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TETIANA BRODIAZHENKO

RelA-SpoT Homolog enzymes as effectors of Toxin-Antitoxin systems

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

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS 7

LIST OF ABBREVIATIONS 8

INTRODUCTION 10

REVIEW OF LITERATURE 11

1. Magic spot alarmone nucleotide (p)ppGpp 11
   - 1.1. Discovery of the (p)ppGpp alarmone or the 'magic spot' 11
   - 1.2. Biological functions of (p)ppGpp: control of metabolism, growth rate, virulence and antibiotic tolerance 12
     - 1.2.1. Direct transcriptional regulation by (p)ppGpp though allosteric control of the RNA polymerase: the *Escherichia coli* paradigm 14
     - 1.2.2. Indirect transcription regulation by (p)ppGpp through control of nucleotide metabolism: the *Bacillus subtilis* paradigm 15
     - 1.2.3. Regulation of protein synthesis by (p)ppGpp through regulation of expression of ribosome hibernation factors 16
   - 1.3. Regulation of protein synthesis by (p)ppGpp through inhibition of translational GTPases 16
2. RelA-SpoT homolog (RSH) protein family 17
   - 2.1. Evolutionary diversity of RelA-SpoT Homolog (RSH) protein family 17
   - 2.2. Biological functions and molecular mechanisms of long RSHs Rel, RelA and SpoT 20
     - 2.2.1. Housekeeping Small Alarmone Synthetase (SAS) RSH enzymes 21
     - 2.2.2. toxSAS FaRel and Tas1: growth inhibition by (pp)pApp 22
     - 2.2.3. toxSAS FaRel2, PhRel, PhRel2 and CapRel: growth inhibition by tRNA CCA end pyrophosphorylation 23
   - 2.3. Small Alarmone Hydrolases (SAH) 24
3. Toxin-antitoxin (TA) systems 25
   - 3.1. Classification of TA systems 25
   - 3.2. Molecular mechanisms of controlled growth inhibition employed by TA systems 27
   - 3.3. Specificity and promiscuity of toxin neutralization amongst class II TA systems 29
   - 3.4. Biological functions of TA systems 30

AIMS OF THE STUDY 33

MATERIALS AND METHODS 34
1. Toxicity neutralization assay 34
2. Metabolic labelling 34
3. *In vitro* transcription-translation assays 35
3.1. Preparation of cell-free translational extracts 35
3.2. Cell-free transcription-translation reactions and luciferase assay 36

RESULTS AND DISSCUSSION 38
I. toxSAS FaRel2, PhRel, PhRel2 and CapRel pyrophosphorylate tRNA CCA end to abrogate the tRNA aminoacylation (Paper I) 38
II. Panacea: a hyperpromiscuous antitoxin protein domain involved in neutralization of diverse toxin domains (Paper II) 40
III. Elimination of ribosome inactivating factors improves the efficiency of protein production in yeast and B. subtilis in vitro translational lysates (Paper III) 46

CONCLUSIONS 49

REFERENCES 50

SUMMARY IN ESTONIAN 61

ACKNOWLEDGMENTS 63

PUBLICATIONS 65

CURRICULUM VITAE 125

ELULOOKIRJELDUS 127

LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following original publications which are referred to by their Roman numerals:

I. Kurata T, **Brodiazhenko T**, Alves Oliveira SR, Roghanian M, Sakaguchi Y, Turnbull KJ, Bulvas O, Takada H, Tamman H, Ainelo A, Pohl R, Rejman D, Tenson T, Suzuki T, Garcia-Pino A, Atkinson GC, Hauryliuk V. RelA-SpoT Homolog toxins pyrophosphorylate the CCA end of tRNA to inhibit protein synthesis. Molecular Cell. 2021 Aug 5; 81(15):3160-3170.e9.

II. Kurata T, Saha CK, Buttress JA, Mets T, **Brodiazhenko T**, Turnbull KJ, Awoyomi OF, Oliveira SRA, Jimmy S, Ernits K, Delannoy M, Persson K, Tenson T, Strahl H, Hauryliuk V, Atkinson GC. A hyperpromiscuous antitoxin protein domain for the neutralization of diverse toxin domains. Proceedings of the National Academy of Sciences of the United States of America. 2022 Feb 8; 119(6):e2102212119.

III. **Brodiazhenko T**, Johansson MJO, Takada H, Nissan T, Hauryliuk V, Murina V. Elimination of Ribosome Inactivating Factors Improves the Efficiency of *Bacillus subtilis* and Saccharomyces cerevisiae Cell-Free Translation Systems. Frontiers in Microbiology. 2018 Dec 18; 9:3041.

Author's contributions:

In paper I and II I have performed metabolic labelling assays and as well as plate toxicity-neutralization assays. I participated in writing the original draft and preparation of figures. In paper III I have prepared and characterised bacterial and yeast cell-free translation systems. I performed all the experiments presented in the paper except for the polyribosome profiling. I participated in writing the original draft and preparation of figures.

LIST OF ABBREVIATIONS

AA	Amino Acid
ADPr	ADP-ribose
BCAA	Branched-Chain Amino Acids
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUF	Domain of Unknown Function
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HD	Hydrololase domain
HPLC	High Performance Liquid Chromatography
HPF	Hibernation Promoting Factor
LA	Luria Agar
LB	Lysogeny broth
MS	Magic Spot
mRNA	messenger RNA
ppApp	adenosine 5'-diphosphate 3'-diphosphate
pppApp	adenosine 5'-triphosphate 3'-diphosphate
(p)ppGpp	guanosine-5'-(tri)diphosphate 3'-diphosphate
ppGpp	guanosine-5'-diphosphate 3'-diphosphate
pppGpp	guanosine-5'-triphosphate 3'-diphosphate
(p)ppGpp^0	bacterial strain lacking (p)ppGpp
IF2	Initiation Factor 2
PanA	Panacea Antitoxin
PanAT	Panacea Antitoxin-Toxin system
PanT	Panacea Toxin
PP	Pyrophosphate group
RC	RNA control locus
RLU	relative luminescence unit
RMF	ribosome modulation factor
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNAP	RNA polymerase
ROS	Reactive Oxygen Species
RSH	RelA/SpoT Homolog
SAH	Small Alarmone Hydrolase
SAS	Small Alarmone Synthetase
SD	Shine-Dalgarno
SR	Stringent Response

ssDNA	single stranded DNA
SYNTH	Synthetase domain
TA	Toxin-Antitoxin
TCA	Trichloroacetic acid
toxSAS	toxic Small Alarmone Synthetase
TLC	Thin Layer Chromatography
tRNA	Transfer RNA
T6SS	type 6 secretion system
WT	Wild-Type

