuela Castro-Camargo, and Kenn Gerdes. 2013. “(P)PpGpp Controls Bacterial Persistence by Stochastic Induction of Toxin-Antitoxin Activity.” *Cell* 154 (5): 1140–50. https://doi.org/10.1016/j.cell.2013.07.048.

Maitra, Amarnath, Irina Shulgina, and V. James Hernandez. 2005. “Conversion of Active Promoter-RNA Polymerase Complexes into Inactive Promoter Bound Complexes in E. Coli by the Transcription Effector, PpGpp.” *Molecular Cell* 17 (6): 817–29. https://doi.org/10.1016/j.molcel.2005.02.026.

Makman, R. S., and E. W. Sutherland. 1965. “Adenosine 3’,5’-Phosphate in Escherichia Coli.” *The Journal of Biological Chemistry* 240: 1309–14.

Markham, R., and J. D. Smith. 1952. “The Structure of Ribonucleic Acid. I. Cyclic Nucleotides Produced by Ribonuclease and by Alkaline Hydrolysis.” *The Biochemical Journal* 52 (4): 552–57. https://doi.org/10.1042/bj0520552.

Mechold, Undine, Michael Cashel, Kerstin Steiner, Daniel Gentry, and Horst Malke. 1996. “Functional Analysis of a RelA/SpoT Gene Homolog from Streptococcus Equisimilis.” *Journal of Bacteriology* 178 (5): 1401–11. https://doi.org/10.1128/jb.178.5.1401-1411.1996.

Mechold, Undine, Katarzyna Potrykus, Helen Murphy, Katsuhiko S Murakami, and Michael Cashel. 2013. “Differential Regulation by PpGpp versus PppGpp in Escherichia Coli.” *Nucleic Acids Research* 41 (12): 6175–89. https://doi.org/10.1093/nar/gkt302.

Merlie, J. P., and L. I. Pizer. 1973. “Regulation of Phospholipid Synthesis in Escherichia Coli by Guanosine Tetraphosphate.” *Journal of Bacteriology* 116 (1): 355–66.

Meyer, Hanna, Manuel Liebeke, and Michael Lalk. 2010. “A Protocol for the Investigation of the Intracellular Staphylococcus Aureus Metabolome.” *Analytical Biochemistry* 401 (2): 250–59. https://doi.org/10.1016/j.ab.2010.03.003.

Mitkevich, Vladimir A., Andrey Ermakov, Alexandra A. Kulikova, Stoyan Tankov, Viktoriya Shyp, Aksel Soosaar, Tanel Tenson, Alexander A. Makarov, Mans Ehrenberg, and Vasili Hauryliuk. 2010. “Thermodynamic Characterization of PpGpp Binding to EF-G or IF2 and of Initiator TRNA Binding to Free IF2 in the Presence of GDP, GTP, or PpGpp.” *Journal of Molecular Biology* 402 (5): 838–46. https://doi.org/10.1016/j.jmb.2010.08.016.

Mittenhuber, G. 2001. “Comparative Genomics and Evolution of Genes Encoding Bacterial (p)PpGpp Synthetases/Hydrolases (the Rel, RelA and SpoT Proteins).” *Journal of Molecular Microbiology and Biotechnology* 3 (4): 585–600.

Mobley, Harry L.T., Rachel R. Spurbeck, and Rebecca J. Tarrien. 2012. “Enzymatically Active and Inactive Phosphodiesterases and Diguanylate Cyclases Are Involved in Regulation of Motility or Sessility in Escherichia Coli CFT073.” *MBio* 3 (5). https://doi.org/10.1128/mBio.00307-12.

Modolell, J., B. Cabrer, A. Parmeggiani, and D. Vazquez. 1971. “Inhibition by Siomycin and Thiostrepton of Both Aminoacyl-TRNA and Factor G Binding to Ribosomes.” *Proceedings of the National Academy of Sciences of the United States of America* 68 (8): 1796–1800. https://doi.org/10.1073/pnas.68.8.1796.

Mouery, Kyle, Bethany A. Rader, Erin C. Gaynor, and Karen Guillemin. 2006. “The Stringent Response Is Required for Helicobacter Pylori Survival of Stationary Phase, Exposure to Acid, and Aerobic Shock.” *Journal of Bacteriology* 188 (15): 5494–5500. https://doi.org/10.1128/JB.00366-06.

Müller, Claudia M., Laura Conejero, Natasha Spink, Matthew E. Wand, Gregory J. Bancroft, and Richard W. Titball. 2012. “Role of RelA and SpoT in Burkholderia Pseudomallei Virulence and Immunity.” *Infection and Immunity* 80 (9): 3247–55. https://doi.org/10.1128/IAI.00178-12.

