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UNIVERSITY OF TARTU
Press

Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on June 25th, 2020 by the council of Institute of Technology, Faculty of Science and Technology, University of Tartu.

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Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia on August 19th, 2020 at 12:15.

Publication of this dissertation is granted by the University of Tartu, Estonia.

ISSN 2228-0855
ISBN 978-9949-03-410-9 (print)
ISBN 978-9949-03-411-6 (pdf)

University of Tartu Press
www.tyk.ee

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS 7

ABBREVIATIONS 8

INTRODUCTION 10
1. Post-transcriptional regulation by RNA-binding proteins 11
   1.1 Chaperone-like regulation by Hfq 11
   1.2 Translation regulation of mRNA by CsrA 12
2. Nucleotide second messengers: master regulators of bacterial physiology 13
   2.1 cAMP 15
   2.2 c-di-GMP 15
   2.3 (p)ppGpp 16
      2.3.1 The stringent response: (p)ppGpp over-production upon acute stress 17
      2.3.2 ppGpp during normal growth: the role of basal (p)ppGpp levels 18
      2.3.3 Regulation of transcription by (p)ppGpp 19
      2.3.4 (p)ppGpp targets in addition to RNAP 21
3. RSH enzymes and the stringent response 23
   3.1 Monofunctional RSH enzyme RelA 23
   3.2 Bifunctional RSH enzyme SpoT 26
   3.3 Bifunctional RSH enzyme Rel 26
   3.4 Monofunctional RSH SAS enzymes 27
   3.5 Monofunctional RSH enzyme Metazoan SpoT homolog protein 1, Mesh1 29
4. Role of (p)ppGpp in bacterial virulence and inhibition of the stringent response as a therapeutic strategy 30
   4.1 Antibiotics that target translation 30
   4.2 ppGpp analogues 31
   4.3 Peptide 1018 31

AIMS OF THE STUDY 32

MATERIALS AND METHODS 33
1. Enzymatic assays 33
2. Electromobility shift assay (EMSA) 33
3. Preparation of recombinant proteins 34
   3.1 E. faecalis RelQ 34
   3.2 S. aureus RelP 34

RESULTS AND DISCUSSION 35
1. Biochemical characterization of *E. faecalis* RelQ 35
   1.1 *E. faecalis* RelQ activity is not induced by 70S ribosomes or tRNA, and is inhibited by model mRNA(MF) 36

1.2 *E. faecalis* RelQ has a preference to GDP substrate and pppGpp activator 37
1.3 Relacin does not inhibit *E. faecalis* RelQ 38
2. RelQ combines two archetypical regulatory mechanisms: nucleotide messenger-mediated signaling and RNA binding 39
3. Structural and biochemical characterization of *S. aureus* RelP 45

CONCLUSIONS 48

REFERENCES 49

SUMMARY IN ESTONIAN 58

ACKNOWLEDGEMENTS 59

PUBLICATIONS 61

CURRICULUM VITAE 98

ELULOOKIRJELDUS 100

LIST OF ORIGINAL PUBLICATIONS

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

II. Negative Allosteric Regulation of *Enterococcus faecalis* Small Alarmone Synthetase RelQ by single-stranded RNA (2017). **Beljantseva, J**., Kudrin, P., Andresen, L., Shingler, V., Atkinson, G. C., Tenson, T., Hauryliuk, V Proc Natl Acad Sci USA. 2017 Apr 4;114(14):3726–3731. http://doi.org/10.1073/pnas.1617868114.

III. Structural Basis for (p)ppGpp Synthesis by the *Staphylococcus aureus* Small Alarmone Synthetase RelP (2018). Manav, C.M., **Beljantseva, J**., Bojer, M.S., Tenson, T., Ingmer, H., Hauryliuk, V., Brodersen, D.E. J Biol Chem 2018 Mar 2;293(9):3254–3264. http://doi.org/10.1074/jbc.RA117.001374.

Author's contribution:

I. Performed several enzyme kinetics experiments for *E. faecalis* RelQ and *E. coli* RelA.
II. Performed all of the EMSA experiments and most of the biochemical experiments, analyzed results and participated in writing the manuscript.
III. Performed all of the biochemical and EMSA experiments, analyzed results and participated in writing the manuscript.

ABBREVIATIONS

ACP	Acyl Carrier Protein
ACT	Aspartokinase, Chorismate mutase, TyrA
ASF	A-site finger rRNA element
ATP	adenosine triphosphate
bp	base pair
c-di-GMP	cyclic di-guanosine monophosphate
cAMP	cyclic adenosine monophosphate
CC	conserved cysteine domain
Crp	cAMP receptor protein
CsrA	Carbon storage regulator A
CTD	carboxy terminal domain
DRACALA	Differential Radial Capillary Action of Ligand Assay
EF-G	Elongation factor G
EF-Tu	Elongation factor thermo unstable
EMSA	Electrophoretic Mobility Shift Assay
GDP	guanosine diphosphate
Gmk	GMP kinase
GTP	guanosine triphosphate
HD	hydrolysis domain
HprT	hypoxantine-guanine phosphoribosyltransferase
IF2	Initiation factor 2
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
LdcI	Lysine decarboxylase, inducible
Lrp	Leucine-responsive regulatory protein
Mesh1	metazoan SpoT homolog 1
mRNA	messenger RNA
NTD	amino terminal domain
PNPase	polynucleotide phosphohydrolase
PolyP	Polyphosphate
ppGpp	guanosine penta- and tetraphosphate
PPi	inorganic pyrophosphate
RBP	RNA-binding protein
RBS	RNA binding site/ribosome binding site
RNAP	RNA polymerase
rRNA	ribosomal RNA
RSH	RelA/SpoT homologue
SAH	small alarmone hydrolase
SAS	small alarmone synthetase

sRNA	small RNA
SYNTH	synthesis domain
tRNA	transfer RNA
TA	toxin-antitoxin
TGS	threonyl-tRNA synthetase, GTPase, SpoT
TLC	thin layer chromatography
βMe	β-mercaptoethanol

INTRODUCTION

Bacteria are the most skillful organisms to adapt to and withstand diverse environmental conditions. A wide variety of both biotic and abiotic stressors are influencing their life. Therefore, bacteria have evolved highly coordinated and interconnected molecular networks that integrate different and simultaneous extracellular signals into adequate physiologic responses. These physiologic responses are mediated by regulation of gene expression at transcriptional, post-transcriptional and protein activity levels as well as via highly controlled synthesis and degradation of dedicated small signaling molecules that regulate the aforementioned processes.

One of the most global bacterial stress survival mechanisms – the stringent response – is mediated by an alarmone nucleotide (p)ppGpp. This molecule is synthesized in response to nutrient and other stresses and is able to regulate not only bacterial growth and survival, but virulence and host evasion during pathogenesis. Representatives of RelA-SpoT Homolog (RSH) family of proteins control the (p)ppGpp levels. These enzymes are highly conserved among bacteria due to their evolutionary importance and the universal nature of the stringent response in bacteria. In this thesis, I contribute to knowledge on RSH enzymes by taking a glimpse into working mechanisms of Small Alarmone Synthetases (SAS) – so far not so well studied RSH representatives. Specifically, I have investigated the enzymatic characteristics of two SAS proteins: *Enterococcus faecalis* RelQ and *Staphylococcus aureus* RelP. The distinctive features of the working principles found for these proteins underlay their role in fine-tuning the stringent response, and possibly in post-transcriptional regulation – as we have discovered *E. faecalis* RelQ has an RNA-binding property that is directly connected to its enzymatic function.

1. Post-transcriptional regulation by RNA-binding proteins

Post-transcriptional control of gene expression is important for bacteria to adapt to changing surroundings. It can be achieved by regulatory RNA-binding proteins (RBPs), which can modulate translation of the target mRNA in several ways. For example, they can protect RNA from RNases by shielding the target mRNA, compete with ribosomes for binding the ribosome binding site (RBS), act as a chaperone by providing a platform for the interaction of target RNA with its effector or modulate transcription termination/antitermination structure formation. The best-studied examples of these proteins are Hfq and CsrA, which represent the two archetypical mechanisms of this regulation – chaperone-like regulation or formation of stable complex with target RNA, respectively [1].

1.1 Chaperone-like regulation by Hfq

Hfq is a conserved RNA-binding protein which was initially identified in *Escherichia coli* as an essential host factor for replication of bacteriophage Qß [2, 3]. Later it was discovered to be important for the bacterium itself, as loss of Hfq results in reduced growth rate, attenuated stress resistance and diminished virulence [4]. Hfq was shown to play an important role in biofilm formation, motility, catabolite repression control and others [5]. Although Hfq regulates gene expression post-transcriptionally via several different mechanisms, probably its main function is facilitation of transient intermolecular base-pairing of regulatory small RNAs (sRNAs) with the target mRNAs [6]. By doing so, Hfq affects translation and turnover rates of many cellular mRNAs, additionally providing protection for RNA molecules from chemical and enzymatic degradation.

The ability of Hfq to bind several molecules simultaneously can be attributed to its ring-like homohexameric structure with three distinct RNA-binding interfaces: distal, proximal and lateral (**Fig. 1B**). Each domain has disparate binding specificity [7, 8]. The proximal part preferentially binds AU-rich motifs, the distal site has poly(A) binding specificity and the lateral site preferentially binds U-rich sequences and double-stranded RNA segments. Hfq binding can trigger changes in secondary structure of the target RNA which would render parts of it more accessible for base-pairing as well as promote the recruitment of protein partners such as RNase E, Rho and polynucleotide phosphohydrolase (PNPase). Given these diverse functional features, Hfq is capable of using different strategies to regulate mRNA and controversially either inhibit or promote translation [9]. For example, by facilitating regulatory sRNA binding to the RBS region of the mRNA, Hfq sequesters ribosome entry and therefore inhibits translation initiation. This is often followed by degradation of sRNA:mRNA hybrid by RNase E and/or PNPase [10, 11]. Conversely, Hfq promotes translation initiation,

for example by releasing the RBS from the secondary structure therefore making it accessible to ribosome binding. Finally, due to the shared binding preference of Hfq and Rnase E toward AU rich motifs, Hfq also can oppose the RNA decay by shielding RNA from RNase cleavage [12].

1.2 Translation regulation of mRNA by CsrA

CsrA (Carbon storage regulator A) is another widely conserved RNA-binding protein found in more than 1,500 bacterial species [13]. It is a global regulator that is post-transcriptionally affecting more than 100 genes involved in carbon metabolism, production of virulence factors, and bacterial motility [14]. The general outcome of its action is activation of exponential phase functions and repression of stationary phase processes [15, 16].

The most common mode of action for CsrA is direct competition with ribosome binding to the target mRNA [17] (**Fig. 1A**). mRNA binding specificity of CsrA proteins is dependent on both primary sequence and secondary structure: the binding consensus sequence of CsrA is RUACARGGAUGU, with ACA and GGA motifs being 100% conserved and GGA being a part of a hairpin structure [17]. Since GGA motif is a component of Shine-Dalgarno sequence, CsrA competes with ribosome binding to target mRNA resulting in reduced translation initiation and consequent mRNA degradation [18–20].

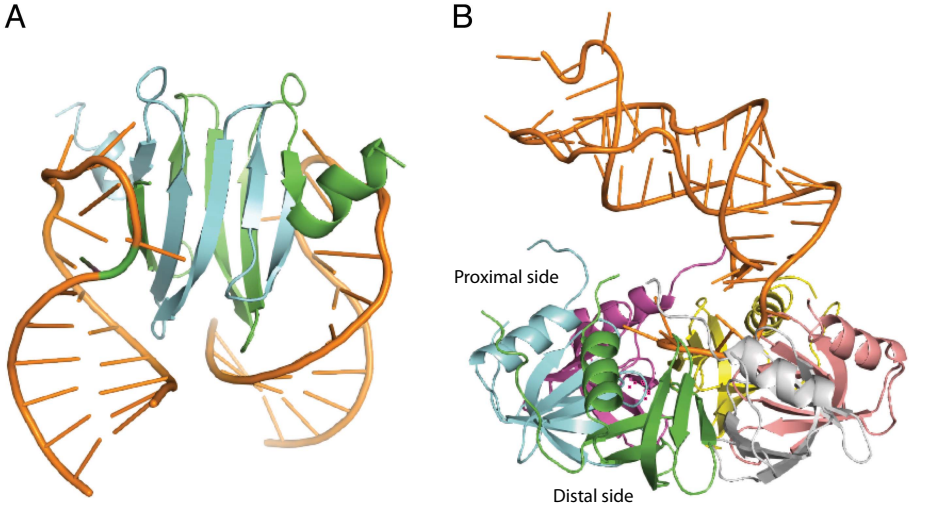

Figure 1. The structure of CsrA dimer in complex with *hcnA* mRNA (A; PDB accession code 2JPP) and Hfq hexamer in complex with RydC sRNA (B; PDB accession code 4V2S). For both structures mRNA is shown in orange and protein subunits in other colors**.** Adapted from [21].

2. Nucleotide second messengers: master regulators of bacterial physiology

The common strategy used by bacteria to respond to changing surroundings is modulation of the intracellular concentration of nucleotide-based second messengers which, transduce signals from the environment (mediated by extracellular first messengers) into according cellular responses. Since bacteria simultaneously sense and respond to an array of stimuli, they employ a wide diversity of these regulatory molecules. The underlying principles for different second messengers are similar: nucleotide messengers are synthesized or degraded by distinct enzymatic activities and they exert their functions by allosterically binding to and regulating the effector molecules (most commonly proteins). The effector molecules, in turn, interact with a molecular target, which finally is a part of output function of the second messenger. The working principle of nucleotide second messengers is outlined in **Fig. 2**. As in bacteria signaling molecules are found to regulate biofilm formation and production of virulence factors, interest towards nucleotide signaling has been growing and repertoire of the second messengers expands.