INTRODUCTION

Bacteria use numerous mechanisms to cope with adverse conditions. Nucleotide signalling molecules called alarmones are important players in bacterial adaptation. The well-studied alarmone guanosine (penta)tetraphosphate (p)ppGpp regulates cellular metabolism and growth in response to nutrient limitations. The less-understood adenosine (penta)tetraphosphate (p)ppApp is both used as a toxin poisoning cells though secretion system and as an effector of intracellular toxin-antitoxin (TA) systems. RelA-SpoT Homologue (RSH) enzymes mediated both the synthesis and degradation of alarmones.

Using computational tool FlaGs (standing for Flanking Genes) we have identified five unusual families of single-domain RSH enzymes – so-called Small Alarmone Synthetases (SASs) – that were encoded in two-gene operons, something that serves as a hallmark of TA systems. As these SAS were exceedingly toxic when expressed in *Escherichia coli*, and therefore we have dubbed these as toxic SAS, toxSAS. We have uncovered the mechanism of toxSAS-mediated growth defect. FaRel toxSAS from *Cellulomonas marina* inhibits the growth by synthetising (p)ppApp, while the other toxSAS are not able to produce alarmones. Instead, they abrogate protein synthesis through pyrophosphorylation of the 3'CCA end of tRNA.

PhRel2 toxSAS antitoxin, ATphRel2, it is a two-domain protein. Using FlaGs we discovered that of the two domains, initially referred as Domain of Unknown Function 4065, DUF4065, is present in numerous antitoxins in TA systems of bacteria, archaea and bacteriophages. We have experimentally validated several DUF4065-containing TA systems and characterised the mechanism of toxicity employed by the nonhomologous toxins. We have renamed the DUF4065 domain to Panacea, after the Greek goddess of universal cure. Our results suggests that Panacea is an adaptable domain that can evolve to neutralize and become specific for a range of various toxin domains. We dubbed this feature of Panacea as hyper-promiscuity.

Finally, in my PhD thesis work I have studied an adaptation mechanism that is employed by both eukaryotes and bacteria to throttle their translational capacity when nutrients are not available by forming inactive ribosomal dimers. In bacteria formation of ribosomal dimers is induced by (p)ppGpp alarmone nucleotide, which, in turn, induces the expression of ribosome dimerization or hibernation factor (HFP). My main focus was developing more active translation lysates from yeast and bacterial strains that lack the capacity to form ribosomal dimers, the hypothesis being that these will have higher concentration of active ribosomes and, therefore, will yield more active lysates. My experiments demonstrated that yes, indeed, genetic ablation of ribosomal dimerization results in improved activity of lysates.

REVIEW OF LITERATURE

1. Magic spot alarmone nucleotide (p)ppGpp

Like all living organisms, bacteria sense the environment and respond to plethora of stresses by adjusting their physiology accordingly (Cashel, 1975; Fernández-Coll L., 2020; Hobbs and Boraston, 2019; Potrykus and Cashel, 2008). One of the central bacterial stress pathways is the stringent response (SR) (Hauryliuk et al., 2015). The stringent response mediates the bacterial adaptation to nutrient limitation as well as to in response to abiotic environmental stresses like heat shock (Anderson et al., 2021; Schäfer H., 2020). More than six decades ago alarmone nucleotides ppGpp and pppGpp – collectively referred to as (p)ppGpp – or the "magic spots" were discovered to be produced in *Escherichia coli* cells as a response to amino acids limitation (Cashel and Gallant, 1969). The first physiological role of the SR to be characterised was inhibition of stable RNA (rRNA and tRNA) synthesis, coordinated with induction of expression of genes involved in amino acid biosynthesis and stress tolerance (Potrykus and Cashel, 2008). However, decades of research have established that in addition to transcription, (p)ppGpp targets multiple other processes in the cell, such as translation, ribosome assembly, metabolism, and impact all the aspects of cell physiology including adaptation to nutrient limitation, antibiotic resistance and virulence (Travis and Schumacher, 2021).

1.1. Discovery of the (p)ppGpp alarmone or the 'magic spot'

Investigations of the molecular mechanisms of the stringent response started with identification of so-called RNA control locus (RC) – a chromosomal locus that is crucial for regulation of ribosomal RNA synthesis in response to amino acid starvation. While a mutant *E. coli* RC harbouring the RC^{rel} ("relaxed") allele are unable to suppress rRNA synthesis upon amino acid starvation, "stringent" (RC^{st}) strains readily repress rRNA production when starved (Stent and Brenner, 1961). Several years later Cashel and Gallant discovered that amino acid starvation leads to appearance of two previously unknown compounds – dubbed as the magic spots I and II, MS I and II – when cellular nucleotide pools are resolved on Thin Layer Chromatography (TLC) (Cashel and Gallant, 1969). Analysis of MS I revealed that it is a highly pyrophosphorylated nucleotide that contains guanine, ribose and phosphate in molar ratios 1:1:4 (Cashel, 1970), and the following chemical structure of MSI/ppGpp was put forward: guanosine-5'-diphosphate 3'-diphosphate. Soon after the structure of pppGpp/MS II was also established, demonstrating that it a close relative of ppGpp – guanosine-5'-triphosphate-3'-diphosphate (Erlich H., 1971). Finally, it was uncovered that pppGpp is synthesised from GTP (**Figure 1**), and the reaction is catalysed by the product of RC locus – a protein called RelA – on 'starved' ribosomes harbouring deacylated tRNA in the A-site (Haseltine and Block, 1973).