Murray, K. Daniel, and Hans Bremer. 1996. “Control of SpoT-Dependent PpGpp Synthesis and Degradation in Escherichia Coli.” *Journal of Molecular Biology* 259 (1): 41–57. https://doi.org/10.1006/jmbi.1996.0300.

Nanamiya, Hideaki, Koji Kasai, Akira Nozawa, Choong Soo Yun, Takakuni Narisawa, Kana Murakami, Yousuke Natori, Fujio Kawamura, and Yuzuru Tozawa. 2008. “Identification and Functional Analysis of Novel (p)PpGpp Synthetase Genes in Bacillus Subtilis.” *Molecular Microbiology* 67 (2): 291–304. https://doi.org/10.1111/j.1365-2958.2007.06018.x.

Neidhardt, F. C. 1966. “Roles of Amino Acid Activating Enzymes in Cellular Physiology.” *Bacteriological Reviews* 30 (4): 701–19.

Neidhardt, Frederick C. 1964. “The Regulation of RNA Synthesis in Bacteria.” *Progress in Nucleic Acid Research and Molecular Biology* 3 (C): 145–81. https://doi.org/10.1016/S0079-6603(08)60741-2.

Ochi, K, J C Kandala, and E Freese. 1981. “Initiation of Bacillus Subtilis Sporulation by the Stringent Response to Partial Amino Acid Deprivation.” *The Journal of Biological Chemistry* 256 (13): 6866–75. http://www.ncbi.nlm.nih.gov/pubmed/6113248.

Ogura, T., and S. Hiraga. 1983. “Mini-F Plasmid Genes That Couple Host Cell Division to Plasmid Proliferation.” *Proceedings of the National Academy of Sciences of the United States of America* 80 (15): 4784–88. https://doi.org/10.1073/pnas.80.15.4784.

Oki, Toshikazu, Akihiro Yoshimoto, Seiji Sato, and Akira Takamatsu. 1975. “Purine Nucleotide Pyrophosphotransferase from Streptomyces Morookaensis, Capable of Synthesizing PppApp and PppGpp.” *BBA – Enzymology* 410 (2): 262–72. https://doi.org/10.1016/0005-2744(75)90228-4.

Page, Rebecca, and Wolfgang Peti. 2016. “Toxin-Antitoxin Systems in Bacterial Growth Arrest and Persistence.” *Nature Chemical Biology* 12 (4): 208–14. https://doi.org/10.1038/nchembio.2044.

Pandey, Deo Prakash, and Kenn Gerdes. 2005. “Toxin-Antitoxin Loci Are Highly Abundant in Free-Living but Lost from Host-Associated Prokaryotes.” *Nucleic Acids Research* 33 (3): 966–76. https://doi.org/10.1093/nar/gki201.

Parker, Jack, Robert J. Watson, James D. Friesen, and Niels P. Fiil. 1976. “A Relaxed Mutant with an Altered Ribosomal Protein L11.” *MGG Molecular & General Genetics* 144 (1): 111–14. https://doi.org/10.1007/BF00277313.

Patacq, Clément, Nicolas Chaudet, and Fabien Létisse. 2018. “Absolute Quantification of PpGpp and PppGpp by Double-Spike Isotope Dilution Ion Chromatography-High-Resolution Mass Spectrometry.” *Analytical Chemistry* 90 (18): 10715–23. https://doi.org/10.1021/acs.analchem.8b00829.

Paul, Brian J, Melanie M Barker, Wilma Ross, David A Schneider, Cathy Webb, John W Foster, and Richard L Gourse. 2004. “DksA: A Critical Component of the Transcription Initiation Machinery That Potentiates the Regulation of RRNA Promoters by PpGpp and the Initiating NTP.” *Cell* 118 (3): 311–22. https://doi.org/10.1016/j.cell.2004.07.009.

Paul, Brian J, Melanie B Berkmen, and Richard L Gourse. 2005. “DksA Potentiates Direct Activation of Amino Acid Promoters by PpGpp.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (22): 7823–28. https://doi.org/10.1073/pnas.0501170102.

Payne, Shelley M., and Bruce N. Ames. 1982. “A Procedure for Rapid Extraction and High-Pressure Liquid Chromatographic Separation of the Nucleotides and Other Small Molecules from Bacterial Cells.” *Analytical Biochemistry* 123 (1): 151–61. https://doi.org/10.1016/0003-2697(82)90636-4.

Persky, Nicole S., Daniel J. Ferullo, Deani L. Cooper, Hayley R. Moore, and Susan T. Lovett. 2009. “The ObgE/CgtA GTPase Influences the Stringent Response to Amino Acid Starvation in Escherichia Coli.” *Molecular Microbiology* 73 (2): 253–66. https://doi.org/10.1111/j.1365-2958.2009.06767.x.