Structurally they can be categorized into cyclic and linear nucleotides. To name some examples, among cyclic representatives most extensively studied second messengers are cyclic adenosine monophosphate (cAMP) and cyclic di-guanosine monophosphate (c-di-GMP), and among linear ones guanosine penta- and tetraphosphate or (p)ppGpp (**Fig. 3**) [22]. An overview of these three messenger nucleotides will be given in the next sections of this thesis, with the emphasis on the (p)ppGpp, considering it is far the most global messenger nucleotide. Among other nucleotides, which will not be commented here are newcomers like c-di-AMP and c-AMP-GMP or less studied linear messengers ppApp [23] and ppGp [24]. All of the messenger compounds listed above are purine-based. The reason for the bias in favor of purines and not of pyrimidines most probably lies in their chemical properties. The two-ring system of purines creates a greater potential for stronger interactions with the receptors when compared to single-ring pyrimidines. The structure of purines is more favorable for forming stacking interactions and has more potential for hydrogen-bonding contacts. Therefore, receptors have probably evolved to create binding pockets for purine-based messengers rather than for pyrimidine-based molecules [25].

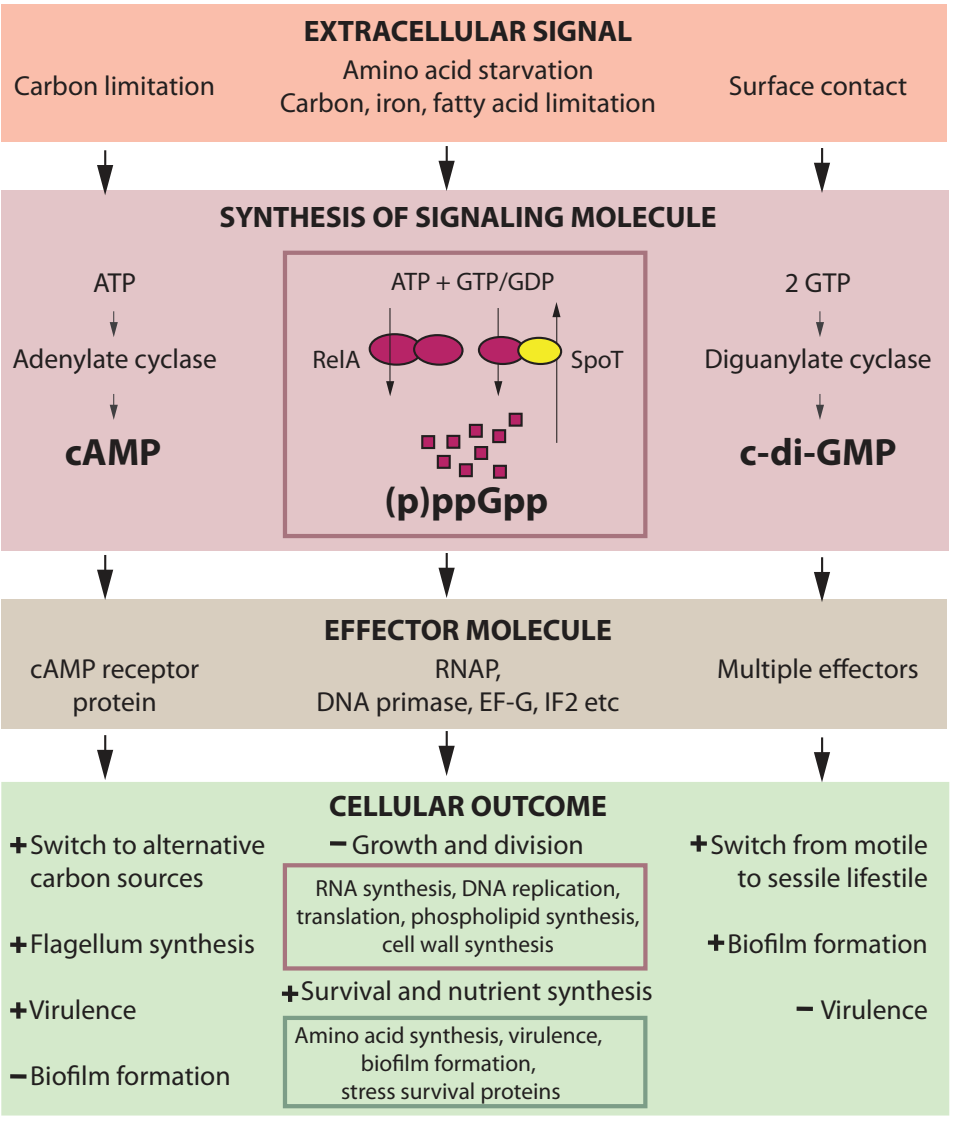

Figure 2. Underlying principles of control exerted by nucleotide second messengers as exemplified by three most well-studied representatives: cAMP (left), (p)ppGpp (center) and c-di-GMP (right). Messenger nucleotides are synthesized in response to extracellular stimuli and consequently bind to their effector molecule (most commonly – protein enzyme). As a result of this interaction, the activity of the effector molecule is changed, finally leading to reorganization of cellular physiology.

2.1 cAMP

The first nucleotide second-messenger to be discovered was cAMP, which was identified in 1950's as mediator of hormone-induced changes in metabolism of eukaryotes [26, 27]. In the 1960's it was shown for the first time that cAMP is also produced in *E. coli* [28]. Now, after decades of extensive research, it is considered as classic textbook paradigm for second messenger signaling in bacteria and is known to be involved in catabolite repression in *E. coli* and other species. cAMP is synthesized by adenylate cyclase CyaA in response to carbon limitation (**Fig. 3**, left row). Accumulating cAMP binds to its receptor protein Crp [29] – a global transcription factor of *E. coli*. This binding triggers activity of Crp, which is to interact with specific promoters and stimulate transcription of genes involved in catabolism of alternative sugars, for example, the *lac* operon [29, 30]. Additionally, cAMP regulates virulence, flagellum biosynthesis and biofilm formation [31].

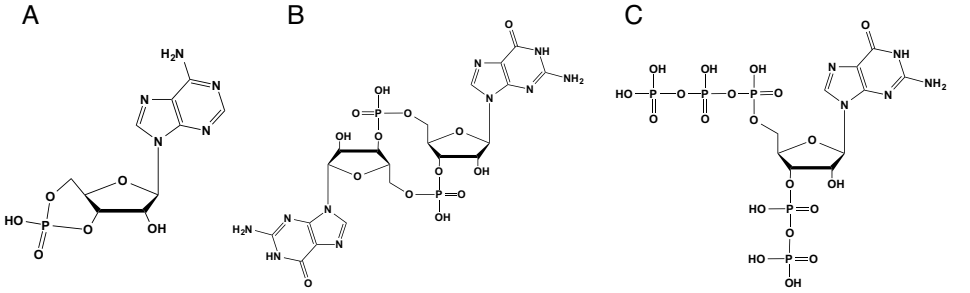

Figure 3. Chemical structures of cAMP (A), c-di-GMP (B) and pppGpp (C).

2.2 c-di-GMP

Since its discovery in mid-1980's, c-di-GMP has become recognized as an ubiquitous signaling molecule [32]. The most prominent function of c-di-GMP is to regulate transition between two lifestyles of bacteria – motile single cells and adhesive multi-cellular communities [33]. For better survival and withstanding environmental cues bacteria mostly live in surface-associated way. These bacteria undergo drastic changes in their physiology, transcription profile and metabolism when compared to the genetically identical free-living bacteria. c-di-GMP is considered to be the influencer of these changes. Accumulation of high c-di-GMP concentrations induces expression of components of extracellular matrix such as adhesive proteins, extracellular polysaccharides and curli fimbriae, and, therefore, promotes biofilm formation and adhesive lifestyle. Conversely, cells that use flagella or pili for their movement have low c-di-GMP concentrations [33]. c-di-GMP is metabolized by three protein domains – GGDEF, EAL and HD-GYP, names of which stem from the conserved amino acid motifs in their active sites.

Dimerization of two GGDEF domains is needed for the synthesis of c-di-GMP from two GTP molecules. EAL or HD-GYP are responsible for c-di-GMP degradation. Opposing cyclase and phosphodiesterase activities of these proteins lead to tight regulation of transition between sessile and moving lifestyles of bacteria [34]. Although not much is known about the extracellular trigger for c-di-GMP accumulation, there is growing evidence that surface contact itself can serve as activating signal for c-di-GMP production [35, 36].

2.3 (p)ppGpp

(p)ppGpp was discovered by analyzing the changes in intracellular nucleotide pools upon amino acid starvation in *E. coli*, where Cashel and Gallant have identified two unusual spots on a thin layer chromatogram, TLC [37]. These "magic spots" were thereafter shown to be hyperphosphorylated guanosine nucleotides, alarmones ppGpp and pppGpp, collectively referred to as (p)ppGpp. Subsequent analyses showed that the two messengers are synthesized on idling step of protein synthesis that is explained later (§ 3.1) [38] in a chemical reaction of pyrophosphate transfer from ATP to the 3' position of GTP or GDP, resulting in pppGpp or ppGpp, respectively [39] (**Fig. 4**). The (p)ppGpp is a global second messenger that affects numerous processes in bacterial cell, with physiological effects ranging from growth rate regulation to virulence [40]. Its main role is to mediate the global stress survival program of bacteria – the so-called "stringent response" – a prime hallmark of which is accumulation of the alarmones and inhibition of production of so-called "stable RNA", i.e. ribosomal RNA (rRNA) and transfer RNA (tRNA) [41]. During acute stringent response (p)ppGpp levels were estimated to reach millimolar concentrations and are needed to survive unfavorable environmental conditions [42]. Under the balanced growth, the alarmones are present in the cell at the basal levels of 10's of µM which are necessary for coordination of normal metabolism [43].

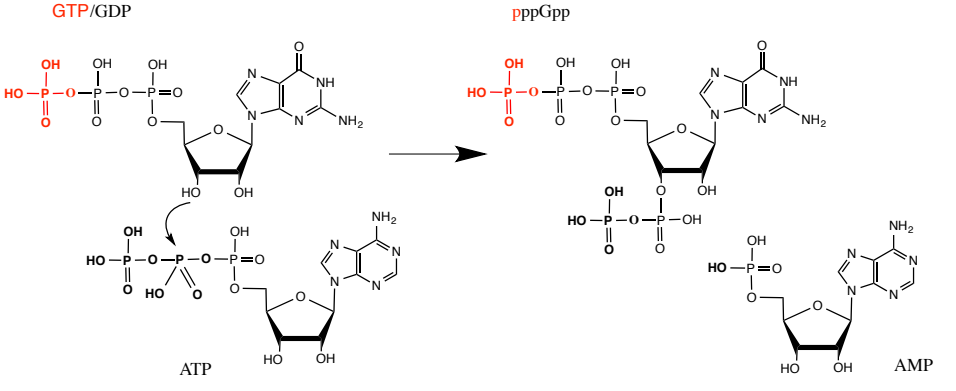

Figure 4. (p)ppGpp synthesis from ATP and GTP/GDP.

Although for a long time (p)ppGpp-mediated signaling was considered to be unique for bacteria, in early 2000s (p)ppGpp was identified in chloroplasts of plants, where it has been shown to accumulate in response to biotic and abiotic stresses [44]. The presence of the (p)ppGpp-mediate signaling in chloroplasts can be explained by the antient origin of chloroplasts from endosymbiotic event between photosynthetic cyanobacteria and an eukaryotic organism. In addition to the role in stress response, (p)ppGpp has been shown to influence growth and development of plants [45, 46] as well as photosynthesis [47]. Intriguingly, in chloroplasts (p)ppGpp is also accumulated in response to increase in Ca^{2+} concentrations, showing possible connection between eukaryotic and prokaryotic signaling in chloroplasts [48]. This thesis will focus solely on bacterial (p)ppGpp signaling.

2.3.1 The stringent response: (p)ppGpp over-production upon acute stress

The first identified and studied phenotypic trait of the stringent response was inhibition of stable RNA (tRNA and rRNA) synthesis upon amino acid deprivation. It was indicated to be dependent on the function of a single gene, mutation of which leads to two consequences [41]. First, it results in so called "relaxed" phenotype, in which, as opposed to the "stringent", synthesis of stable RNA is resumed independently of amino acid deprivation. The gene was therefore designated as *relA*. Second, the "relaxed" strains are not able to accumulate (p)ppGpp, indicating the connection between the stringent response and (p)ppGpp production. Eventually, the protein product of *relA* gene was identified to be the enzymatic catalyst responsible for (p)ppGpp synthesis.

Later studies showed that not only amino acid starvation can trigger stringent response, but deprivation of other nutrients like carbon [49–51], fatty acid [52], iron [49–51] or non-nutrient condition like heat shock [53], can initiate (p)ppGpp production. Moreover, inhibition of stable RNA is now only one of many more (p)ppGpp-dependent cellular responses. These include inhibition of nucleotide, cell envelope, lipid and phospholipid biosynthesis, DNA replication as well simultaneous activation of amino acid production and expression of proteins needed for stress survival [54]. This all leads to overall reorganization of cellular resources resulting in repression of growth and division and promotion of survival and nutrient synthesis [40]. Several decades of research have changed the stringent response into global regulatory system that integrates many stress signals into cellular responses at different levels.

2.3.2 ppGpp during normal growth: the role of basal (p)ppGpp levels

(p)ppGpp can exert important regulatory effects at concentrations that are way below those needed for activation of stringent response. It is shown to act as a rheostat affecting expression of different genes at its different concentrations [55]. A transcriptional study of two regulons affected by ppGpp – Leucine-responsive regulatory protein Lrp and RpoS (RNAP sigma factor that regulates expression of genes responsible for entry into stationary phase and general stress response genes) – has demonstrated that that these factors are expressed at different stages of the stringent response activation [56]. Specifically, Lrp is activated in the early stage when cells are still actively growing and measured ppGpp concentrations at that time were only below 100 pmol ml^{-1} OD^{-1}. RpoS, on the other hand, is expressed when the ppGpp concentration reached above 400 pmol ml^{-1} OD^{-1}, i.e. when upon growth arrest [56]. Therefore, it was concluded that beginning of starvation as a first aid bacteria tries to restore intracellular amino acid pools, but if more severe conditions are met, then survival responses are activated.

Although (p)ppGpp is usually connected to survival under harsh conditions, it has been also indicated as an important coordinator of normal metabolism during balanced growth conditions. This coordination is exerted by the basal levels of the alarmone, which is always present in the bacterial cell during normal growth at the concentration of around 50 μM [53]. Already in 1980's the basal levels of (p)ppGpp were demonstrated as growth rate regulators in *E.coli*, when it was shown that (p)ppGpp levels and growth rate anti-correlate [57]. More recently this topic has been revisited, and (p)ppGpp was proven to be the major growth rate regulator as in cells where (p)ppGpp is absent growth-rate control is abolished during balanced growth [58].