The other *E. coli* enzyme mediating in both (p)ppGpp synthesis and degradation is SpoT (**Figure 1**) (Sy, 1977). Together with RelA, SpoT is a founding member of the RelA SpoT Homolog (RSH) protein family (Atkinson et al., 2011). While the (p)ppGpp synthetase (SYNTH) activity of RelA is specifically induced by only one metabolite limitation signal – amino acid starvation – SYNTH activity of SpoT is activated by numerous is inputs, such as carbon (Hernandez, 1991; Murray, 1996), fatty acid (Seyfzadeh et al., 1993) and iron (Murray, 1996) limitations. In addition to relatively weak SYNTH activity, SpoT is possesses a strong (p)ppGpp hydrolysis (HD) activity, thus balancing the (p)ppGpp production by RelA (Xiao et al., 1991). Finally, pppGpp is converted to ppGpp trough the catalytic action of guanosine-5'-triphosphate-3'-diphosphate pyrophosphatase (GppA), an enzyme found in many Gram-negative species such as *E. coli* (Mechold et al., 2013).

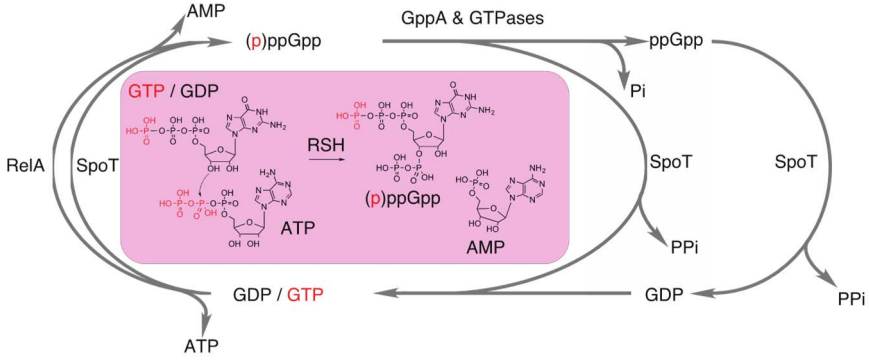

Figure 1. The cycle of (p)ppGpp synthesis and degradation in *E. coli*. Guanosine tetraphosphate ppGpp/MSI, is synthesized from ATP and GDP, while guanosine pentaphosphate, pppGpp/MSII, is formed from ATP and GTP, respectively. The (p)ppGpp synthesis is catalysed by strong SYNTH activity of RelA and weak SYNTH activity of SpoT, potent HD activity of SpoT responsible for (p)ppGpp degradation. Adapted from (Hauryliuk et al., 2015).

In the following chapters I will focus on the role of (p)ppGpp as a master regulator of bacterial metabolism and gene expression.

1.2. Biological functions of (p)ppGpp: control of metabolism, growth rate, virulence and antibiotic tolerance

The alarmone has numerous molecular targets in bacterial cell, with some of mechanisms of regulation differing between Gram-positive and Gram-negative species. The targets are summarised on **Figure 1**. In this chapter I will give an overview of (p)ppGpp-mediated control of cellular metabolism and gene expression, and in the following chapters I will discuss the individual regulatory pathways one by one.

While acute starvation or heat shock leads to a spike in (p)ppGpp alarmone levels that causes will inhibition of bacterial growth and protein synthesis, with the cell entering a 'survival mode' (Svitil A. L., 1993), the low basal level of (p)ppGpp under balanced growth condition is essential for maintenance efficient cellular metabolism and adjustment of it to growth rate (Fernández-Coll L., 2020). An example of (p)ppGpp playing a key role in metabolic control, is. direct regulation of GTP synthesis though the alarmone binding to HprT and GmK *B. subtilis* (Anderson B. W., 2019; Kriel et al., 2012).

Bacteria profoundly modulate their gene expression in response to changing environmental conditions (Fernández-Coll L., 2020; Hauryliuk et al., 2015; Yoshida and Wada, 2014), with expression growth-related genes being down-regulated and the survival program engaged under stress (Potrykus and Cashel, 2008; Ross et al., 2016). The effect of (p)ppGpp on gene expression is well-studied in Gamma proteobacteria (currently Pseudomonadota by (Oren, 2021)) such as *E. coli* (Haugen, 2008). In this bacterium (p)ppGpp controls expression of more than 700 genes involved in translation machinery, rRNA synthesis, amino acid biosynthesis and others (Durfee, 2008; Haugen, 2008; Ross et al., 2013; Traxler, 2008). While acting on the same target genes, ppGpp is more potent regulator than pppGpp (Bruhn-Olszewska et al., 2018; Mechold et al., 2013).