Pesavento, Christina, and Regine Hengge. 2009. “Bacterial Nucleotide-Based Second Messengers.” *Current Opinion in Microbiology*. Curr Opin Microbiol. https://doi.org/10.1016/j.mib.2009.01.007.

Poole, Keith. 2012. “Bacterial Stress Responses as Determinants of Antimicrobial Resistance.” *The Journal of Antimicrobial Chemotherapy* 67 (9): 2069–89. https://doi.org/10.1093/jac/dks196.

Potrykus, Katarzyna, and Michael Cashel. 2008. “(P)PpGpp: Still Magical?” *Annual Review of Microbiology* 62 (January): 35–51. https://doi.org/10.1146/annurev.micro.62.081307.162903.

Qin, Xuan, and Xin Wang. 2018. “Quantification of Nucleotides and Their Sugar Conjugates in Biological Samples: Purposes, Instruments and Applications.” *Journal of Pharmaceutical and Biomedical Analysis*. Elsevier B.V. https://doi.org/10.1016/j.jpba.2018.06.013.

Ramisetty, Bhaskar C M, Dimpy Ghosh, Maoumita Roy Chowdhury, Ramachandran S Santhosh, Maoumita Roy Chowdhury, and Ramachandran S Santhosh. 2016. “What Is the Link between Stringent Response, Endoribonuclease Encoding Type II Toxin-Antitoxin Systems and Persistence?” *FRONTIERS IN MICROBIOLOGY* 7: 1882. https://doi.org/10.3389/fmicb.2016.01882.

Raué, H A, and M Cashel. 1975. “Regulation of RNA Synthesis in Escherichia Coli. III. Degradation of Guanosine 5’-Diphosphate 3’-Diphosphate in Cold-Shocked Cells.” *Biochimica et Biophysica Acta* 383 (3): 290–304. http://www.ncbi.nlm.nih.gov/pubmed/1090305.

Rhaese, H. J., and R. Groscurth. 1979. “Apparent Dependence of Sporulation on Synthesis of Highly Phosphorylated Nucleotides in Bacillus Subtilis.” *Proceedings of the National Academy of Sciences of the United States of America* 76 (2): 842–46. https://doi.org/10.1073/pnas.76.2.842.

Rhaese, Hans-Jurgen., Reinhardt Grade, and Herbert Dichtelmuller. 1976. “Studies on the Control of Development. Correlation of Initiation of Differentiation with Synthesis of Highly Phosphorylated Nucleotides in Bacillus Subtilis.” *European Journal of Biochemistry* 64 (1): 205–13. https://doi.org/10.1111/j.1432-1033.1976.tb10289.x.

Rhizobium, Growth-promoting Endophyte. 2013. “Complete Genome Sequence of the Sesbania Symbiont and Rice.” *Nucleic Acids Research* 1 (1256879): 13–14. https://doi.org/10.1093/nar.

Rodionov, D. G., A. G. Pisabarro, M. A. De Pedro, W. Kusser, and E. E. Ishiguro. 1995. “β-Lactam-Induced Bacteriolysis of Amino Acid-Deprived Escherichia Coli Is Dependent on Phospholipid Synthesis.” *Journal of Bacteriology* 177 (4): 992–97. https://doi.org/10.1128/jb.177.4.992-997.1995.

Römling, Ute. 2008. “Great Times for Small Molecules: C-Di-AMP, a Second Messenger Candidate in Bacteria and Archaea.” *Science Signaling*. Sci Signal. https://doi.org/10.1126/scisignal.133pe39.

Römling, Ute, and Roger Simm. 2009. “Prevailing Concepts of C-Di-GMP Signaling.” *Contributions to Microbiology* 16: 161–81. https://doi.org/10.1159/000219379.

Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, et al. 1987. “Regulation of Cellulose Synthesis in Acetobacter Xylinum by Cyclic Diguanylic Acid.” *Nature* 325 (6101): 279–81. https://doi.org/10.1038/325279a0.

Ross, Wilma, Patricia Sanchez-Vazquez, Albert Y. Chen, Jeong Hyun Lee, Hector L. Burgos, and Richard L. Gourse. 2016. “PpGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response.” *Molecular Cell* 62 (6): 811–23. https://doi.org/10.1016/j.molcel.2016. 04.029.

Ross, Wilma, Catherine E. Vrentas, Patricia Sanchez-Vazquez, Tamas Gaal, and Richard L. Gourse. 2013. “The Magic Spot: A PpGpp Binding Site on E. Coli RNA Polymerase Responsible for Regulation of Transcription Initiation.” *Molecular Cell* 50 (3): 420–29. https://doi.org/10.1016/j.molcel.2013.03.021.

Sanchez-Vazquez, Patricia, Colin N. Dewey, Nicole Kitten, Wilma Ross, and Richard L. Gourse. 2019. “Genome-Wide Effects on Escherichia Coli Transcription from PpGpp Binding to Its Two Sites on RNA Polymerase.” *Proceedings of the National Academy of Sciences of the United States of America* 116 (17): 8310–19. https://doi.org/10.1073/pnas.1819682116.