Bacterial strains that are completely unable to synthesize (p)ppGpp, known as $(p)ppGpp^0$ strains (genes encoding the proteins for (p)ppGpp synthesis are deleted), are used to study the functions of basal levels of the alarmone [59]. Characterization of transcriptional profile of $(p)ppGpp^0$ in *E. faecalis* showed up-regulation of genes involved in energy generation under starvation conditions, however during balanced growth alternative carbon sources were used for energy production. Phenotypically the $(p)ppGpp^0$ strain showed switch from normal homolactic to heterofermentative metabolism, which subsequently led to H_2O_2 production [60]. These observations indicate that basal levels of (p)ppGpp are important for keeping the balanced metabolism in *E. faecalis* and are required for fully efficient stringent response activation.

(p)ppGpp inhibits GTP production by targeting enzymes in GTP synthesis pathway [61, 62]. In *Bacillus subtilis* and *E. faecalis* strong inhibition of HprT (hypoxantine-guanine phosphoribosyltransferase) and Gmk (GMP kinase) is established with IC_{50} ranging from 11 to 80 μM. Moreover, complete absence of (p)ppGpp results in increased GTP levels which lead to severe inhibitory effect and possibly cell death (so called death by GTP) and it happens independently of

stress conditions [61, 62]. Collectively, these observations substantiate that GTP homeostasis is regulated by basal levels of (p)ppGpp.

Regulation of GTP levels by (p)ppGpp also contributes to survival of nutrient downshifts as dysregulation of GTP metabolism in (p)ppGpp^0 causes severe auxotrophic requirements for specific amino acids. *B. subtilis* (p)ppGpp^0 shows strong auxotrophic requirements for BCAA, valine and threonine and also moderate requirements for histidine, arginine and tryptophane. At the same time strain where the basal levels are present shows strong auxotrophy only for valine and moderate requirements for leucine, isoleucine and methionine [63, 64]. This auxotrophy is connected to the elevated levels of GTP in (p)ppGpp^0 strain, which, in turn, either directly and/or through the activation of transcriptional regulator CodY represses the transcription of the genes encoding the enzymes involved in amino acid biosynthesis.

Finally, it is shown that basal levels of (p)ppGpp confer bacterial tolerance to antibiotics vancomycin, ampicillin and norfloxacin [43, 59, 60].

2.3.3 Regulation of transcription by (p)ppGpp

With extensive effects on bacterial metabolism and physiology, the (p)ppGpp is capable of regulating the expression of hundreds of genes [65, 66]. The (p)ppGpp mediated stringent transcription control can be exerted in two ways. In many bacterial species, the alarmone acts indirectly through altering cellular GTP level (see below). In proteobacteria (p)ppGpp alters transcription through direct interaction with RNAP. The classical example is *E. coli* RNAP: (p)ppGpp directly binds to the RNAP (Fig. 5) and regulates its activity either positively or negatively depending on the kinetic properties of the target promoter sequences [67]. In cooperation with transcription factor DksA, (p)ppGpp inhibits transcription from promoters that form short-lived complexes with RNAP, such as those of stable RNA genes [68]. These promoters are characterised by the presence of GC-rich discriminator region, suboptimal –35 and –10 elements and shorter –10/–35 spacer length (16 bp vs optimal 17 bp) [69]. ppGpp and DksA also act together to activate other promoters (e.g. promoters of amino acid biosynthesis genes) either indirectly by the increase of available RNAP levels, which are freed from rRNA promoters or directly by stimulating the association rate of RNAP [70].

The molecular mechanism of DksA-mediated RNAP regulation remained a mystery for more than two decades. It was suggested that DksA exerts its effects by protruding its coiled coil into the secondary channel of RNAP and coordinates (p)ppGpp-bound Mg^{2+} ions therefore stabilizing RNAP:ppGpp interaction [71]. Recent structural analyses supported this idea, directly demonstrating the role of DksA in forming the (p)ppGpp binding site of the RNAP (**Fig. 5**). In the presence of DksA, ppGpp has a second binding site (site 2) on RNAP in addition to the one that was characterized previously (site 1) [72]. The two sites are located 60 Å

apart from each other with site 1 being at the β'-ω subunit interface and site 2 intriguingly at the DksA-β' interface (**Fig. 5**) [72, 73].

In many other bacterial species, e.g. Firmicutes, the alarmone production regulates transcription indirectly through altering GTP levels in the cell. A prime example of a bacterial species in which (p)ppGpp does not bind to RNAP is *B. subtilis* [74, 75]. Transcriptional control in these organisms is mediated by the reciprocal change in the GTP/ATP pools caused by GDP/GTP conversion to ppGpp/ pppGpp during the stringent response. (p)ppGpp reduces accumulation effects of GTP in two ways. First, GTP is consumed upon production of the alarmone. Second, (p)ppGpp directly inhibits IMP degydrogenase, the first enzyme in the GTP synthesis pathway. The latter scenario leads to IMP accumulation, which is also a precursor for ATP and therefore ATP production is promoted. Thus increase in ATP and decrease in GTP levels lead to changes in transcriptional profile for promoters sensitive to concentration of initiating nucleotide causing repression of transcription driven by promoters initiating from GTP nucleotide and upregulation of stringent promoters initiating from ATP [61, 74].

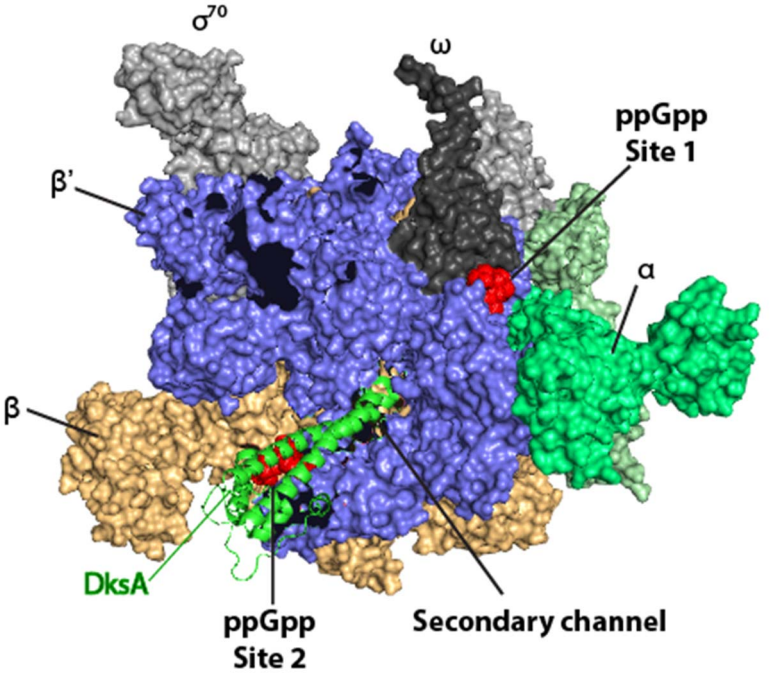

Figure 5. Structure of *E. coli* RNA polymerase in complex with DksA and two ppGpp molecules. DksA bound in the secondary channel is shown in green, the two ppGpp molecules are shown in red. Adapted from [73], PDB accession code 5VSW.

2.3.4 (p)ppGpp targets in addition to RNAP

Although global regulatory functions of (p)ppGpp are exerted through its effect on transcription profile of the cell, there are many other core cellular processes that are affected by the alarmone. (p)ppGpp is capable of directly binding and inhibiting proteins involved in such processes like protein biosynthesis (translation), DNA replication, acid stress response and polyphosphate metabolism [76].

Translational GTPases EF-G [77], EF-Tu [78] and IF2 [79] are all direct targets of (p)ppGpp. Due to structural similarity between GTP and (p)ppGpp, the nucleotides orthosterically bind the GTP-binding active site of GTPases [80]. Therefore, (p)ppGpp interferes with elongation and initiation steps of the translation, e.g. binding to IF2 prevents 30S initiation complex formation [79]. Investigations of the affinities of (p)ppGpp and GTP towards GTPases showed that (p)ppGpp does not bind these enzymes better than the native substrate, GTP, indicating that inhibition by (p)ppGpp is transient and takes place at its peak concentrations during the stringent response [81]. An Isothermal Titration Calorimetry (ITC) study showed that (p)ppGpp has higher affinity towards IF2 than to EF-G, suggesting that the alarmone primarily affects initiation step of translation, rather than translocation [82].

(p)ppGpp was also suggested to regulate protein degradation by affecting polyphosphate (polyP) metabolism. PolyP is an anionic polymer of hundreds of phosphate residues, which is synthesized from ATP by polyphosphate kinase and degraded to inorganic phosphate by exopolyphosphatase [83, 84]. PolyP is accumulated in the cell during nutrient downshifts and in cooperation with ATP dependent Lon protease which degrades ribosomal proteins and thereby restores amino acid supply of the cell [85]. (p)ppGpp is responsible for accumulation of polyP as it inhibits the polyphosphatase activity [86].

Besides regulation of protein synthesis and degradation, (p)ppGpp is also involved in inhibition of DNA replication elongation by directly targeting DNA primase (DnaG) – an essential component of replication complex. Interestingly replication forks arrested by (p)ppGpp do not recruit recombination protein RecA indicating that the forks are not disrupted. This implies that (p)ppGpp is important for maintaining genomic stability during nutrient downshifts [87].

(p)ppGpp also regulates acid stress response by modulating activity of inducible lysine decarboxylase (LdcI) in *E. coli* [81]. LdcI is activated in response to low-pH environments. It increases cytoplasmic pH by consuming a proton as it catalyzes decarboxylation of L-lysine to cadaverine and carbon dioxide [88]. Cadaverine is then transported out of the cell in exchange for lysine. LdcI is found to co-crystalize with (p)ppGpp and its activity is strongly inhibited by the alarmone in mildly acidic conditions [89]. Although still bound to LdcI, (p)ppGpp is unable to inhibit LdcI at low pH. These findings suggest that (p)ppGpp coordinates acid stress and stringent response by preventing amino acid consumption by decarboxylases when pH is normalized [90].

Despite the intensive and continuous study of the topic it was only very recently that comprehensive search for (p)ppGpp targets was undertaken. Two approaches – Differential Radial Capillary Action of Ligand Assay (DRACALA) [91] and capture-compound mass spectrometry [92] – were used in two different studies to systematically screen for the potential (p)ppGpp effectors. The latter approach, being advantageous, identified 56 hits including almost all previously known (p)ppGpp targets. Many of the new targets fell into the previously determined "classes" of (p)ppGpp binders, suggesting the conserved nature of the affected processes. These are de-novo and salvage pathways of nucleotide metabolism, translation, ribosome biogenesis etc. For example, in addition to previously described representatives of (p)ppGpp targets from nucleotide metabolism pathway – like HprT, Gmk, GuaB – two new binders were identified in this class. PurF – the first enzyme in *de novo* purine synthesis pathway was shown to be inhibited by (p)ppGpp *in vitro*, and *E. coli* strain with mutated PurF was shown to over-produce purine nucleotides as compared to wild-type strain [91]. A new possible player in purine salvage pathway, YgdH, is another potential (p)ppGpp target identified by DRACALA assay. Interestingly, DRACALA assays have also identified potential novel (p)ppGpp degradation proteins. Although repertoire of the (p)ppGpp targets has greatly expanded, physiological relevance of these new targets still remains to be validated by dedicated follow-up studies.

3. RSH enzymes and the stringent response

The (p)ppGpp levels are regulated by the members of RelA-SpoT Homolog [93] protein family. The protein family name comes from their representatives in *E. coli*, RelA and SpoT. While RSH proteins are conserved among bacteria, their structure and repertoire differ among bacterial species. Structurally they can be divided to long and short RSHs [94]. Long RSHs are comprised of regulatory C-terminal domain region (CTD) and catalytic N-terminal domain region (NTD) (**Fig. 6**). The exact function of C-terminus remains unknown. Short RSHs however lack these regulatory domains and possess only either synthetase or hydrolase domains, and are referred either Small Alarmone Synthetases (SASs) or Small Alarmone Hydrolases (SAHs), respectively [95–98].

In representatives of Gamma- and Betaproteobacteria there are two long RSH proteins, one of which has major (p)ppGpp synthetic (RelA) and the other major (p)ppGpp hydrolytic activity (SpoT) [94]. Most common bacterial RSH representative is bifunctional Rel, present (responsible for (p)ppGpp metabolism) in most of the bacterial species [94]. Bifunctional Rel can sometimes be accompanied by one or two monofunctional SAS proteins, especially in Firmicutes.

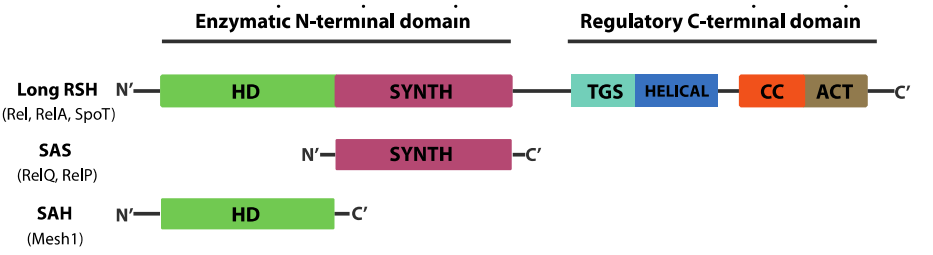

Figure 6. Domain structure of RSH proteins. Long RSH proteins are composed of six domains: (p)ppGpp hydrolysis domain (HD), (p)ppGpp synthesis domain (SYNTH), TGS (Threonyl-tRNA synthetase, GTPase, SpoT [99]), Helical, Conserved Cysteines and ACT (Aspartokinase, Chorismate mutase, TyrA) [94].

3.1 Monofunctional RSH enzyme RelA

RelA is the best-studied RSH representative and most of what is known about the mechanism of stringent response activation is based on the studies on *E. coli* RelA. Although it possesses SYNTH and HD domains, enzymatically it is only capable of (p)ppGpp synthesis, with the HD domain being inactivated [100]. The (p)ppGpp production by RelA is triggered by amino acid starvation [38] and heat shock [101], and it is further allosterically activated by the (p)ppGpp itself [100]. Despite of several decades of study, there are still ongoing discrepancies concerning the molecular mechanism of RelA action.