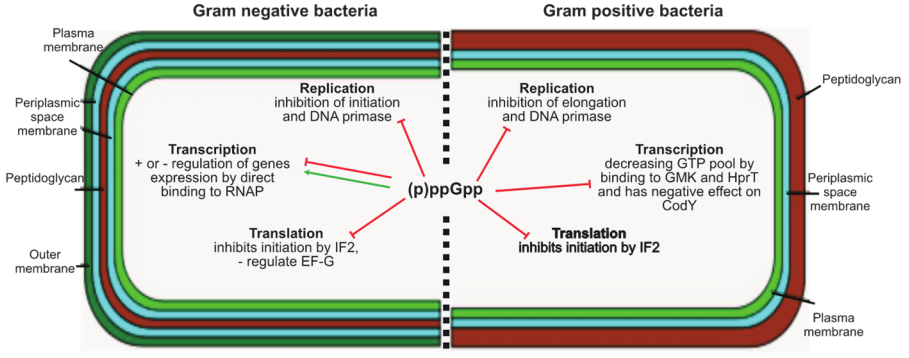

Figure 2. Main targets of (p)ppGpp in Gram-positive and Gram-negative bacteria. By binding to the DNA primase (p)ppGpp suppresses DNA replication both in Gram-negative or at the and Gram-positive bacteria (Giramma et al., 2021; Maciag et al., 2010). To modulate transcription in a promotor-specific manner, in Gram-negative bacteria (p)ppGpp binds directly to RNAP together with the DksA (Paul et al., 2005; Ross et al., 2013); (p)ppGpp-mediated repression or activation of target gene are determined by the discriminator elements in the promoter region (Wagner, 2002). Additionally, Gram-negative bacteria (p)ppGpp regulates transcription indirectly by affecting the sigma factor usage (Traxler, 2011). In Gram-positive bacteria (p)ppGpp regulates transcription in-directly through depletion of the GTP pool (Paul et al., 2004). Furthermore, low GTP levels cause CodY de-repression, promoting the transcription of genes involved in amino acid biosynthesis, nutrient transport and virulence (Kriel et al., 2012). Both in Gram-negative and Gram-positive bacteria (p)ppGpp competes with GTP by directly binding to and inhibiting translational GTPases, such as initiation factor 2 (IF2) and elongation factor G (EF-G) (Milon, 2006). This results in suppression of protein synthesis upon (p)ppGpp accumulation. Adapted from (Gaca et al., 2015).

In addition to controlling metabolism and growth rate, (p)ppGpp plays a key role in bacterial virulence and antibiotic tolerance (Fernández-Coll L., 2020). Genetic disruption of the RSH genes in *Salmonella enterica* serovar Typhimurium results in the so-called (p)ppGpp^0 strain which is avirulent and non-invasive in infection models (Pizarro-Cerdá J., 2004). Similarly, (p)ppGpp is implicated in virulence of opportunistic pathogenic *E. coli* (Aberg A., 2009; Aberg A., 2008; Cabrer-Panes J. D., 2020). (p)ppGpp was also suggested to be implicated in formation of the antibiotic-tolerant sub-population of bacterial culture – the so-called persisters (Svenningsen M. S., 2019). However, the mechanism underlying persister formation and the exact role of (p)ppGpp in the process are still unclear (Kaldalu N., 2020). Since the stringent response and (p)ppGpp-mediated signalling are important in bacterial virulence and antibiotic tolerance, RSH enzymes are currently being explored as potential targets for development of novel antibacterials (Fernández-Coll L., 2020).

1.2.1. Direct transcriptional regulation by (p)ppGpp though allosteric control of the RNA polymerase: the *Escherichia coli* paradigm

Mechanistic studies of transcriptional regulation in *E. coli* have resulted in uncovering the regulatory paradigm of (p)ppGpp-mediated transcriptional regulation through direct alarmone binding to RNA polymerase (RNAP), which modulates transcription initiation activity in a promoter-specific way (Haugen, 2008). Importantly, in addition to RNAP itself, this regulation involves a transcriptional factor – DksA, a negative regulator of rRNA expression and positive regulator of amino acid biosynthesis promoters (Edwards, 2011). When tested in reconstituted transcription system, DksA dramatically potentiates the regulatory effect of (p)ppGpp on RNAP (Paul et al., 2004). Structural studies have revealed the mechanistic underpinnings of this synergistic regulation. (p)ppGpp binds to two separates sites on *E. coli* RNA polymerase (**Figure 3**) (Ross et al., 2016) The (p)ppGpp binging site 1 of *E. coli* RNAP is located at the interface of β' and ω subunits (Ross et al., 2013) and does not require DksA (Ross et al., 2016). The Site 1 is responsible for effects on transcription initiation *in vivo* when ppGpp concentrations are low, such as during growth in rich medium or early starvation response. Conversely, the Site 2 is formed by both the DksA and RNAP β' subunit and when (p)ppGpp concentrations are high, the alarmone can populate Site 2 in addition to high-affinity Site 1 (Ross et al., 2016). Site 1 is well-conserved in Pseudomonadota but not in other bacterial phyla (Ross et al., 2013). Similarly, distribution of DksA homolog is also limited to proteobacterial species (Ross et al., 2016).

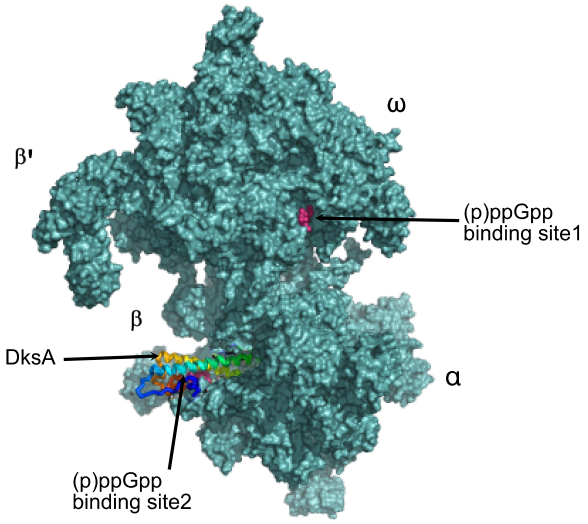

Figure 3. Location of (p)ppGpp Sites 1 and 2 on *E. coli* RNAP with 1 and 2. RNAP (marine green), (p)ppGpp (pink), DksA (rainbow). Adapted from (Ross et al., 2016), PDB code 5VSW.

1.2.2. Indirect transcription regulation by (p)ppGpp through control of nucleotide metabolism: the *Bacillus subtilis* paradigm

Gram-positive bacteria – such as Firmicute (currently Bacillota by (Oren, 2021)) *B. subtilis* (p)ppGpp – employ an alternative, indirect, mechanism to control transcription during stringent response (Krasny and Gourse, 2004). In these species the alarmone binds and inhibits enzymes which are involved in GTP synthesis, such as the guanylate kinase (GMK) (converts GMP to GDP), HprT (converts hypoxanthine to IMP and guanine to GMP) GuaB (converts IMP to XMP) (Lopez, 1981), which results in decreased GTP levels (Kriel et al., 2012). Conversely, in the absence of (p)ppGpp-mediated suppression of GTP biosynthesis, (p)ppGpp^0 *B. subtilis* strain has decreased viability due to abnormally high levels of GTP (Kriel et al., 2014). Since GTP acts as an initiator nucleotide (NTPi) for rRNA promoters in *B. subtilis*, the reduction of the GTP concentration leads to repression of the rRNA production (Krasny and Gourse, 2004; Liu et al., 2015).