Santarella-Mellwig, Rachel, Josef Franke, Andreas Jaedicke, Matyas Gorjanacz, Ulrike Bauer, Aidan Budd, Iain W. Mattaj, and Damien P. Devos. 2010. “The Compartmentalized Bacteria of the Planctomycetes-Verrucomicrobia-Chlamydiae Superphylum Have Membrane Coat-Like Proteins.” Edited by Sandra L. Schmid. *PLoS Biology* 8 (1): e1000281. https://doi.org/10.1371/journal.pbio.1000281.

Schaik, Willem van, Julie Prigent, and Agnès Fouet. 2007. “The Stringent Response of Bacillus Anthracis Contributes to Sporulation but Not to Virulence.” *Microbiology* 153 (12): 4234–39. https://doi.org/10.1099/mic.0.2007/010355-0.

Schumacher, M. A., K. M. Piro, W. Xu, S. Hansen, K. Lewis, and R. G. Brennan. 2009. “Molecular Mechanisms of HipA-Mediated Multidrug Tolerance and Its Neutralization by HipB.” *Science* 323 (5912): 396–401. https://doi.org/10.1126/science.1163806.

Scott, Janelle M., and W. G. Haldenwang. 1999. “Obg, an Essential GTP Binding Protein of Bacillus Subtilis, Is Necessary for Stress Activation of Transcription Factor σ(B).” *Journal of Bacteriology* 181 (15): 4653–60. https://doi.org/10.1128/jb.181.15.4653-4660.1999.

Seifar, Reza Maleki, Cor Ras, Amit T. Deshmukh, Katelijne M. Bekers, Camilo A. Suarez-Mendez, Ana L.B. da Cruz, Walter M. van Gulik, and Joseph J. Heijnen. 2013. “Quantitative Analysis of Intracellular Coenzymes in Saccharomyces Cerevisiae Using Ion Pair Reversed Phase Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry.” *Journal of Chromatography A* 1311 (October): 115–20. https://doi.org/10.1016/j.chroma.2013.08.076.

Shan, Yue, Autumn Brown Gandt, Sarah E. Rowe, Julia P. Deisinger, Brian P. Conlon, and Kim Lewis. 2017. “ATP-Dependent Persister Formation in *Escherichia Coli*.” Edited by Karen Bush. *MBio* 8 (1). https://doi.org/10.1128/mBio.02267-16.

Shrout, Joshua D., David L. Chopp, Collin L. Just, Morten Hentzer, Michael Givskov, and Matthew R. Parsek. 2006. “The Impact of Quorum Sensing and Swarming Motility on Pseudomonas Aeruginosa Biofilm Formation Is Nutritionally Conditional.” *Molecular Microbiology* 62 (5): 1264–77. https://doi.org/10.1111/ j.1365-2958.2006.05421.x.

Silva, Anisia J., and Jorge A. Benitez. 2006. “A Vibrio Cholerae Relaxed (RelA) Mutant Expresses Major Virulence Factors, Exhibits Biofilm Formation and Motility, and Colonizes the Suckling Mouse Intestine.” *Journal of Bacteriology* 188 (2): 794–800. https://doi.org/10.1128/JB.188.2.794-800.2006.

Sobala, Michał, Bożena Bruhn-Olszewska, Michael Cashel, and Katarzyna Potrykus. 2019. “Methylobacterium Extorquens RSH Enzyme Synthesizes (p)PpGpp and PppApp in Vitro and in Vivo, and Leads to Discovery of PppApp Synthesis in Escherichia Coli.” *Frontiers in Microbiology* 10 (APR): 859. https://doi.org/10.3389/fmicb.2019.00859.

Sokawa, Y, E Nakao-Sato, and Y Kaziro. 1970. “RC Gene Control in Escherichia Coli Is Not Restricted to RNA Synthesis.” *Biochimica et Biophysica Acta* 199 (1): 256–64. https://doi.org/10.1016/0005-2787(70)90714-8.

Sokawa, Yoshihiro, Eiko Nakao, and Yoshito Kaziro. 1968. “On the Nature of the Control by RC Gene in E. Coli: Amino Acid-Dependent Control of Lipid Synthesis.” *Biochemical and Biophysical Research Communications* 33 (1): 108–12. https://doi.org/10.1016/0006-291X(68)90263-5.

Spira, B, N Silberstein, E Yagil, and Ezra Yagil. 1995. “Synthesis in Cells of Escherichia Coli Starved for Pi. These Include : Guanosine 3 J, 5 J -Bispyrophosphate (PpGpp) Synthesis in Cells of Escherichia Coli Starved for P I” 177 (14): 4053–58.