Soon after the discovery of (p)ppGpp, its synthesis by RelA was shown to be induced by stalled ribosomes with cognate uncharged tRNAs in the A-site [38]. According to prevailing theory of RelA's mechanism of activation, amino acid starvation leads to increased abundance of deacylated tRNAs in the cell, which bind the vacant ribosomal A-site and consequently stall the translation; these stalled ribosomes are a prerequisite for RelA binding and activation (**Fig. 7**). Some recent studies, however, proposed that RelA first binds the deacylated tRNA in the cytoplasm, and this complex is then loaded on the ribosome, eventually triggering the (p)ppGpp synthesis [102, 103].

There is also uncertainty in how the catalysis step is carried out – does it take place while RelA is bound to the ribosome or when it is free in the cytoplasm? To date, three models have been put forward. Wendrich and collages proposed so-called "hopping" model according to which RelA gets activated by stalled ribosomal complex, synthesizes one (p)ppGpp molecule and the act of catalysis fuels RelA's dissociation from the ribosome, letting the enzyme "hop" between the ribosomes [104]. Two other studies using single molecule tracking in live cells resulted in somewhat contradictory results. English and colleagues proposed an "extended hopping model" [105]. Specifically, according to this model RelA produces multiple (p)ppGpp molecules off the ribosome per dissociation event. Conversely, Li and colleagues concluded that RelA performs several acts of (p)ppGpp synthesis while bound to the ribosome [106].

Three recent cryogenic electron microscopy (cryo-EM) structures of *E. coli* RelA bound to the "starved" ribosomal complexes have provided crucial insights into the molecular mechanism of amino acid starvation sensing by RelA [95–98, 107]. When bound to the ribosome, RelA assumes a boomerang-like open conformation with its catalytic N-terminal domain region extending into the solvent not contacting with the ribosome while the C-terminal domain region protrudes into the intersubunit space of the ribosome, making multiple contacts both with tRNA and rRNA **(Fig. 8)**. Specifically, the TGS domain of RelA contacts with CCA acceptor end of the tRNA thereby inspecting the aminoacylation state of the tRNA [98]. The rest of the protein protrudes deeper the intersubunit space. The Helical linker domain is wrapped around the acceptor arm of tRNA and ACT domain interacts with elbow region of tRNA. The CC and ACT domains make most contacts with the ribosome, localized on ASF of the 23S rRNA. These contacts position deacylated tRNA in a distorted A/T-like conformation. Surprisingly, RelA does not contact the ribosomal protein uL11, while biochemical experiments show the requirement of uL11 for activation of RelA upon amino acid starvation [108].

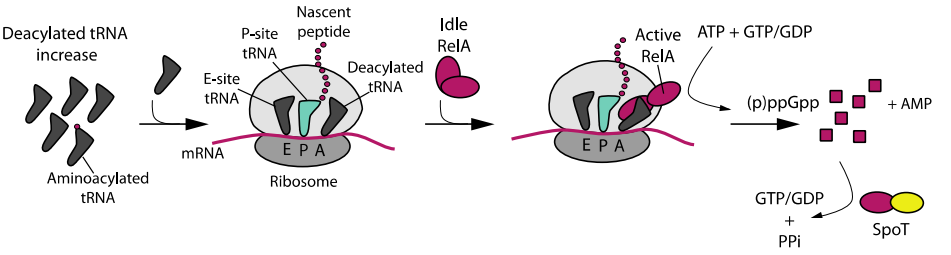

Figure 7. Mechanism of RelA activation by starved ribosomal complexes upon amino acid starvation. During amino acid downshift, the pool of deacylated tRNA increases and tRNAs stall the translation by binding to the ribosomal A-site. These stalled ribosomes are sensed and bound by stringent factor RelA. When binding to the 50S ribosomal subunit, RelA adopts an "open" active conformation making contacts both with rRNA and deacylated A-site tRNA, resulting in a distorted confirmation of the latter tRNA. Synthesis of (p)ppGpp is induced. When conditions become favorable, SpoT hydrolyses (p)ppGpp back to GTP or GDP and pyrophosphate (PPi) is released.

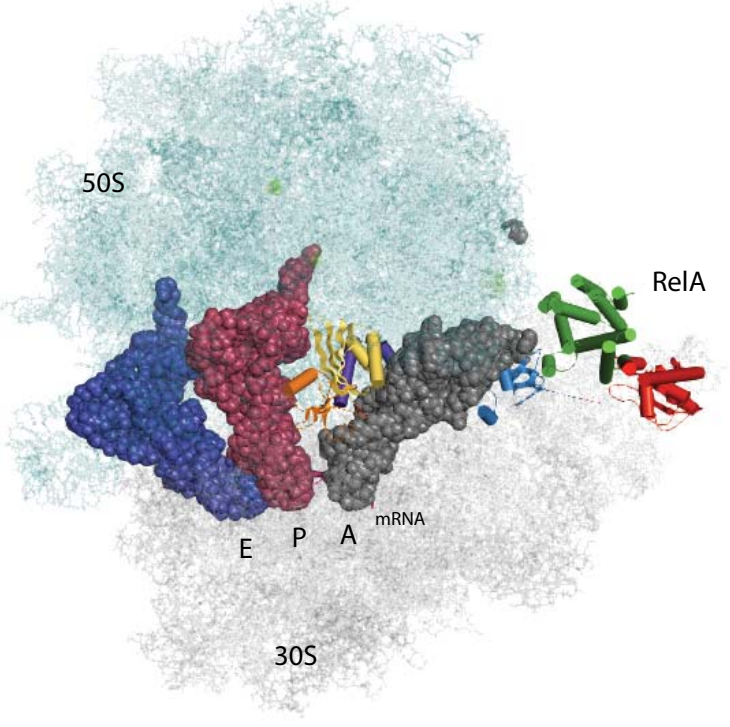

Figure 8. Structure of RelA bound on the ribosome. RelA is wrapped around A-site tRNA (dark grey) with its N-terminal Synthetase (red) and Hydrolase (green) domains being located outside of the ribosome structure. C-terminal part of RelA consisting of TGS (light blue), Helical linker (purple), ACT (yellow) and CC (orange) domains protrude into the intersubunit space of the ribosome. 50S ribosomal subunit is depicted in cyan, 30S subunit in light grey. E-site and P-site tRNA-s are in blue and pink respectively. PDB accession code is 5IQR. Adapted from [98].

3.2 Bifunctional RSH enzyme SpoT

The second RSH protein responsible for (p)ppGpp metabolism in Gamma- and Betaproteobacteria is SpoT. As opposed to RelA, SpoT is capable of both synthesizing and hydrolyzing (p)ppGpp [49]. Synthetic activity of SpoT is however rather weak compared to RelA, but intriguingly SpoT is capable of responding to various stress stimuli aside from amino acid starvation. These include limitations of several nutrients: carbon, phosphate, iron and fatty acids [49–51]. Another important outcome of (p)ppGpp synthesis by SpoT is maintaining basal levels of the alarmone during normal growth conditions. Basal levels produced by SpoT are in turn connected to the expression of virulence in many pathogenic bacteria, as only Δ*relA* Δ*spoT* double knockout strains – and not single mutants Δ*relA* – show attenuated virulence phenotypes [109]. The most important and essential function of SpoT is (p)ppGpp hydrolysis since deletion of *spoT* gene in *relA*-positive background leads to lethality, caused by uncontrolled accumulation of (p)ppGpp abolishing the cell growth [49]. It is worth mentioning that the double Δ*relA* Δ*spoT* deletion is not lethal, but leads to the $ppGpp^0$ phenotype, which is auxotrophic for multiple amino acids [49]. The essentiality of the SpoT-mediate detoxification prompted the idea of SpoT being a potential therapeutic target for developing new antibacterials. However, no attempts to specifically inhibit hydrolytic activity of SpoT have been made – or at least not reported so far – possibly, partially due to the difficulty of purification of the protein and lack of any structural data that could guide the *in silico* design of small molecule inhibitors. All of the currently available biochemical investigations of SpoT are made with either partially purified protein [110, 111] or a truncated version [52].

Although biochemical investigations of SpoT are very limited due to failure to successfully purify the protein, some interaction partners of SpoT are identified and mechanisms of its regulation elucidated. For example, Acyl Carrier Protein (ACP) is shown to interact with SpoT and activate its (p)ppGpp synthetic activity [52]. Thereby ACP, being a sensor of lipid metabolic status of the cell, signals inhibition of fatty acid synthesis to SpoT and stringent response is thereafter activated. Conversely, GTPase CgtA, was shown to bind SpoT in order for low (p)ppGpp levels to be maintained during exponential growth. Thus, repression of stringent response is assured in nutrient-replete conditions [112–114].

Catalytic site of SpoT contains conserved metal chelating His-Asp (HD) motif [115] and (p)ppGpp hydrolytic activity of SpoT is dependent on concentrations of Mn^{2+} ions [116].

3.3 Bifunctional RSH enzyme Rel

Although RelA and SpoT are, due to historic reasons, the most well-studied representatives of RSH family, the majority of bacterial species use single bifunctional enzyme, named Rel, for both synthesis and degradation of (p)ppGpp. Phylogenetic studies showed that Rel is ancestral to RelA and SpoT with latter

ones being products of *rel* gene duplication followed by functional diversification of the two copies [94, 117]. Combining both enzymatic activities for synthesis and degradation of (p)ppGpp in one protein makes Rel a fascinating study subject. Despite decades of research, it is yet unclear as to how the two opposing activities are regulated in such a manner that futile reaction cycles are avoided.

Several studies suggest reciprocal character of synthetic and hydrolytic activities of Rel protein. Hogg and colleagues showed that the NTD domain region of Rel from *Streptococcus dysgalactiae* subsp. *equisimilis* adopts two distinct conformations representing "hydrolase-ON/synthetase-OFF" and "hydrolase-OFF/synthetase-ON" states of the protein [118]. They suggest that substrate binding to the either of the active sites triggers conformational switch leading to deactivation of the opposing site.

Biochemical experiments with *M. tuberculosis* [119], *Thermus thermophilus* [120] and *B. subtilis* [121] Rel showed that, similarly to *E. coli* RelA, the (p)ppGpp synthesis by these enzymes is activated by deacylated tRNA on the ribosomal complex. This activation is accompanied by almost complete loss of (p)ppGpp hydrolysis activity – this again prompts the presence of regulatory communication between active sites of Rel [119]. Although biochemical investigations of *M. tuberculosis* Rel have showed that in this organism enzyme is activated by deacylated tRNA in the ribosomal A-site, microbiological experiments have shown that in live bacteria complete removal of nutrients is needed to activate the stringent response [122]. Other bacteria were also found to require alternative stimuli to activate (p)ppGpp production by Rel. For example, in *Helicobacter pylori* stringent response is elicited upon carbon starvation and acid stress [123]. In *Caulobacter crescentus* (p)ppGpp is produced in response to combined carbon and nitrogen limitations [124]. Rel protein in *Myxococcus xanthus* can detect limitations of amino acid, carbon, phosphate and nitrogen [125]. The diversity in signals that activate stringent response in different bacteria can be accounted for their need to cope with different lifestyles.

3.4 Monofunctional RSH SAS enzymes

Until the last decade, bi-functional Rel was considered as the sole RSH in Firmicutes responsible for both production and degradation of (p)ppGpp. However, in contrast to previous findings that in *B. subtilis* and *C. glutamicum* [63, 126] inactivation of *rel* gene led to (p)ppGpp^0 phenotype, it was shown for *S. mutans* that Δ*rel* mutant was still able to produce (p)ppGpp [127]. Latter observation indicated that in *S. mutans* there are other sources of (p)ppGpp besides Rel. Indeed, *S. mutans* genome (UA159) was found to contain two ORFs encoding single-domain ppGpp synthetases homologous to RelA/SpoT synthetase domain but lacking the hydrolase domain. These Small Alarmone Synthetase enzymes were shown to produce (p)ppGpp [128]. More recently extensive phylogenetic studies of RSH enzymes across the tree of life showed that SAS proteins are

ubiquitous in Firmicutes [94], making the typical repertoire of RSHs in Firmicutes – one long bifunctional Rel and one or two short monofunctional SAS proteins, usually named RelQ and RelP, with the latter one being absent in some species. Surprisingly another class of SAS named RelV is also found in a representative of Gammaproteobacterium *Vibrio cholerae* [129]. Such extensive presence of SAS enzymes suggests their evolutionary advantage and raises the question of their special function in bacteria. Although there is still vast uncertainty in the contribution of SAS factors to bacterial physiology, recent studies have shed some light onto mechanism of their action and their possible biological roles [55].

Recent structural data showed that *B. subtilis* RelQ forms a symmetric oval-shaped homotetrameric structure that binds two pppGpp molecules at the interface of subunits (**Fig. 9**). Biochemical data supports that bound pppGpp molecules in the cleft act as allosteric activators of the *B. subtilis* RelQ and that tetrameric structure of the protein is essential for its enzymatic activity [130].

The activity of SAS enzymes is believed to be regulated on transcriptional level, meaning there is a direct correlation between increase in (p)ppGpp levels and the abundance of the enzyme. SAS proteins are up-regulated in response to different stress stimuli. For example, in *B. subtilis* expression of SAS1 (RelQ) is promoted in response to cell wall damage [93], *B. subtilis relP* transcription is upregulated by alkaline shock [131], in *S. aureus* by cell wall stress [132], in *Streptococcus mutans* by oxygen stress [133]. SAS proteins are not only activated during stress, but constitutively produce basal levels of (p)ppGpp during favorable growth conditions [43, 128]. As earlier, basal levels of (p)ppGpp are necessary for keeping normal metabolism of the cell during unrestrained growth as well as needed for fully efficient mounting of the stringent response, also for GTP homeostasis, virulence and resistance to cell wall antibiotics [43, 61].