Additionally, in Bacillota (p)ppGpp-mediated depletion of the GTP acts by affecting transcriptional repressor CodY (Geiger, 2014). Decrease of the GTP level destabilises the interaction between CodY and DNA (Sonenshein, 2007). Destabilisation CodY-DNA complex leads to de-repression of transcription of numerous genes involved in metabolism (Belitsky, 2013; Slack, 1995), including the ones responsible for the synthesis of the branched-chain amino acids (BCAA) (isoleucine, leucine, and valine) (Molle, 2003), as well as threonine and arginine (Belitsky, 2013).

1.2.3. Regulation of protein synthesis by (p)ppGpp through regulation of expression of ribosome hibernation factors

Protein synthesis is an energy-costly process which is repressed under conditions of stress and nutrient limitation (Yoshida and Wada, 2014). In bacteria the large (50S) and the small (30S) ribosomal subunits form together functional 70S ribosome. In eukaryotes, 60S and 40S ribosomal subunits form 80S ribosomes (Rodnina, 2018). Formation of inactive ribosomal dimers – 100S (in bacteria) or 110S (in eukaryotes) – is one of the mechanisms that mediates translational repression upon stress (Yoshida and Wada, 2014). In Gamma-pseudomonadota such as *E. coli* three factors are involved in the 100S formation: ribosome modulation factor (RMF), short version of the hibernation promoting factor (HPF), and YfiA (Izutsu, 2001; Lazzarini, 1971; Wada A., 1990). Most of other bacterial lineages encode only one factor, a long version of HPF (Yoshida and Wada, 2014). When environmental conditions improve, the ribosome dimers dissociate back from inactive 100S form to active 70S ribosomes (Aiso, 2005; Yamagishi, 1993). For example, 100S dissociate when stationary phase cells are transferred to fresh nutrient-rich growth media (Aiso, 2005). Transcription of *rmf* gene in *E. coli* is controlled by (p)ppGpp, thus directly connecting ribosomal dimerization to the stringent response (Kaczanowska, 2007). Similarly, in cyanobacteria (like *Synechococcus*) increased level of (p)ppGpp during transition from light condition to dark control long *hpf* expression to suppress translation by forming 100S ribosome dimers.

1.3. Regulation of protein synthesis by (p)ppGpp through inhibition of translational GTPases

Translation initiation in bacteria involves three initiation factors (IFs): IF1, translational GTPase IF2 and IF3 (Rodnina, 2018; Vinogradova et al., 2020). In its GTP-bound form IF2 promotes the association of initiator tRNA (fMet-$tRNA_i^{Met}$) to small ribosomal subunit (30S) to initiate the protein synthesis (Goyal, 2015; Milon, 2010). By competing with IF2's natural substrate, GTP, (p)ppGpp inhibits the activity of translational GTPase, and therefore, suppresses translation initiation (Milon, 2006; Vinogradova et al., 2020). While ppGpp can bind and inhibit other translational GTPases as well – such as EF-G which catalyses ribosomal elongation and recycling – affinity of ppGpp to IF2 is higher than to EF-G, which renders IF2 the primary target (Mitkevich, 2010). The ppGpp concentration can ruse to millimolar concertation upon acute stringent response and exceed that of GTP (Varik et al., 2017), resulting in efficient suppression of initiation translation initiation. Finally, (p)ppGpp also binds to and inhibits GTPases RsgA, RbgA, Era and ObgE that are involved in ribosomal assembly, thus throttling the ribosomal production upon nutrient limitation (Corrigan et al., 2016).

2. RelA-SpoT homolog (RSH) protein family

More than 50 years is past after discovery of stringent response by (Stent and Brenner, 1961) and effector molecule (p)ppGpp (Cashel and Gallant, 1969). The question which remains open was synthesis of (p)ppGpp. How is it synthesized? What kind of enzymes are involved in synthesis? To answer these questions, we should have a look at the previous studies. First authors who demonstrated that synthesis of (p)ppGpp requires deacylated tRNA, 30S and 50S ribosomal subunits and messenger RNA as a template were Haseltine and Block in 1973. They also proved that ppGpp and pppGpp are not synthesized in vitro if translation machinery (ribosome) is occupied by protein production (Haseltine and Block, 1973).The stringent elements like RelA responsible for production of (p)ppGpp (Cashel, 1975) and SpoT for its hydrolyzation (Sy, 1977) respectively.

The relA and spoT genes are not the only ones which are involved in (p)ppGpp metabolism. These proteins are part a protein family which is called RelA-SpoT homolog or RSH family. In the next chapter will be described the evolution and variety of RSH proteins in the tree of life.

2.1. Evolutionary diversity of RelA-SpoT Homolog (RSH) protein family

RelA/SpoT Homologue (RSH) proteins regulate the concentration of the alarmone nucleotides ppGpp and pppGpp. Members of the RSH superfamily can be divided into long RSHs (Rel, RelA and SpoT) and short RSHs, with the latter being sub-divided in Small Alarmone Synthetases (SAS) and Small Alarmone Hydrolases (SAH). The long RSHs are large multidomain proteins, while the short RSHs typically contain only one domain– either (p)ppGpp synthesis (STYNTH) or hydrolysis (HD) domain (Atkinson et al., 2011) (**Figure 5**). The N-terminal enzymatic region (NTD) of long RSHs consists of SYNTH (sometimes referred to as pseudo-SYNTH in the case of SYNTH-inactive RelA) and HD (sometimes referred to as pseudo-HD in the case of HD-inactive *Moraxellaceae* SpoT) domains. The regulatory multi-domain carboxy-terminal (CTD) domain region is comprised of Thr-tRNA synthetase, GTPase and SpoT domain (TGS), the Helical domain, the Zing Finger Domain (ZFD) [alternatively referred to as Conserved Cysteine, CC], and, finally, the RNA Recognition Motif (RRM) [alternatively referred to as Aspartokinase, Chorismate mutase and TyrA, ACT] domains (Atkinson et al., 2011).