Stallings, Christina L., Nicolas C. Stephanou, Linda Chu, Ann Hochschild, Bryce E. Nickels, and Michael S. Glickman. 2009. “CarD Is an Essential Regulator of RRNA Transcription Required for Mycobacterium Tuberculosis Persistence.” *Cell* 138 (1): 146–59. https://doi.org/10.1016/j.cell.2009.04.041.

Steinchen, Wieland, Jan S. Schuhmacher, Florian Altegoer, Christopher D. Fage, Vasundara Srinivasan, Uwe Linne, Mohamed A. Marahiel, and Gert Bange. 2015. “Catalytic Mechanism and Allosteric Regulation of an Oligomeric (p)PpGpp Synthetase by an Alarmone.” *Proceedings of the National Academy of Sciences of the United States of America* 112 (43): 13348–53. https://doi.org/10.1073/ pnas. 1505271112.

Sun, Dawei, Gina Lee, Jun Hee Lee, Hye Yeon Kim, Hyun Woo Rhee, Seung Yeol Park, Kyung Jin Kim, et al. 2010. “A Metazoan Ortholog of SpoT Hydrolyzes PpGpp and Functions in Starvation Responses.” *Nature Structural and Molecular Biology* 17 (10): 1188–94. https://doi.org/10.1038/nsmb.1906.

Sussman, A J, and C Gilvarg. 1969. “Protein Turnover in Amino Acid-Starved Strains of Escherichia Coli K-12 Differing in Their Ribonucleic Acid Control.” *The Journal of Biological Chemistry* 244 (22): 6304–6. http://www.ncbi.nlm.nih.gov/pubmed/ 4900514.

Szalewska-Palasz, Agnieszka, Linda U.M. Johansson, Lisandro M.D. Bernardo, Eleonore Skärfstad, Ewa Stec, Kristoffer Brännström, and Victoria Shingler. 2007. “Properties of RNA Polymerase Bypass Mutants: Implications for the Role of PpGpp and Its Co-Factor DksA in Controlling Transcription Dependent on Σ54.” *Journal of Biological Chemistry* 282 (25): 18046–56. https://doi.org/10.1074/jbc. M610181200.

Takada, Hiraku, Mohammad Roghanian, Julien Caballero-Montes, Katleen Van Nerom, Pavel Kudrin, Fabio Trebini, Rikinori Murayama, Genki Akanuma, Abel Garcia-Pino, and Vasili Hauryliuk. 2020. “Ribosome Association Primes the Stringent Factor Rel for Recruitment of 1 Deacylated TRNA to Ribosomal A-Site 2 3.” *BioRxiv*, January, 1–24. https://doi.org/10.1101/2020.01.17.910273.

Taylor, Clare M., Mark Beresford, Harry A.S. Epton, David C. Sigee, Gilbert Shama, Peter W. Andrew, and Ian S. Roberts. 2002. “Listeria Monocytogenes RelA and Hpt Mutants Are Impaired in Surface-Attached Growth and Virulence.” *Journal of Bacteriology* 184 (3): 621–28. https://doi.org/10.1128/JB.184.3.621-628.2002.

Tswett, M. S. 1905. “On a New Category of Adsorption Phenomena and on Its Application to Biochemical Analysis.” *Proceedings of the Warsaw Society of Naturalists, Biology Section* 14 (6): 20–39.

Unterholzner, Simon J, Brigitte Poppenberger, and Wilfried Rozhon. 2013. “Toxin–Antitoxin Systems.” *Mobile Genetic Elements* 3 (5): e26219. https://doi.org/10. 4161/mge.26219.

Varik, Vallo, Sofia Raquel Alves Oliveira, Vasili Hauryliuk, and Tanel Tenson. 2017. "HPLC-Based Quantification of Bacterial Housekeeping Nucleotides and Alarmone Messengers PpGpp and PppGpp." *Scientific Reports* 7 (1): 11022. https://doi.org/10.1038/s41598-017-10988-6.

Vinella, Daniel, Christian Albrecht, Michael Cashel, and Richard D'Ari. 2005. "Iron Limitation Induces SpoT-Dependent Accumulation of PpGpp in Escherichia Coli." *Molecular Microbiology* 56 (4): 958–70. https://doi.org/10.1111/j.1365-2958.2005.04601.x.

Vrentas, Catherine E., Tamas Gaal, Melanie B. Berkmen, Steven T. Rutherford, Shanil P. Haugen, Dmitry G. Vassylyev, Wilma Ross, and Richard L. Gourse. 2008. "Still Looking for the Magic Spot: The Crystallographically Defined Binding Site for PpGpp on RNA Polymerase Is Unlikely to Be Responsible for RRNA Transcription Regulation." *Journal of Molecular Biology* 377 (2): 551–64. https://doi.org/10.1016/j.jmb.2008.01.042.