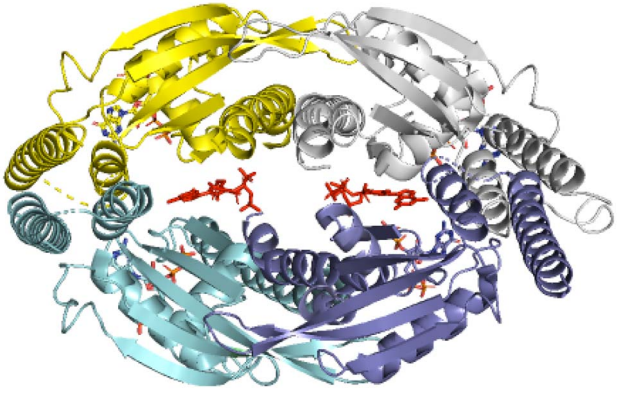

Figure 9. Tetrameric structure of *B. subtilis* RelQ with two allosteric pppGpp molecules in the central cleft (shown in red). PDB accession code 5DED, adapted from [130].

3.5 Monofunctional RSH enzyme Metazoan SpoT homolog protein 1, Mesh1

Intriguingly, RSH enzymes were identified in animals. The so-called metazoan SpoT homolog 1 (Mesh1) protein possesses conserved Mn^{2+} binding catalytically active HD domain and structurally is very similar to bacterial SAH enzymes [134]. Mesh1 has been shown to degrade (p)ppGpp *in vitro* as well as to complement the lack of SpoT in *E. coli* cells. The physiological substrates and the role of these proteins in animals is however elusive as there is no (p)ppGpp and no proteins that synthesize the alarmones in metazoa. Nevertheless, the deletion of *mesh1* leads to substantial changes in physiology of Drosophila. Δ*mesh1* mutants showed retarded body growth and impaired response to amino acid starvation [134]. These findings indicate the importance of Mesh1 in starvation responses in higher organisms prompting functional conservation of the protein. One recent study embarked on a search for potential substrates of Mesh1 in mammalian cells and it was proposed that NADPH could be the natural substrate of the protein [135]. However, an independent study contradicted these results by showing no changes in NADPH levels in *E. coli* cells upon expression of Mesh1 [136].

4. Role of (p)ppGpp in bacterial virulence and inhibition of the stringent response as a therapeutic strategy

Many pathogenic bacteria rely on (p)ppGpp signaling to coordinate the expression of their virulence genes and to confer survival in the host organisms [109]. For example, (p)ppGpp induces bacterial adherence of enterohemorrhagic and uropathogenic *E. coli* strains, the virulence trait of these bacteria is colonization on epithelial cells of intestine and urinary tract, respectively [137, 138]. Absence of (p)ppGpp in *Salmonella enterica* leads to inability to invade and replicate in intestinal epithelial cells [139]. Finally, the viability during host invasion requires functionality of the stringent response. For example in case of *F. tularensis*, *H. pylori* and *S. aureus* (p)ppGpp is needed for macrophage survival [109].

Recently the connection of (p)ppGpp to formation of so-called persister cells became a hotly debated topic. According to widely accepted model (p)ppGpp accumulation leads to activation of toxin-antitoxin (TA) systems, which, in turn, was proposed to be connected to persistence [140]. However, the experimental papers supporting that model has been questioned [141–143], resulting in the retraction of the original papers [140, 144, 145].

Since the growing body of evidence supports the involvement of (p)ppGpp-mediated signaling in bacterial virulence and survival within host organisms during infection makes the stringent response a potential therapeutic target. In recent years several classes of compounds were tested as potential stringent response inhibitors: i) antibiotics acting via inhibition of translation leading to inhibition of Rel/RelA RSH enzymes, ii) compounds that directly inhibit synthetic activity of RSH proteins and iii) compounds that catalytically degrade (p)ppGpp.

4.1 Antibiotics that target translation

Antibiotics thiostrepton and tetracycline directly inhibit Rel/RelA activation by starved ribosomes [146, 147]. Tetracycline blocks tRNA incorporation to the A-site of the ribosome and deacylated tRNA in the A-site is a prerequisite for activation of the Rel/RelA, thus leading to efficient inhibition of the (p)ppGpp synthesis [104, 148].

Thiostrepton inhibits translation by interfering with the binding and action of several translational GTPases such as EF-G, EF-Tu and IF2 [149–151]. The inhibition is mediated by the antibiotic binding to the cleft between helices 43 and 44 of the 23S rRNA and ribosomal protein uL11 [152]. Since uL11 is indispensable for activity of RelA [108], it was suggested that thiostrepton inhibits (p)ppGpp synthesis by interfering with the function of uL11 and was shown to do such *in vitro* [38, 148].

In addition to specific inhibition of RSHs activation by ribosomal complexes, all antibiotics that target protein synthesis universally abrogate the stringent response indirectly since blocking of translation leads to accumulation of aminoacyl-tRNAs and, consequently, decrease in the concentration of deacylated

tRNAs. Due to its fast uptake chloramphenicol is most widely used antibiotic as inhibitor of the stringent response in microbiological experiments [153, 154].

4.2 ppGpp analogues

Synthetic activity of RSH proteins was also targeted directly by using synthetic analogues of (p)ppGpp that would bind to the catalytic site of Rel/RelA proteins and outcompete the GTP/GDP substrates [155]. The (p)ppGpp molecule is a poor starting point for developing an antimicrobial: it is large, unstable and highly charged, therefore unable to penetrate cell membrane.

First such compound was Wexselblatt's bisphosphonate or (10) [155]. In this compound pyrophosphate moieties at 3' and 5' positions are substituted with non-hydrolysable methylene-bisphosphonate groups. Although in the test tube it showed moderate inhibitory effect on Rel proteins, the IC_{50} in mM range is physiologically irrelevant. Another problem is that as this compound is highly hydrophilic it is inactive against live bacteria [155].

Further modifications of compound (10) led to development of ppGpp analogue inhibitor Relacin [156]. Its structure contains less charged due to the use of diglycine moieties instead of the pyrophosphate groups and the guanine base is carrying 2-N-isobutyryl protecting group. The resultant molecule is less hydrophilic and it was shown to have inhibitory effect *in vivo* as well as *in vitro*, but only at physiologically irrelevant millimolar concentrations [156, 157].

4.3 Peptide 1018

One more compound that was suggested to inhibit the (p)ppGpp-mediated signaling is anti-biofilm peptide 1018 [53]. Since this compound is active against broad-spectrum of bacteria, it was suggested to exert its activity by affecting some widely conserved bacterial mechanism. Since the cells treated with 1018 fail to accumulate (p)ppGpp, it was suggested that 1018 directly binds to the (p)ppGpp and marks it for degradation [53].

However, the follow-up studies questioned the proposed mechanism of 1018 as a specific stringent response inhibitor [158, 159]. Firstly, peptide 1018 does not inhibit biofilm formation specifically, but eradicates planktonic cells with the same efficiency. Secondly, the control peptide, with the inverted amino-acid sequence of 1018 (named respectively 8101) had even better antibacterial efficiency than 1018 itself [158]. Moreover, the antibacterial efficiency of 1018 does not seem to rely on specific degradation of (p)ppGpp, since it is equally potent against wild type and $ppGpp^0$ (i.e. lacking RSH enzymes) strain [159]. Finally, the inverted 8101 version co-precipitates with ppGpp equally well as the original 1018 [158]. Later Bryson and colleagues [160] re-confirm the inefficacy of both Relacin and peptide 1018 in targeting the stringent response inhibition as neither of these compounds were able to reverse tolerance and/or growth defect phenotype exhibited by elevated (p)ppGpp levels in *S. aureus*

AIMS OF THE STUDY

The topic in this study is investigation of the biochemical properties of two SAS proteins:

- *E. faecalis* RelQ (Papers I and II)
- *S. aureus* RelP (Paper III)

Specifically, the focus of the study was identification of the cellular components responsible for regulation of the enzymatic activity of those proteins and characterize the enzymatic properties of the RSHs in question.

MATERIALS AND METHODS

All materials and methods are described in detail in publications I, II and III. The most important methods are outlined here.

1. Enzymatic assays

Enzyme experiments for both *E. faecalis* RelQ and *S. aureus* RelP were performed at 37 °C in HEPES:Polymix buffer [161]: 25 mM Hepes pH 7.5, 0.5 mM CaCl, 95 mM KCl, 5 mM NH_4Cl, 8 mM putrescine, 1 mM spermidine, 5 mM K_3PO_4 pH 7.3 with 5 mM Mg^{2+} supplemented with 1 mM beta-Mercaptoethanol. Reaction mixtures containing 250 nM *E. faecalis* RelQ or *S. aureus* RelP and 300 or 200 µM ^{3}H-GDP were pre-incubated for 2 min at 37 °C in the presence of fixed or increasing concentrations of different effectors: (p)ppGpp, metal ions, Relacin, mRNA coding for a Met-Phe (MF) dipeptide, 5'-GGCAAGGAGGUA AAAAUGUUCAAA-3'. *E. faecalis* RelQ was also tested in presence of starved ribosomal complex or its separate components (0.5 µM *E. coli* 70S, 2 µM $tRNA^{Phe}$ and $tRNA^{Met}$, 2 µM model mRNA encoding the Met-Phe (MF) dipeptide) **(Fig. 10)**. Thereafter reaction is activated by 1 mM ATP.

After reaction initiation by ATP, the 5 µl aliquots were taken throughout the course of the reaction and quenched with 4 µl of 70% formic acid supplemented with a cold nucleotide standard (10 mM GDP and 10 mM GTP) used for UV shadowing after resolution on PEI-TLC plates (Macherey-Nagel). Nucleotides were resolved in 0.5 mM KH_2PO_4 pH 3.5 buffer, after which the plates dried and then cut into sections as guided by UV shadowing. ^{3}H radioactivity was quantified by scintillation counting in Optisafe-3 scintillation mixture (PerkinElmer/ Fisher). Progression of the reaction was quantified as substrate (^{3}H-GDP) to product (^{3}H-ppGpp) conversion, [^{3}H-ppGpp/(^{3}H-ppGpp + ^{3}H-GDP)].

2. Electromobility shift assay (EMSA)

10 µl of total reactions were performed in Hepes:Polymix buffer with at 37 °C. Before the reaction mixtures were assembled, stock mRNA was incubated for 2 min at 65 °C to melt possible secondary structures. Reaction mixtures were assembled by adding fixed or increasing concentrations of *E. faecalis* RelQ or *S. aureus* RelP to the mRNA (final concentration 0.15 µM), followed by the addition of RelQ and 4 U/µl of RiboLock RNase inhibitor (Thermo Fisher Scientific). In case of *E. faecalis* RelQ, combinations of nucleotides (final concentrations: 1 mM ATP, 1 mM GDP and 100 µM ppGpp) were added prior to addition of the enzyme. The reaction mixture is then incubated for 10 min at 37 °C, after which 5 µl of loading dye (40% sucrose supplemented with

bromphenol blue) was added per 10 µl (i.e. 1.5 pmol of mRNA) and the samples resolved on 12–15% Tris:borate:EDTA (TBE) gels run at 4 °C (120–140 V) for 1.5–2 hours. Gels were stained with SYBR Gold nucleic acid stain (LifeTechnologies) for 10 minutes prior to visualization using a Typhoon Trio Variable Mode Imager (Amersham Biosciences).

3. Preparation of recombinant proteins

3.1 *E. faecalis* RelQ

E. faecalis RelQ protein was purified from *E. coli* BL21(DE3) chemical competent cells transformed with pET28a plasmid encoding C-terminally 6His-tagged RelQ. Cells were grown in 400 ml LB medium under antibiotic selective pressure. Protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD_{600} 0.6 and grown for additional 2 hours at 30 °C. Cells were harvested by centrifugation for 15 minutes at 3000 rpm and diluted in cell opening buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, 10% glycerol, 1 mM β-Mercaptoethanol (βMe), pH 8) with addition of 1 µg/ml DNase-1 and 1 mM PMSF. Cells were lysed by Stansted Pressure Cell Homogeniser FPG12800 after which cell debris were removed by centrifugation for 30 min at 15 000 rpm. Supernatant was loaded on the 1 ml His-trap Ni^{2+} column (GE Healthcare) equilibrated with Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 25 mM Imidazole, 10% glycerol, 1 mM βMe, pH 8. After additional wash by increasing ionic strength to 1 mM NaCl, protein was finally eluted with increasing imidazole concentration over 20 minutes, until achieving 100% Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 300 mM Imidazole, 10% glycerol, 1 mM βMe, pH 8. Fractions containing pure protein were pooled and concentrated against Storage buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM EDTA, 5 % glycerol) using Amicon Ultra Centrifugal Filters. Protein concentration was measured by Bradford protein assay.

3.2 *S. aureus* RelP

Protein expression and purification procedure was the same as for *E. faecalis* RelQ with the exception of buffers used:

- Cell opening/Washing buffer: 50 mM Tris-HCl pH 8, 500 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole, 1 mM βMe
- Elution buffer: 50 mM Tris-HCl pH 8, 500 mM NaCl, 5 mM $MgCl_2$, 300 mM imidazole, 1 mM βMe
- Storage buffer: 30 mM Tris-HCl pH 8, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM βMe, 5% glycerol.

RESULTS AND DISCUSSION

1. Biochemical characterization of *E. faecalis* RelQ

Enterococcus faecalis is a Firmicute bacteria encoding both long bifunctional RSH Rel and short monofunctional RSH RelQ [59]. This bacteria is commensal in our gut, but is also opportunistic pathogen causing several infections ranging from urinary tract infections to severe conditions like endocarditis or bacteremia [162]. Commonly these diseases are hospital-acquired and this can be accounted for the ability of *E. faecalis* to withstand many adverse conditions like prolonged starvation, exposure to sanitizers and most importantly to antibacterial treatment. *E. faecalis* has intrinsic and acquired resistance towards many commonly used antibiotics like penicillin, clindamycin, aminoglycosides and most of cephalosporins [163, 164]. This bacterium accumulates (p)ppGpp in response to stresses like alkaline and heat shock, and vancomycin treatment. Importantly, resistance to vancomycin was shown to be dependent on functionality of RelQ protein [43, 59, 60]. Therefore, given the clinical relevance, we focused to dissect the mechanism of *E. faecalis* SAS enzyme through biochemical investigations.