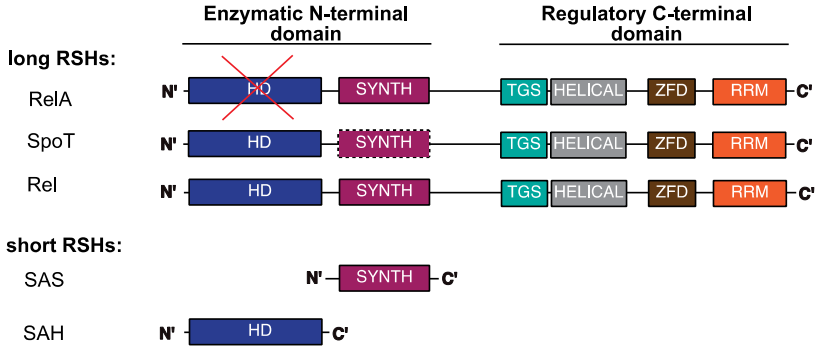

Figure 5. Domain organization of long and short RSH proteins. Long RSH proteins are made up of six domains: (p)ppGpp hydrolysis domain (HD), (p)ppGpp synthesis domain (SYNTH), TGS (Threonyl-tRNA synthetase, GTPase, SpoT), Helical, ZFD and RRM. Grey boxes shown the N-terminal region (NTD), blue boxes shown the C-terminal domain region (CTD). Th the case of RelA the HD domain is enzymatically inactive (sometimes referred to as pseudo-SYNTH), which is marked with a red cross. The SYNTH domain for SpoT has weak enzymatic activity, marked with dashed line. Adapted from (Hauryliuk et al., 2015).

The pair of the SYNTH-only stringent factor RelA and bifunctional – that is capable of both synthesises and degradation of (p)ppGpp – SpoT is found exclusively in Gamma- and Beta-pseudomonadota (Atkinson et al., 2011; Mittenhuber, 2001) (**Figure 4**). The most of other bacterial genomes carry one bifunctional long RSH: the ancestral stringent factor Rel (Atkinson et al., 2011; Mittenhuber, 2001) (**Figure 4**). Just as in the course of evolution RelA has lost its HD function and became a SYNTH-only factor, in *Moraxellaceae* SpoT has lost its (originally weak) SYNTH function (Williams, 2010). The loss of RelA resulting in SpoT-only bacteria is a is rare event, that seemingly has occurred in *Acidithiobacillus ferrooxidans*, *Candidatus Ruthia magnifica*, *Methylovorus* SIP and *Nitrosomonas europaea*. In these species it seems that SpoT, similarly to bifunctional Rel, is the sole bifunctional (p)ppGpp synthetase and hydrolase (Atkinson et al., 2011). Members of the PVC superphylum (plantomycetes, verrucomicrobia and chalmydiales) seem to lack long RSHs altogether (Atkinson et al., 2011; Mittenhuber, 2001). These are mainly intracellular endosymbionts and pathogens like *Neorickettsia*, *Rickettsia*, *Bifidobacterium* and *Anaplasma* (Atkinson et al., 2011). Long RSH genes are also undetectable in genomes of *Mycoplasma* spp., three of *Spirochetes* species as well as *Thermoanaerobacter* X514 of *Clostridiales*.

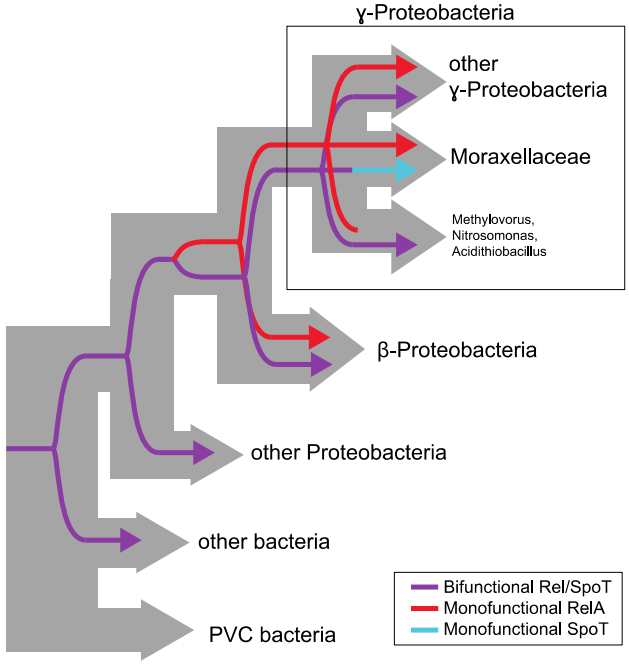

Figure 5. Evolution of long RSH proteins in different bacterial clades. While γ- and β-Proteobacteria encode pair of long RSHs – RelA (a strong ribosome-associated (p)ppGpp synthetase) and SpoT (bifunctional RSH capable of both hydrolysis and synthesis of (p)ppGpp), most of the clades encode only one long RHS, bifunctional Rel. On the lineage to γ- and β-Proteobacteria (currently Pseudomonadota) Rel underwent gene duplication, giving rise to RelA and SpoT. In some lineages no long RSHs are present (chalmydiales, verrucomicrobia and plantomycetes, PVC). Adapted from (Atkinson et al., 2011).