Wade, Joseph T., Kevin Struhl, Stephen J.W. Busby, and David C. Grainger. 2007. "Genomic Analysis of Protein-DNA Interactions in Bacteria: Insights into Transcription and Chromosome Organization." *Molecular Microbiology*. Mol Microbiol. https://doi.org/10.1111/j.1365-2958.2007.05781.x.

Wagner, Michael, and Matthias Horn. 2006. "The Planctomycetes, Verrucomicrobia, Chlamydiae and Sister Phyla Comprise a Superphylum with Biotechnological and Medical Relevance." *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2006.05.005.

Walsh, K., and D. E. Koshland. 1984. "Determination of Flux through the Branch Point of Two Metabolic Cycles. The Tricarboxylic Acid Cycle and the Glyoxylate Shunt." *Journal of Biological Chemistry* 259 (15): 9646–54.

Walter, Justin D., Margaret Hunter, Melanie Cobb, Geoff Traeger, and P. Clint Spiegel. 2012. "Thiostrepton Inhibits Stable 70S Ribosome Binding and Ribosome-Dependent GTPase Activation of Elongation Factor G and Elongation Factor 4." *Nucleic Acids Research* 40 (1): 360–70. https://doi.org/10.1093/nar/gkr623.

Wang, Boyuan, Peng Dai, David Ding, Amanda Del Rosario, Robert A. Grant, Bradley L. Pentelute, and Michael T. Laub. 2019. "Affinity-Based Capture and Identification of Protein Effectors of the Growth Regulator PpGpp." *Nature Chemical Biology* 15 (2): 141–50. https://doi.org/10.1038/s41589-018-0183-4.

Wegrzyn, Grzegorz. 1999. "Replication of Plasmids during Bacterial Response to Amino Acid Starvation." *Plasmid*. Academic Press Inc. https://doi.org/10.1006/plas.1998.1377.

Wendrich, Thomas M., and Mohamed A. Marahiel. 1997. "Cloning and Characterization of a RelA / SpoT Homologue from Bacillus Subtilis." *Molecular Microbiology* 26 (1): 65–79. https://doi.org/10.1046/j.1365-2958.1997.5511919.x.

Wendrich, Thomas M, Gregor Blaha, Daniel N Wilson, Mohamed a Marahiel, and Knud H Nierhaus. 2002. "Dissection of the Mechanism for the Stringent Factor RelA." *Molecular Cell* 10 (4): 779–88. http://www.ncbi.nlm.nih.gov/pubmed/12419222.

Wexselblatt, Ezequiel, Jehoshua Katzhendler, Raspudin Saleem-Batcha, Guido Hansen, Rolf Hilgenfeld, Gad Glaser, and Roee R Vidavski. 2010. "PpGpp Analogues Inhibit Synthetase Activity of Rel Proteins from Gram-Negative and Gram-Positive Bacteria." *Bioorganic & Medicinal Chemistry* 18 (12): 4485–97. https://doi.org/10.1016/j.bmc.2010.04.064.

Wexselblatt, Ezequiel, Yaara Oppenheimer-Shaanan, Ilana Kaspy, Nir London, Ora Schueler-Furman, Eylon Yavin, Gad Glaser, Joshua Katzhendler, and Sigal Ben-Yehuda. 2012. "Relacin, a Novel Antibacterial Agent Targeting the Stringent Response." *PLoS Pathogens* 8 (9): e1002925. https://doi.org/10.1371/journal.ppat.1002925.

Winther, Kristoffer Skovbo, Mohammad Roghanian, and Kenn Gerdes. 2018. "Activation of the Stringent Response by Loading of RelA-TRNA Complexes at the Ribosomal A-Site." *Molecular Cell* 70 (1): 95-105.e4. https://doi.org/10.1016/j.molcel.2018.02.033.

Xiao, H, M Kalman, K Ikehara, S Zemel, G Glaser, and M Cashel. 1991. "Residual Guanosine 3',5'-Bispyrophosphate Synthetic Activity of RelA Null Mutants Can Be Eliminated by SpoT Null Mutations." *The Journal of Biological Chemistry* 266 (9): 5980–90. http://www.jbc.org/content/266/9/5980.abstract.

Yegian, C. D., G. S. Stent, and E. M. Martin. 1966. "Intracellular Condition of Escherichia Coli Transfer RNA." *Proceedings of the National Academy of Sciences of the United States of America* 55 (4): 839–46. https://doi.org/10.1073/pnas.55.4.839.

Zborníková, Eva, Zdeněk Knejzlík, Vasili Hauryliuk, Libor Krásný, and Dominik Rejman. 2019. "Analysis of Nucleotide Pools in Bacteria Using HPLC-MS in HILIC Mode." *Talanta* 205 (December): 120161. https://doi.org/10.1016/J.TALANTA.2019.120161.