When I started working on the project, most of what was known about SAS enzymes was based on transcriptional studies [60], and the enzymatic mechanisms of SAS were unexplored. Given the domain organization of SAS enzymes, we reasoned that they have different enzymatic properties from that of long RSHs. SAS lack the C-terminal regulatory domain region (CTD) of long RSHs, which in case of *E. coli* RelA mediates ribosome binding and RelA activation. The lack of CTD suggests that SAS RSHs are being regulated differently, specifically that SAS enzymes act independently of the ribosome.

In our laboratory we have established a biochemical experimental setup for studying enzymatic properties of long RSH *E. coli* RelA [100]. This system mimics the native intracellular signal that induces the activation of RelA in starved cells, i.e. the stalled ribosomal complexes. In our system, we reconstitute the said complexes from purified 70S *E. coli* ribosomes, model mRNA, and native deacylated *E. coli* tRNA. Using radioactive nucleotide substrates combined with separation of nucleotides on TLC and consequent scintillation counting, we applied this system to follow the synthesis of (p)ppGpp by *E. faecalis* RelQ. The aim was to answer the following questions:

1. Is the enzymatic activity of *E. faecalis* RelQ regulated by starved ribosomal complexes or/and individual components of the complex: ribosomes, mRNA or tRNA?
2. Is *E. faecalis* RelQ allosterically regulated by ppGpp/pppGpp similarly to *E. coli* RelA?
3. What is the preferred nucleotide substrate of RelQ: GDP or GTP?

1.1 *E. faecalis* RelQ activity is not induced by 70S ribosomes or tRNA, and is inhibited by model mRNA(MF)

E. coli RelA is virtually inactive in the absence of 70S ribosomes and it is strongly activated by the starved ribosomal complexes consisting of 70S ribosomes, model mRNA(MF) coding MF dipeptide, deacylated initiator $tRNA_i^{Met}$ in A-site and $tRNA^{Phe}$ in the P-site. As predicted, the enzymatic activity of *E. faecalis* RelQ is insensitive to addition of 70S ribosomes (**Fig. 10**). Surprisingly, when the starved complexes are added, the enzymatic activity of RelQ is almost completely abolished. This observation raises the question: which individual component of the RelA-activating starved ribosomal complex responsible for the inhibition of RelQ activity? By testing the effects of each component separately we demonstrated that it is the mRNA(MF) that is responsible for the effect. This phenomenon was later investigated in detail, see section '*2. RelQ combines two archetypical regulatory mechanisms: nucleotide messenger-mediated signaling and RNA binding*', below.

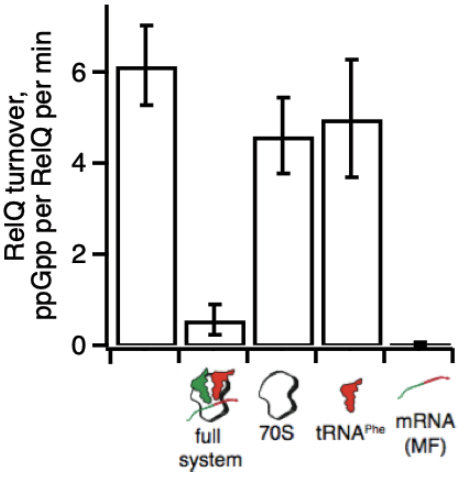

Figure 10. ppGpp synthesis activity of *E. faecalis* RelQ in the presence of ATP and GDP and substrates alone (first bar), starved ribosomal complex (second bar) and individual components of the ribosomal complex (70S ribosomes, $tRNA^{Phe}$ and mRNA(MF)) as indicated on the figure.

The synthesis activity of multi-domain *E. coli* RSH RelA is allosterically activated by the product, ppGpp/pppGpp, with the latter being the primary effector [100, 165]. Single-domain *E. faecalis* RSH RelQ displays the same effect: addition of 100 μM ppGpp alleviates the lag-effect of the ppGpp production time course by *E. faecalis* RelQ, just like it does in the case of *E. coli* RelA (**Fig. 11**). In an excellent agreement with our results, later an independent report from Gert Bange lab demonstrated that pppGpp positively regulates activity of *B. subtilis* RelQ by binding to the central regulatory cleft of the protein [130].

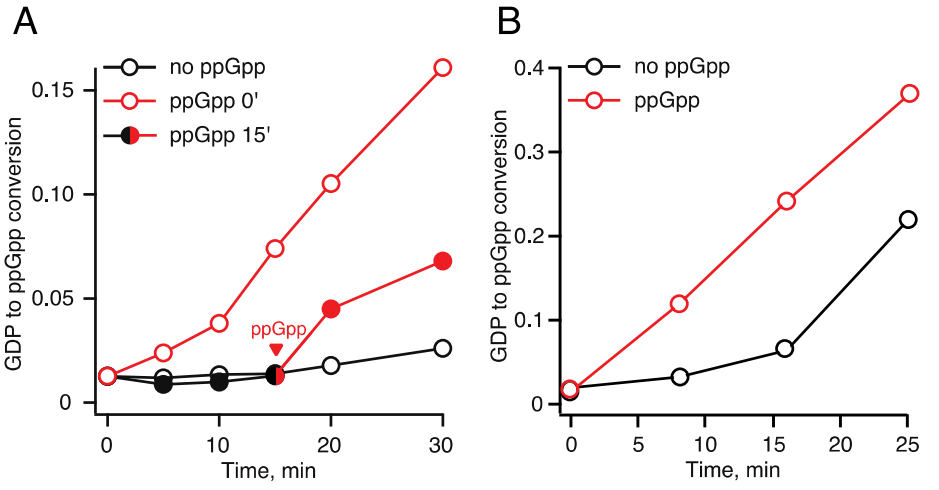

Figure 11. ppGpp activates synthetic activity of *E. coli* RelA (A) and *E. faecalis* RelQ (B). Empty black circles represent the time course of ppGpp synthesis without addition of ppGpp, empty red circles – with ppGpp added. On panel A ppGpp is added at the 15 minute timepoint.

1.2 *E. faecalis* RelQ has a preference to GDP substrate and pppGpp activator

Our next question was what is the preferred substrate of RelQ (GDP or GTP) and what is the preferred activator (ppGpp or pppGpp). We have determined the kinetic properties (k_{cat} and K_m) of *E. faecalis* RelQ enzyme, and estimated the relative efficiency of GTP and GDP utilization as substrates by calculating the specificity constant k_{cat}/K_m (**Table 1**). We preformed our enzyme kinetics experiments in the presence of either 100 μM ppGpp or pppGpp.

RelQ has a moderate preference (i.e. higher k_{cat}/K_m) toward GDP over GTP as a substrate (**Fig. 12**), but the nature of the added alarmone fine-tunes the extent of this preference (**Table 1**). Specifically, in the presence of ppGpp RelQ has an approximately 2-fold-higher preference toward GDP over GTP: $k_{cat}/K_m(GDP^{ppGpp}) = 2.08 \pm 0.82\ mM^{-1}s^{-1}$ versus $k_{cat}/K_m(GTP^{ppGpp}) = 1.19 \pm 0.43\ mM^{-1}s^{-1}$. Addition of pppGpp promotes the preference towards GDP approximately 4-fold: $k_{cat}/K_m(GDP^{pppGpp}) = 3.90 \pm 1.28\ mM^{-1}s^{-1}$ versus $k_{cat}/K_m(GTP^{pppGpp}) = 0.87 \pm 0.30\ mM^{-1}s^{-1}$. Collectively, our results demonstrate that *E. faecalis* RelQ prefers GDP as a substrate and pppGpp as an activator.

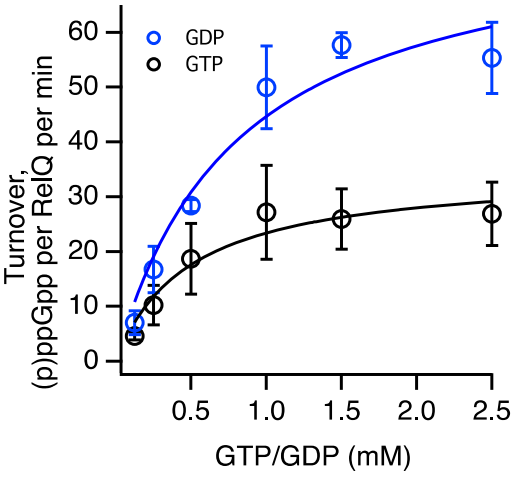

Figure 12. *E. faecalis* RelQ utilizes GDP more readily as a substrate. Increasing concentrations of substrates (GTP or GDP) were supplied to *E. faecalis* RelQ and turnover rates of (p)ppGpp synthesis (^{3}H ppGpp synthesized per RelQ per minute) were obtained in the absence of (p)ppGpp.

Table 1. Kinetic constants for ppGpp and pppGpp production by *E. faecalis* RelQ in the presence of ppGpp or pppGpp.

Substrate	Cofactor	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
GTP	ppGpp	0.58 ± 0.07	0.49 ± 0.17	1.19 ± 0.43
	pppGpp	0.74 ± 0.1	0.85 ± 0.27	0.87 ± 0.3
GDP	ppGpp	1.34 ± 0.2	0.65 ± 0.24	2.08 ± 0.82
	pppGpp	0.81 ± 0.07	0.21 ± 0.07	3.9 ± 1.28

1.3 Relacin does not inhibit *E. faecalis* RelQ

Since the (p)ppGpp-mediated signaling is implicated in virulence of pathogenic bacteria, in recent years it became a target for development of new antibacterials [166], such as ppGpp analogue Relacin [156]. In case of several bacterial species the basal (p)ppGpp levels were shown to be responsible for virulence/pathogenicity and antibacterial tolerance [109]. Since in Firmicute bacteria SAS proteins are responsible for the maintenance of these basal (p)ppGpp levels, it is essential to efficiently target those RSH enzymes when developing new antibacterials that are aimed to compromise the (p)ppGpp-mediated signaling. However, so far only long ribosome-dependent RSH proteins have been tested for inhibition by Relacin [156].

We have tested the efficiency of *E. faecalis* RelQ inhibition by Relacin, adding increasing concentrations of Relacin to ppGpp synthesis reactions (**Fig. 13**). As a positive control, we used *E. coli* RelA. Consistently with the results of Wexselblatt and collogues [156], *E. coli* RelA is dose-dependently inhibited by Relacin. However, *E. faecalis* RelQ is virtually immune to Relacin. Even addition of 5 mM Relacin – a concentration that completely inhibited RelA – have no significant effect. We concluded that bacterial species that encode SAS RSHs might not be successfully targeted by compounds that were optimized to inhibit long RSH. Further analyses are needed to assess if SAS representatives from other bacteria will also be insensitive to Relacin.

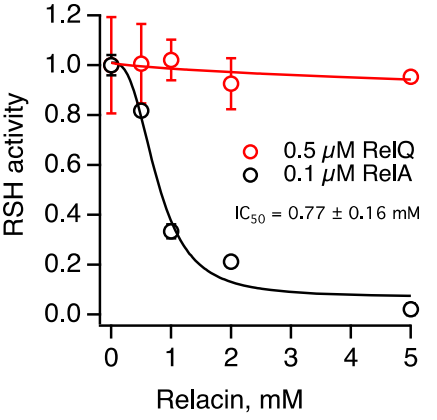

Figure 13. While the enzymatic activity of *E. coli* RelA is moderately inhibited by Relacin *E. faecalis* RelQ is immune to the compound. To calculate the RSH activity, the turnover rate (^{3}H ppGpp synthesized per RelQ per minute) in the presence of Relacin was divided by that in the absence of Relacin, total ppGpp accumulation in the absence of Relacin is set to 1.

2. RelQ combines two archetypical regulatory mechanisms: nucleotide messenger-mediated signaling and RNA binding

When we analyzed biochemical properties of *E. faecalis* RelQ we discovered that mRNA(MF) inhibits the synthesis of (p)ppGpp by RelQ (**Fig. 10**). This kind of effect has never been observed for other RSH proteins, thus prompting a more detailed investigation. We reasoned that the biological purpose of this RelQ:mRNA interaction could be either regulation the enzymatic activity of RelQ, or, alternatively, RelQ could be acting as an RNA-binding protein, similarly to classical RBPs CsrA and Hfq. Therefore, we decided to characterize the relationship between the RelQ:mRNA and RelQ:(p)ppGpp complex formation on one hand and RelQ's enzymatic activity on the other, using a combination of enzymatic

assays and binding assays – electrophoretic mobility shift assays, EMSA, to study RelQ:mRNA complex formation and Differential Radial Capillary Action of Ligand Assay, DRACALA, to study RelQ:(p)ppGpp complex formation

First, by titrating increasing concentrations of model mRNA(MF) and following its effect on ppGpp production by RelQ, we demonstrated that this RNA a very potent inhibitor. It is able to abolish ppGpp synthesis of 250 nM RelQ at concentration as low as 150 nM (**Fig. 14A**, empty black circles). ppGpp has an opposing effect on RelQ's enzymatic activity, serving as a strong allosteric activator (**Fig. 11B**). When 100 µM of ppGpp is added in mRNA(MF) titration experiments, the alarmone efficiently mitigates the inhibition by mRNA (**Fig. 14A**, empty red circles). The alarmone does not, however, abrogate the inhibition completely, as 1 µM mRNA still inhibits RelQ activity approximately five-fold in the presence of 100 µM ppGpp.

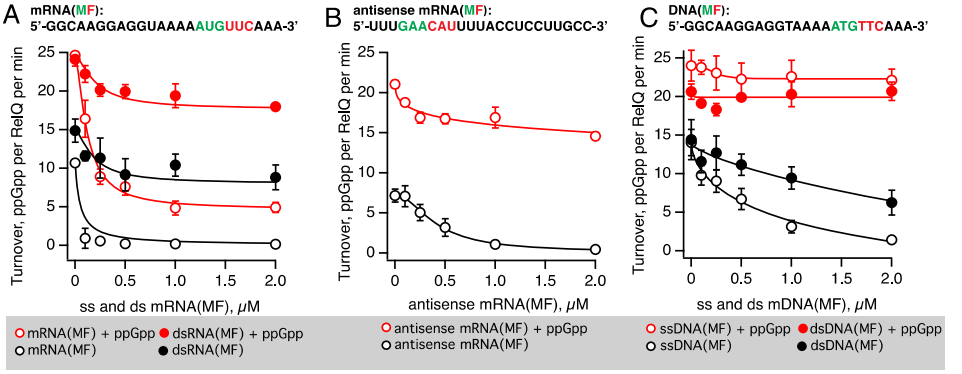

Figure 14. Single-stranded mRNA(MF) potently inhibits ppGpp synthesis by *E. faecalis* RelQ. The inhibition is sequence-specific (**A**, empty black circles**)** and is mitigated by ppGpp (**A**, empty red circles**)**; other derivatives of this mRNA including its double-stranded (**A,** filled circles) and antisense (**B**) versions as well as corresponding single- and double-stranded DNA (**C**) show poor inhibition of RelQ activity. Titrations were performed with increasing concentrations of single-stranded (empty circles) and double-stranded (filled circles) nucleotides in the presence (red circles) or absence (black circles) of 100 µM ppGpp.