The first SASs to be discovered are housekeeping single domain synthetases RelQ and RelP encoded in *B. subtilis* genome (Nanamiya et al., 2008). Subsequently RelQ and RelP were studied in detail (Beljantseva et al., 2017; Manav et al., 2018; Steinchen et al., 2018). Recently our lab discovered that some subfamilies of SASs are encoded in conserved bicistronicity organisation and act as toxic effectors – toxSAS – of toxin-antitoxin (TA) systems (Jimmy et al., 2020). This discovery and TAs in general are discussed in a separate chapter of this thesis. While SAH are relatively well studied, SAHs are less well understood, with, arguably, most well-studied representative being is eukaryotic SAH MESH1 (Atkinson et al., 2011).

2.2. Biological functions and molecular mechanisms of long RSHs Rel, RelA and SpoT

In 1973 Haseltine and Block uncovered the biochemical basis of (p)ppGpp accumulation in *E. coli* upon amino acids starvation by demonstrating that the presence of uncharged (deacylated) tRNA in the ribosomal A site serves as the inducer of RelA's SYNTH enzymatic activity (Haseltine and Block, 1973). Recent cryo-EM reconstructions (Arenz et al., 2016; Brown et al., 2016; Loveland et al., 2016) and biochemical studies (Roghanian et al., 2021; Takada et al., 2021) have uncovered the molecular mechanism of the ribosome-mediated RelA activation in great detail. Upon amino acid starvation, 'hungry' ribosomes with empty A site accumulate as well as deacylated tRNAs cognate to the A-site codon of hungry ribosomes. The regulatory C-terminal domains of RelA recruit the stringent factor to the hungry A-site, which is followed by the recruitment of the deacylated tRNA, leading to full activation of RelA's SYNTH enzymatic activity (**Figure 6**).

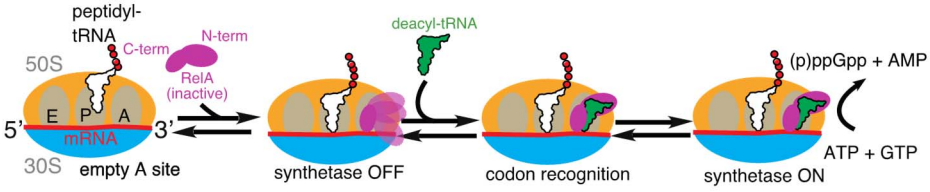

Figure 6. Mechanism of RelA activation by the starved ribosomal complexes. The C-terminal domains of RelA, the ZFD and RRM, bind the vacant A site of the hungry ribosome. The NTD remains outside of the A site. The A-site-cognate deacylated tRNA is then recruited by RelA to the ribosomal at the A site. The allosteric signal for the CTD is transferred to the NTD, activating the SYNTH function of RelA. Adapted from (Loveland et al., 2016).

In addition to starved ribosomal complexes, the SYNTH activity of both RelA (Shyp et al., 2012) and Rel (Takada et al., 2021) enzymes is also stimulated by the very enzymatic product synthesised by these RSHs, the alarmone (p)ppGpp. Detailed mechanistic understanding of this regulatory mechanism was uncovered in 2021 (Roghanian et al., 2021). In the absence of the alarmone, the NTD of RelA/Rel assumes a compact conformation in which the pseudo-HD/HD inhibits the SYNTH domain. (p)ppGpp binding to an allosteric site located in the hinge region that connects pseudo-HD/HD and SYNTH domains drives the transition to an open conformation of the NTD which is – unlike the HD-primed closed one – is compatible with the SYNTH activity (Roghanian et al., 2021). Finally, activation by the alarmone and activation by the ribosomal starved complex synergise, resulting in full activation of the (p)ppGpp production (Roghanian et al., 2021).

As discussed above, bifunctional RSHs Rel and SpoT control (p)ppGpp level in the cell via two active sites – one with hydrolase function and another synthetase (Avarbock et al., 2000; Hogg et al., 2004). SpoT is metalloenzyme, with

Mn^{2+} being essential for its HD activity (Hogg et al., 2004). It is not clear if SpoT is activated by stalled ribosome complexes (Gentry, 1995; Jiang, 2007). While SpoT senses and is regulated by numerous stress conditions like fatty acid (Seyfzadeh et al., 1993; Sinha, 2019), carbon (Xiao et al., 1991) or iron (Vinella, 2005) limitation, the exact mechanism of sensing and activation is unclear. Unlike SpoT – and similarly to RelA – activation of the Rel's SYNTH is mediated by starved ribosomes (Avarbock et al., 2000). Similarly, to SpoT, the hydrolysis function is also strictly dependant on the Mn^{2+} cofactor (Avarbock et al., 2000; Hogg et al., 2004; Van Nerom et al., 2019).

2.2.1. Housekeeping Small Alarmone Synthetase (SAS) RSH enzymes

Short Alarmone Synthetases (SAS) are monofunctional enzyme performing synthesis of alarmone nucleotides (Atkinson et al., 2011; Steinchen et al., 2018). The most well-studied 'housekeeping' SAS representatives are RelQ (also known as SAS1, YjbM) and RelP (also known as SAS2, YwaC) (Atkinson et al., 2011; Beljantseva et al., 2017; Nanamiya et al., 2008; Ruwe et al., 2017; Steinchen et al., 2015; Steinchen et al., 2018). While the enzymatic output of long RSHs is predominantly regulated though binding to and regulation by other components of the cell (such as starved ribosomes), activity the of SAS enzymes is largely regulated on transcriptional level. In *Enterococcus* species treatment with cell wall-targeting antibiotic vancomycin was shown to trigger the transcriptional induction of RelQ expression, resulting in (p)ppGpp overproduction, thus conferring tolerance to the antibiotic (Abranches et al., 2009). Similarly, transcription-driven SAS overexpression is implicated in cell envelope stress response in *Staphylococcus aureus* (Geiger et al., 2014; Tagami, 2012).