Zuo, Yuhong, Yeming Wang, and Thomas A. Seitz. 2013. "The Mechanism of E. Coli RNA Polymerase Regulation by PpGpp Is Suggested by the Structure of Their Complex." *Molecular Cell* 50 (3): 430–36. https://doi.org/10.1016/j.molcel.2013.03.020.

SUMMARY IN ESTONIAN

HPLC põhine regulaatornukleotiidide (p)ppGpp ja ppApp tasemete analüüs bakterirakus

Bakteritel on evolutsiooni käigus välja kujunenud arvukalt kohanemismehhanisme, mis aitavad neil ellu jääda ka karmides keskkonnatingimustes. Keerukad molekulaarsed võrgustikud kontrollivad adaptiivseid füsioloogilisi vastuseid, näiteks antibiootikumiresistentsust, biokile moodustumist ja bakterite minekut uinunud olekusse. Sellised kohanemismehhanismid sõltuvad stressi tajuvate ja sellele reageerivate valkude ensümaatilistest aktiivsustest. Üheks oluliseks komponendiks stressivastuses on signaalmolekulide süntees ja lagundamine. Käesolevas töös uuriti ühte kõige laiemalt levinud adaptiivset mehhanismi, mida nimetatakse poomisvastuseks. Selle mehhanismi puhul on võtmetähtsusega RelA / SpoT homoloogsed (RSH) ensüümid, mis sünteesivad ja lagundavad alarmoon-nukleotiide ppGpp ja ppp(G)pp. Nende nukleotiidide ühiseks nimetamiseks kasutatakse tähistust (p)ppGpp. Need molekulid mõjutavad mitmeid protsesse bakterirakus, näiteks virulentsust ja antibiootikumitolerantsust. Käesoleva töö eesmärgiks oli välja töötada metoodika nukleotiidide, sealhulgas (p)ppGpp, tasemete kvantifitseerimiseks. Rakendades seda metoodikat uuriti nukleotiidide taset bakterite kasvul ning antibiootikumitöötluse käigus.

Nukleotiidide, sealhulgas (p)ppGpp taseme kvantifitseerimiseks töötati välja HPLC-l põhinev meetod. Nukleotiidide kvantifitseerimise meetodid sisaldavad kolme etappi: proovi kogumine, nukleotiidide ekstraheerimine ja kvantifitseerimine. Kogumisetapis filtreeriti bakterikultuur ja nukleotiidide ekstraheerimiseks viidi filter äädikhappesse. (p)ppGpp kvantifitseerimiseks rakendati HPLC metoodikat 5 µm 4,6 x 150 mm tugeval anioonvahetuskolonnil. Teiste nukleotiidide tuvastamiseks ja kvantifitseerimiseks kasutati ioon-paar pöördfaasi (IPRP) kromatograafiat Kinetex C18 2,6 µm 4,6 x 150 mm kolonnil. Kasutades väljatöötatud metoodikaid uuriti nukleotiidide tasemete muutust bakterite stressivastuse korral. Soolekepikesel (*Escherichia coli*) analüüsiti nukleotiidide tasemeid kasvukõvera erinevates faasides ja aminohapete nälja puhul. Aminohapete nälja puhul täheldati kiiret (p)ppGpp taseme tõusu.

Translatsiooni inhibeerivate antibiootikumide (tiostreptooni, klooramfenikooli ja tetratsükliini) mõju (p)ppGpp ja teiste nukleotiidide tasemetele bakterirakus uuriti nii Gram-negatiivsetes kui ka Gram-positiivsetes bakterites, esindajateks vastavalt *E.coli* ja *Bacillus subtilis*. (p)ppGpp kuhjumise indutseerimiseks kasutati eeltöötlust muprirotsiiniga. Seejärel lisati uuritav antibiootikum subinhibeerivas kontsentratsioonis. Mõlema bakteriliigi korral pidurdasid kõik testitud translatsiooni inhibiitorid (p)ppGpp kuhjumist.

Meie uurimisrühma bioinformaatiline analüüs tuvastas, et mõnedes bakteriliikides on RSH ensüümid, millel on ainult (p)ppGpp sünteesi eest vastutav osa. Leiti, et selline ensüüm bakteris *Cellulomonas marina* võib fosforüleerida ka adenosiini, tekitades molekuli ppApp. Koos paralleelselt ilmunud töödega teistest laboritest on alust arvata, et tegemist on uudse regulaatornukleotiidiga. Selle nukleotiidi täpse rolli kindlakstegemine nõuab edasisi uuringuid.

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PUBLICATIONS

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RESEARCH INTERESTS AND EXPERTISE

My research focus is on investigation of the mechanism of bacterial stringent response – a regulatory mechanism mediated by (p)ppGpp alarmone nucleotide that controls bacterial growth, virulence and antibiotic tolerance. My core skill set is measurements of bacterial nucleotides pools using HPLC as well as antibiotic susceptibility assays.