Next, we evaluated the structure and/or sequence specificity of the RelQ inhibition by mRNA by testing a panel of derivatives of the model mRNA(MF). Specifically, we used its antisense (**Fig. 14B**) and double-stranded versions (**Fig. 14A,** filled circles), also corresponding single-stranded and double-stranded DNA (**Fig. 14C**) as well as made mutational analysis of this mRNA to look for the sequence specificity (**Fig. 15**). Taken together, the results of these experiments demonstrated that:

1) RNA is significantly better inhibitor than DNA
2) Single stranded nucleic acids are more efficient inhibitors than double stranded
3) Inhibition by single-stranded RNA is sequence-specific, with a tentative consensus GGAGG
4) ppGpp has a universal protective effect.

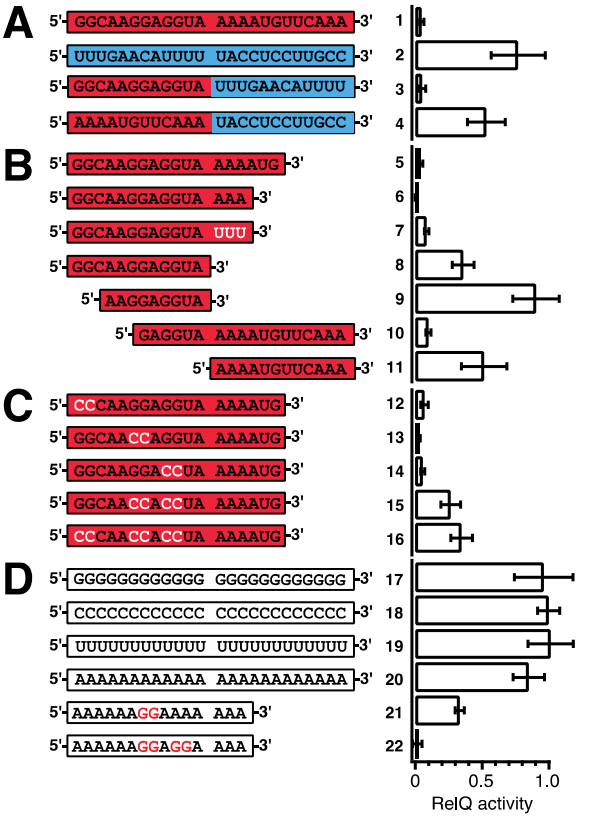

Figure 15. mRNA sequence specificity of RelQ inhibition. As a starting point, we use 24 nucleotide long inhibitory model mRNA(MF) (red) and its ineffective complementary antisense RNA (blue). By swapping the 3' and 5' halves of these RNA molecules, **(A)** we demonstrated that that 5' half of mRNA(MF) is an essential for inhibition of RelQ. Next, we probed the length of the inhibitory RNA **(B)** and identify length requirement of 12–15 nucleotides. Prompted by the fact that the inhibitory mRNA contains Shine-Dalgarno sequence AGGAGG, which is, in turn, reminiscent of the CsrA's target consensus RUACARGGAUGU, we next tested the necessity of this sequence by mutating the GG motifs in inhibitory mRNA **(C)** or adding GG motifs in otherwise inactive polyA sequence **(D)**. We found that only sequence containing GGAGG cluster is able to completely inhibit RelQ activity. To calculate the RelQ activity, the turnover rate (^{3}H ppGpp synthesized per RelQ per minute) in the presence of RNA was divided by that in the absence of RNA.

Our next question was whether the observed inhibition of RelQ by mRNA(MF) takes place through complex formation between the two. Using EMSA I directly demonstrated the complex formation (**Fig. 16A**). Importantly, the complex formation is incompatible with protein's enzymatic activity: simultaneous addition of both of the substrates (GDP and ATP) to EMSA assay results in RNA dissociation from the protein (**Fig. 16B**). However, this effect did not happen when individual substrates are added (**Fig. 16B**) or when other nucleotides, that are not

accepted by the enzyme or the non-hydrolysable ATP analog AMPCPP added in combination with GDP (see paper II, **Fig. S4C**). We reasoned that the very act of ppGpp synthesis, rather than binding of the nucleotides is responsible for dislodging of mRNA from RelQ. In agreement with this idea, catalytically inactive D82G mutant variant of RelQ protein forms complex with mRNA as efficiently as wild-type RelQ, but this complex is insensitive to addition of ATP, GDP or ppGpp (**Fig. 16C**).

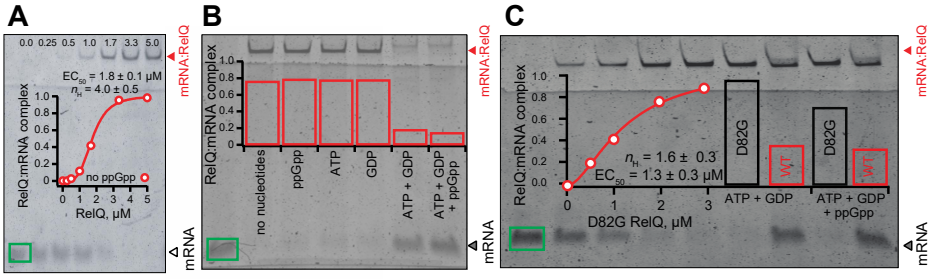

Figure 16. RelQ binding to mRNA and ppGpp synthesis are incompatible. (**A**) Complex formation between 0.15 μM mRNA and increasing concentrations of *E. faecalis* RelQ was monitored by EMSA. (**B**) Simultaneous addition of substrates ATP and GDP disrupts RelQ:mRNA complex, same is not observed when individual nucleotides are added. Addition of 100 μM ppGpp does not have strong effect on this complex as opposed to its strong effect on enzymatic activity of the protein. (**C**) Enzymatically inactive RelQ D82G binds mRNA with same efficiency as wild-type RelQ, however this complex is insensitive to addition of substrates ATP and GDP.

Next we compared the effects of ppGpp and pppGpp on RelQ:mRNA complex formation. In agreement with stronger effect on induction of the enzymatic activity of RelQ [130], pppGpp also destabilizes RelQ:mRNA complex much more potently than ppGpp. Titration of (p)ppGpp in EMSA assay showed that pppGpp potently abrogated mRNA binding to RelQ with an EC_{50} of 35 ± 6 μM (**Fig. 17B**), ppGpp on the other hand is able to disrupt the complex only when titrated up to 1 mM (**Fig. 17A**). Finally, we have analyzed complex formation between pppGpp/ppGpp and RelQ by DRACALA assay. In agreement with the data for *B. subtilis* [167] pppGpp efficiently bound *E. faecalis* RelQ with EC_{50} of 2.1 ± 0.1 μM. ppGpp on the other hand was a poor binder and even in the presence of 20 μM RelQ only 10% of ppGpp was associated with the protein (**Fig. 17C**). ^{32}P-labelled pppGpp was displaced from RelQ by increasing concentrations of mRNA(MF) with an IC_{50} of 2.8 ± 0.1 μM (**Fig. 17D**). Similar effect was observed for ^{32}P-ppGpp (IC_{50} of 5.2 ± 1.9 μM), but not for ^{32}P-ATP (data not shown). These results demonstrate that (p)ppGpp and mRNA binding to *E. faecalis* RelQ are mutually exclusive.

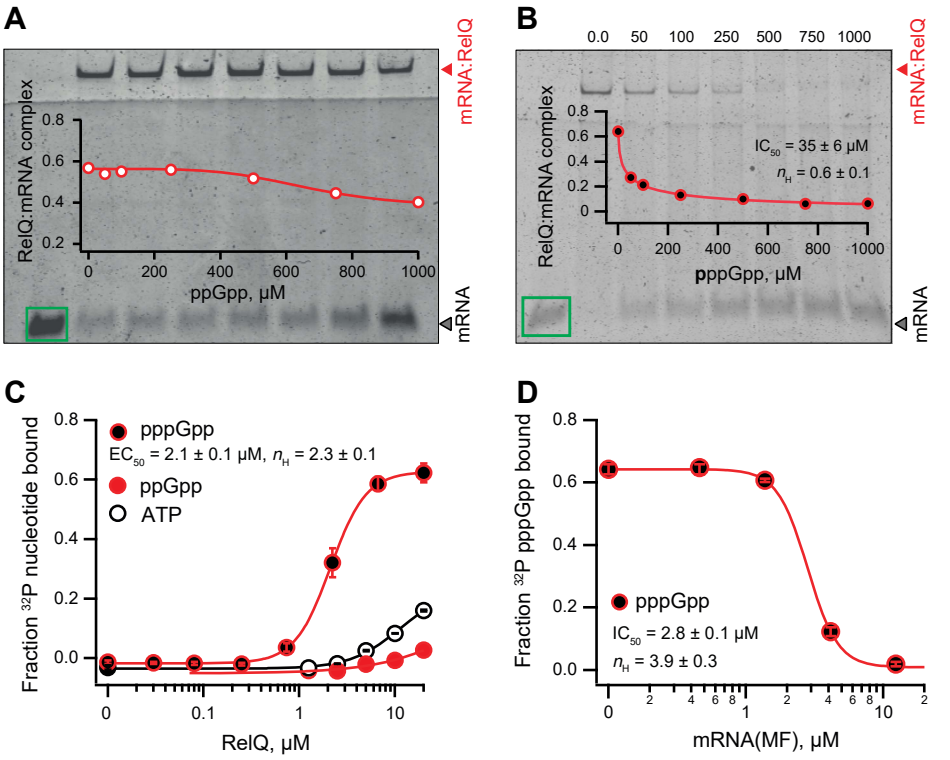

Figure 17. pppGpp and mRNA have destabilizing effect on each other's binding to RelQ, ppGpp has only moderate effect on RelQ:mRNA complex. (**A** and **B**) EMSA analysis of complex between 0.15 μM mRNA(MF) and 2 μM RelQ in presence of increasing concentrations of ppGpp (**A**) and pppGpp (**B**). (**C**) DRaCALA analysis of complex formation between increasing concentrations of RelQ with 50 nM ^{32}P-labelled ATP, ppGpp and pppGpp. (**D**) Increasing concentrations of mRNA(MF) displace ^{32}P-pppGpp from 20 μM RelQ as monitored by DRaCALA.

Given these differences in efficiency of dislodging mRNA:RelQ complex, we also next characterized the protective effect of ppGpp and pppGpp against mRNA-mediated inhibition in enzymatic assays. Using GDP or GTP as a substrate, we titrated ppGpp or pppGpp in the presence of mRNA(MF) (**Fig. 18**). Concentration of mRNA was kept constant at the level that ensured complete inhibition of RelQ's enzymatic activity in the absence of allosteric regulators. When GDP was used as a substrate, pppGpp had the most pronounced protective effect, completely rescuing the inhibition by mRNA(MF) at EC_{50} of 21.4± 15 μM. Other combination of nucleotides show lower protective effect. The combination of GTP with ppGpp fails to rescue any enzymatic activity, which is in good agreement with highest catalytic activity of RelQ attained in the presence of the allosteric activator pppGpp and GDP substrate.

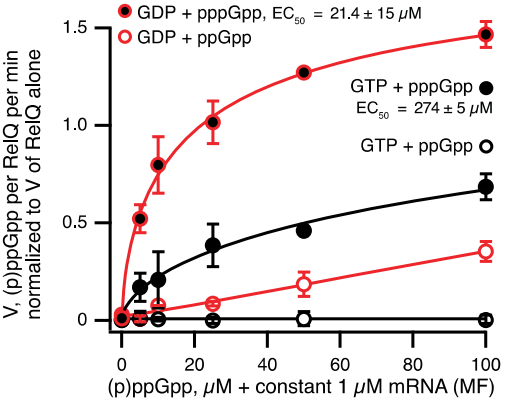

Figure 18. The combination of GDP as a RelQ substrate and pppGpp as allosteric activator provides best protective effect against mRNA(MF) inhibition.

Based on these results, we propose a working model according to which RelQ: RNA interaction acts as a regulatory switch between inactive and active forms of the protein (**Fig. 19**). RelQ is in an inactive state while it is bound to its target mRNA; increase in (p)ppGpp levels allosterically stimulates RelQ's synthetase activity and leads to its dissociation from RNA target. Based on the similarity of consensus sequence GGAGG that we found to be essential for RelQ inhibition to the Shine-Dalgarno sequence AGGAGG we suggest that RelQ can also regulate ribosome-availability of its target mRNA. Therefore, RelQ combines the two post-transcriptional regulatory paradigms – RNA binding similarly to Csr/Hfq and synthesis and responding to second messenger (p)ppGpp. However further analyses are needed to investigate the cellular targets of RelQ.

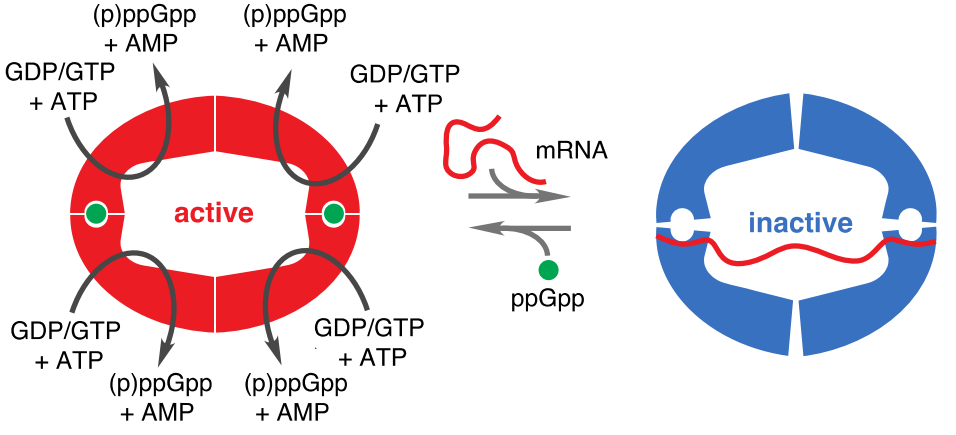

Figure 19. RelQ:RNA interaction as a switch between inactive and active states of the SAS enzyme. *E. faecalis* RelQ is in its inactive form when bound to the mRNA, which possesses a consensus sequence GGAGG. Increasing levels of (p)ppGpp in the cell allosterically stimulate enzymatic activity of RelQ and target mRNA is therefore released from the protein:RNA complex.

3. Structural and biochemical characterization of *S. aureus* RelP

In many representatives of Firmicute bacteria, such as *S. aureus* or *B. subtilis*, RelQ is accompanied by a second SAS protein named, RelP [131, 132]. Despite of high sequence homology between the two (see paper III, **Fig. S3**), presence of second homologous protein prompts its differential function and regulation. Transcriptional studies have shown that RelQ and RelP are expressed at different stages of bacterial growth [131]. Expression of RelQ is induced throughout exponential phase of the growth, but not in stationary phase; conversely, expression of RelP is transiently induced upon the entry into stationary growth phase [131]. While mostly functional studies are carried out for RelQ protein, little is known about RelP. We set out to investigate structural and mechanistic characteristics of RelP protein from *S. aureus* and indeed find some distinctive features of this enzyme.

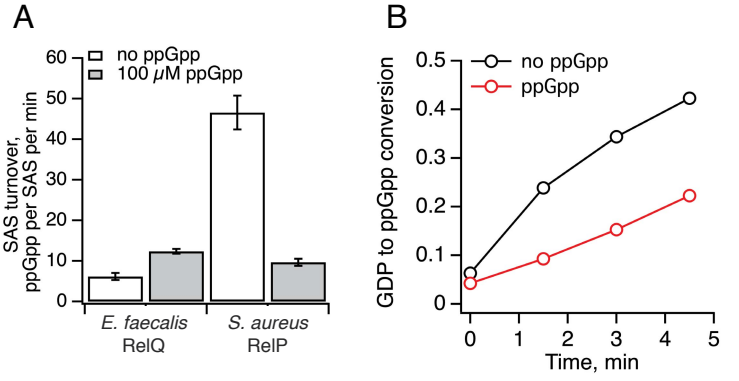

Figure 20. As opposed to *E. faecalis* RelQ, *S. aureus* RelP is inhibited by ppGpp. (**A**) Using the same conditions for both proteins – 200 μM GDP and 1 mM ATP as substrates, 200 nM of either of the enzyme in presence or absence of 100 μM ppGpp – the maximal turnover rates are achieved for *S. aureus* RelP without addition of ppGpp, oppositely it is inhibited by ppGpp. In case of *E. faecalis* RelQ, maximal turnover rates are achieved upon addition of 100 μM ppGpp, but even in the presence of the activator turnover rate of *E. faecalis* RelQ is around 4 times lower than *S. aureus* RelP. (**B**) The characteristic lag-phase in the time-course of ppGpp production of *E. faecalis* RelQ and *E. coli* RelA is not observed for *S. aureus* RelP both in the presence or absence of ppGpp.

Our biochemical assays show *S. aureus* RelP to be considerably more efficient in ppGpp production than *E. faecalis* RelQ (**Fig. 20A**): *S. aureus* RelP has nearly 4 times higher turnover rate (46.6 ± 4.17 reactions per enzyme per minute) compared to *E. faecalis* RelQ (maximal turnover rate of 12.4 ± 0.6 reactions per enzyme per minute) (**Fig. 20A**). Oppositely to *E. faecalis* RelQ (and *E. coli* RelA), which both are activated by (p)ppGpp, *S. aureus* RelP is inhibited by addition of the alarmone (**Fig. 20A** and **B**) and does not show a lag-phase in the

ppGpp production curve (**Fig. 20B**). We investigated the strength of this inhibition for both ppGpp and pppGpp and found ppGpp ($IC_{50}^{ppGpp} = 45 \pm 8$ μM) a bit more potent inhibitor than pppGpp ($IC_{50}^{ppGpp} = 94 \pm 26$ μM) (see paper III, **Fig. 3CD**).

For *E. faecalis* RelQ we also showed that it is bound and inhibited by single stranded mRNA and that this binding is mutually exclusive with allosteric activation by pppGpp. We therefore tested if *S. aureus* RelP is also affected by single-stranded RNA but same effect was not found – RelP is not inhibited by mRNA nor forms protein:RNA complex (see paper III, **Fig. 3E,F**).

The difference in the effect of the (p)ppGpp on the activities of two SAS enzymes can be readily explained by the structural differences of these proteins. Although both of the enzymes form tetrameric structures with the central cleft (**Fig. 9** and **Fig. 21**, respectively), oppositely to *E. faecalis* RelQ, *S. aureus* RelP lacks the (p)ppGpp binding site in the cleft. Therefore, we suggest that (p)ppGpp orthosterically inhibits (p)ppGpp production by *S. aureus* RelP (i.e. inhibition is arising from competition of product binding at the active site). This is confirmed by structural data that showed pppGpp bound to the active site in post-catalytic state. Guanine bases of both GDP (in pre-catalytic state) and ppGpp (in post-catalytic state) stack on a universally conserved Tyr-151 residue. Due to conserved nature of this residue, we investigated if its replacement with alanine (Y151A) will affect the ppGpp synthesis of RelP. Indeed, we observed complete loss of RelP Y151A activity (**Fig. 22B**).

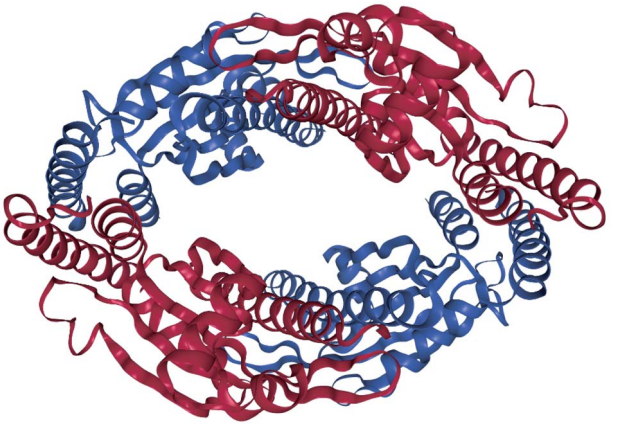

Figure 21. Tetrameric structure of *S. aureus* RelP. PDB accession code 6EX0.

The inhibitory effect of (p)ppGpp on RelP production suggests that RelP cannot be active simultaneously with other RSH proteins, but it is rather activated by a separate stress signal. This suggestion is consistent with *B. subtilis* transcriptomics data [131], that showed dramatic but transient upregulation of RelP in the late exponential phase, only when RelQ production was abrogated. The dramatic

and transient upregulation is also consistent with higher turnover rate of *S. aureus* RelP.

Structural analysis of RelP have identified a four-histidine site at the dimer-dimer interface of RelP tetramer, indicating that RelP might be a metal-binding protein, and that it could be allosterically regulated by metal ions. While this structural element is highly suggestive of zinc-binding site, no Zn^{2+} ion could be located at this site. Nonetheless, we tested the effect of Zn^{2+} on the catalytic rate of RelP, using Ni^{2+} as a specificity control (**Fig. 22A**). Both these metals are not found in the structure of a protein, but both at high concentrations have inhibitory effect on RelP. In the presence of increasing Zn^{2+} concentrations RelP activity shows biphastic concentration response curve: up to 4 µM Zn^{2+} acts as an activator and inhibits RelP at higher concentrations; Ni^{2+} in turn has only inhibitory effect nearly completely abolishing activity of RelP at concentration of 40 µM (**Fig. 22A**). To test if these metals act on a protein through the histidine-site, we substituted the two histidine residues (H73 and H74) to alanine. To our surprise this mutation resulted in complete loss of RelP activity (**Fig. 22B**), suggesting the site essential for structural integrity or/and enzymatic activity of the protein, however not providing us information about its specific purpose in Zn^{2+} binding.

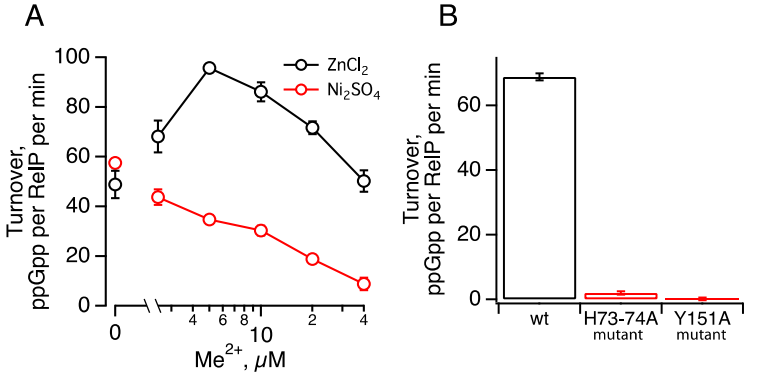

Figure 22. High concentrations of divalent metals Zn^{2+} and Ni^{2+} inhibit ppGpp production by *S. aureus* RelP (**A**). Alanine substitutions of putative Zn^{2+}-binding residues H73 and H74 and universally conserved residue Y151 result in complete loss of *S. aureus* RelP activity (**B**)

Zinc stress has been shown to induce stringent response by RelP in *B. subtilis* [168]. This is consistent with our observation that low concentrations of Zinc induce ppGpp production by RelP. It is therefore appealing to suggest that physiological role of RelP may be responding to oxidative stress induced by chelation of low amounts of metal ions present in the environment. Although the mechanistic details of this effect are still yet to be discovered, our data suggests that in Firmicute bacteria second SAS protein, RelP, could be involved to responses to different set of stress signals than RelQ.

CONCLUSIONS

Results of this thesis reveal several working principles of Small Alarmone Synthetases *E. faecalis* RelQ and *S. aureus* RelP. Specifically, I conclude that:

- *E. faecalis* RelQ preferentially utilizes GDP as a substrate and it is allosterically activated by pppGpp more efficiently than by ppGpp
- *E. faecalis* RelQ is not inhibited by Relacin, currently the most promising ppGpp analogue inhibitor of RSH enzymes
- *E. faecalis* RelQ combines two archetypical regulatory mechanisms: RNA binding and synthesis of alarmone (p)ppGpp
- *S. aureus* RelP is more catalytically efficient enzyme than *E. faecalis* RelQ
- *S. aureus* RelP is not activated by its product (p)ppGpp as *E. coli* RelA or *E. faecalis* RelQ
- *S. aureus* RelP is not inhibited by mRNA(MF) as is *E. faecalis* RelQ
- Enzymatic activity of *S. aureus* RelP is induced in the presence of μM-range concentrations of Zn^{2+} ions.

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SUMMARY IN ESTONIAN

Väikesed bakteriaalse poomisvastuse häälestajad – vaade väikeste alarmooni süntetaaside hingeellu

Bakterid elavad pidevalt muutuvates ja tihtipeale karmides tingimustes. Selleks, et kiiresti reageerida ja edukalt toime tulla muutlike ja ebasoodsate oludega on bakterirakkudel evolutsioneerunud spetsiaalsed keerukad regulatoorsed mehhanismid. Üheks kõige levinumaks selliseks mehhanismiks on "bakteriaalne poomisvastus". Poomisvastus on vahendatud alarmoonmolekuli (p)ppGpp poolt, mida sünteesitakse vastuseks aminohappevaegusele ja muudele stressitingimustele. Selle akumuleerumine rakus soodustab bakterite ellujäämist mõjutades erinevaid rakulisi protsesse nagu transkriptisoon, translatsioon, replikatsioon. (p)ppGpp mängib olulist rolli ka bakterite virulentsuses, biofilmi tekkes ja ka antibiootikumresistentsuses. Seega poomisvastuse uurimine on ka kliinilisest aspektist väga oluline.

(p)ppGpp molekuli tase rakus on reguleeritud bakterites konserveerunud RelA-SpoT homoloogsete (RSH) valkude poolt. Käesolev käsikiri on pühendatud RSH valguperekonna vähemtuntud liikmete uurimisele – *E. faecalis* RelQ ja *S. aureus* RelP. Nimelt uurisin nende valkude ensümaatilisi omadusi ja tõepoolest leidsin, et need omavad erilisi tunnuseid võrreldes teiste uuritud RSH valkudega. Näiteks *S. aureus* RelP on katalüütiliselt efektiivsem, kui *E. faecalis* RelQ ja ta ei ole aktiveeritud produkti (p)ppGpp poolt, nagu *E. coli* RelA või *E. faecalis* RelQ. Teisalt leidsime, et *E. faecalis* RelQ on järjestusspetsiifiliselt inhibeeritud mRNA poolt. Sellist regulatsioonimehhanismi, mis ühendab (p)ppGpp sünteesi ja mRNA sidumist, ei ole siiani täheldatud ühegi teise RSH valgu jaoks.

ACKNOWLEDGEMENTS

My foremost gratitude goes to my supervisor Dr. Vasili Hauryliuk for the support, patience and encouragement throughout my doctoral studies, for his exceptional ability to create highly motivating and productive working environment. I thank my second supervisor prof. Tanel Tenson for accepting me to his group and providing additional support whenever needed.

I am grateful to have worked with prof. Victoria Shingler in Umeå University, who shared her skills in doing EMSA assay without which I would not be able to complete the most important paper of this thesis.

Additionally I would like to thank all the people from Tanel's and Vasili's labs in Tartu and Umeå for sharing their knowledge as well as for having fun in free time. I am especially thankful to Pavel Kudrin, Roshani Payoe, Steffi Jimmy, Liis Andresen, Vallo Varik, Marje and Villu Kasari, Sofia Oliveira, Ievgen Dzhygyr, Victoriia Murina.

Finally I express my gratitude to my family and friends for love and support throughout life. Special thanks goes to my best friend Lucy, who helped me out with the illustrative part of the thesis.

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