SCIENTIFIC DEGREES

2016–2020 PhD student in Biomedical Technology, University of Tartu, Faculty of Science and Technology, Institute of Technology, Tartu, Estonia
Supervisor: Professor Tanel Tenson and Dr. Vasili Hauryliuk
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2014–2016 MSc in Applied Measurement Science, University of Tartu, Faculty of Science and Technology, Institute of Chemistry, Tartu, Estonia
Supervisors: Professor Tanel Tenson, Dr. Vasili Hauryliuk and Vallo Varik
Thesis: Role of the stringent response in antibiotic tolerance of *Escherichia coli*

2009–2014 BSc in Biology, Biology Department, University of Aveiro, Portugal.
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RESEARCH EXPERIENCE

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2. Kudrin P*; Dzhygyr I*; Ishiguro K; Beljantseva J; Maksimova E**; Oliveira SRA**; Varik V; Payoe R; L Konevega A; Tenson T; Suzuki T; Hauryliuk V (2018). The ribosomal A-site finger is crucial for binding and activation of the stringent factor RelA. *Nucleic Acids Research*, 46, Issue 4, 1973–1983
3. Varik V*, **Oliveira SRA***, Tenson T, Hauryliuk V. 2017. HPLC-Based Quantification of Bacterial Housekeeping Nucleotides and Alarmone Messengers ppGpp and pppGpp. *Scientific Reports* 7 (1): 11022. https://doi.org/10.1038/s41598-017-10988-6.
4. Kudrin P*, Varik V*, **Oliveira SRA**, Beljantseva J, Del Peso Santos T, Dzhygyr I, Rejman D, Cava F, Tenson T, Hauryliuk V. 2017. Sub-inhibitory concentrations of bacteriostatic antibiotics induce *relA*-dependent and *relA*-independent tolerance to β-lactams. *Antimicrobial Agents and Chemotherapy*. 61: e02173–16.
5. Varik, V; **Oliveira, SRA**; Hauryliuk, V; Tenson,T (2016). Composition of the outgrowth medium modulates wake-up kinetics and ampicillin sensitivity of stringent and relaxed Escherichia coli. Scientific Reports, 6 (22308), x−x.10.1038/srep22308.

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HARIDUS:

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2014–2016	Magistratuur Tartu Ülikoolis, rakendusliku mõõteteaduse õppekaval. Juhendajad: Professor Tanel Tenson, Dr. Vasili Hauryliuk ja Vallo Varik Magistritöö: Poomisvastuse roll antibiootikumitolerantsuses bakteris *Escherichia coli*
2009–2014	Bakalaureuseõpingud Aveiro Ülikooli bioloogia osakonnas Portugalis. Juhendaja: Isabel Henriques Bakalaureusetöö: Antibiootikumiresistentsus kahepoolmelistes molluskites: võimalik tervishoiurisk

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1. Jimmy S*, Saha CK*, Kurata T*, Stavropoulos C, **Oliveira SRA**, Koh A, Cepauskas A, Takada H, Rejman D, Tenson T, Strahl H, Garcia-Pino A, Hauryliuk V, Atkinson GC. 2020. A widespread toxin-antitoxin system exploiting growth control via alarmone signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 117 (19) 10500-10510.
2. Kudrin P*; Dzhygyr I*; Ishiguro K; Beljantseva J; Maksimova E**; Oliveira SRA**; Varik V; Payoe R; L Konevega A; Tenson T; Suzuki T; Hauryliuk V (2018). The ribosomal A-site finger is crucial for binding and activation of the stringent factor RelA. *Nucleic Acids Research*, 46, Issue 4, 1973–1983

3. Varik V*, **Oliveira SRA***, Tenson T, Hauryliuk V. 2017. HPLC-Based Quantification of Bacterial Housekeeping Nucleotides and Alarmone Messengers ppGpp and pppGpp. *Scientific Reports* 7 (1): 11022. https://doi.org/10.1038/s41598-017-10988-6.
4. Kudrin P*, Varik V*, **Oliveira SRA**, Beljantseva J, Del Peso Santos T, Dzhygyr I, Rejman D, Cava F, Tenson T, Hauryliuk V. 2017. Sub-inhibitory concentrations of bacteriostatic antibiotics induce *relA*-dependent and *relA*-independent tolerance to β-lactams. *Antimicrobial Agents and Chemotherapy.* 61: e02173–16.
5. Varik, V; **Oliveira, SRA**; Hauryliuk, V; Tenson,T (2016). Composition of the outgrowth medium modulates wake-up kinetics and ampicillin sensitivity of stringent and relaxed Escherichia coli. Scientific Reports, 6 (22308), x−x.10.1038/srep22308.

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