X-ray crystallography studies have revealed that *B. subtilis* RelQ and RelP (Steinchen et al., 2015) and *Staphylococcus aureus* RelP (Steinchen et al., 2018) assemble into homotetramer (**Figure 7**). Surprisingly, just like the SYNTH activity of Rel/RelA is regulated by the alarmone, RelQ is also allosterically regulated by pppGpp, so is RelQ – but the molecular mechanism is completely different and evolutionally unrelated: in the case of SAS, the allosteric site is formed on the interface between the subunits (Steinchen et al., 2015). Finally, RelP from *Staphylococcus aureus* was shown to have a putative Zn^{2+} binding site and is inhibited by Zn^{2+} ions in vitro (Manav et al., 2018). The enzyme was proposed to be activated as decrease in the intracellular zinc level in response to oxidative stress, thus ablating the inhibition by the metal ions (Steinchen et al., 2018).

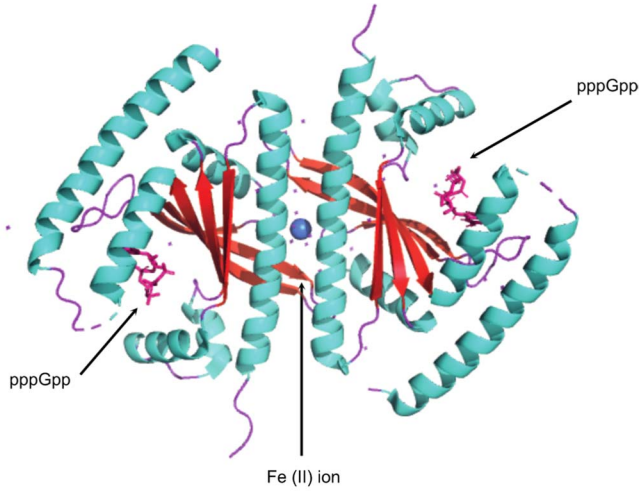

Figure 7. Tetrameric structure of *S. aureus* RelP SAS. Allosteric regulator pppGpp is shown in red. Adapted from (Manav et al., 2018), PDB code 6EX0.

2.2.2. toxSAS FaRel and Tas1: growth inhibition by (pp)pApp

The initial indications of some SAS representatives being specifically toxic for the cell came from a study that focused on a protein pair – SAS gp29 and its antidote protein gp30 – encoded in a mycobacterial bacteriophage Phrann (Dedrick et al., 2017). In was found the gene gp29 product is highly toxic to *Mycobacterium smegmatis* cells, while co-expression of the protein product of the gp29-neighbouring gene gp30 neutralised the toxic effect (Dedrick et al., 2017). As bacterial cells carrying gp29-gp30-encoding Prann prophage were resistant to coinfection by other bacteriophages, such as Tweety and Gaia, Dedrik and colleagues concluded that the gp29 and gp30 gene pair may play role in the phage defence mechanism. However, the mechanism of gp29-mediated toxicity and phage defence were not experimentally established. Note that conserved bicistroinc (two-gene) architecture of gp29-gp30, with one gene being toxic and the oner neutralising the toxic effect, is characteristic for so-called toxin-antitoxin pairs (TA). However, this connection was not made in the original paper (Dedrick et al., 2017).

In 2019 a highly divergent RSH SAS enzyme Tas1 was shown to be is a secreted toxic effector of *Pseudomonas aeruginosa* type 6 secretion system (T6SS) that synthesises (pp)pApp nucleotides – pApp, ppApp and pppApp – using AMP, ADP and ATP as substrates, respectively (Ahmad et al., 2019). Production of (p)ppApp by Tas1 injected into the prey cell via T6SS results in depletion of the ATP pool, with the toxic effect mediated through the inhibition of the activity of PurF enzyme involved in purine synthesis pathway (Ahmad et al., 2019).

In parallel, our lab has developed a computational tool FlaGs (from Flanking Genes) which allows analysing gene neighbourhoods to identify conserved operon

structures (Saha et al., 2021). Since TAs are encoded in conserved bicistronic operons, we used the tool to discover five new TA-like SASs subfamilies: FaRel, PhRel2, FaRel2, PhRel and CapRel (Jimmy et al., 2020). One representative of each subfamily was taken for experimental investigations – *Cellulomonas marina* FaRel, *B. subtilis* la1a PhRel2, *Coprobacillus* sp. D7 FaRel2, *Mycobacterium* phage Phrann PhRel (Gp29), and *Mycobacterium tuberculosis* AB308 CapRel (Jimmy et al., 2020). All of these SAS enzymes have proven to be highly toxic. Similarly, to Tas1, the toxicity of FaRel was shown to be mediated by production of adenosine analogue (p)ppApp, leading to inhibition of translation, transcription and replication (Jimmy et al., 2020).

2.2.3. toxSAS FaRel2, PhRel, PhRel2 and CapRel: growth inhibition by tRNA CCA end pyrophosphorylation

While analysing the nucleotide pool of *E. coli* with expressing toxSAS FaRel2, PhRel, PhRel2 and CapRel families we detected no accumulation of neither (pp)pApp or (p)ppGpp (Kurata et al., 2021; Kurata et al., 2022). This suggested that these toxSAS have a mechanism of toxicity profoundly different from that of FaRel and Tas1. Indeed, when I have performed metabolic labelling assays, I discovered that these toxSAS specifically inhibit protein synthesis (Kurata et al., 2021; Kurata et al., 2022).

Protein synthesis inhibition is a common mechanism of action amongst unrelated TA systems (Harms et al., 2018; Jurenas et al., 2022). A common target of translation-inhibiting TAs is transport tRNA, tRNA, which can be inactivated via cleavage (as exemplified by VapC toxins (Cruz, 2015)), acetylation of amino acid attached to tRNA 3'CCA (as exemplified by GNAT toxins such as AtaT (Jurenas et al., 2017)), or inactivation of the 3'CCA end through the addition of pyrimidines (as exemplified by MenT3 toxin (Cai, 2020)). Through a comprehensive set of experiments, we have established that by translation-inhibiting toxSAS such as FaRel2 inhibit aminoacylation of tRNA via tRNA 3'CCA end pyrophosphorylation, yielding aminoacylation-incompetent PP-tRNA (**Figure 7**) (Kurata et al., 2021).

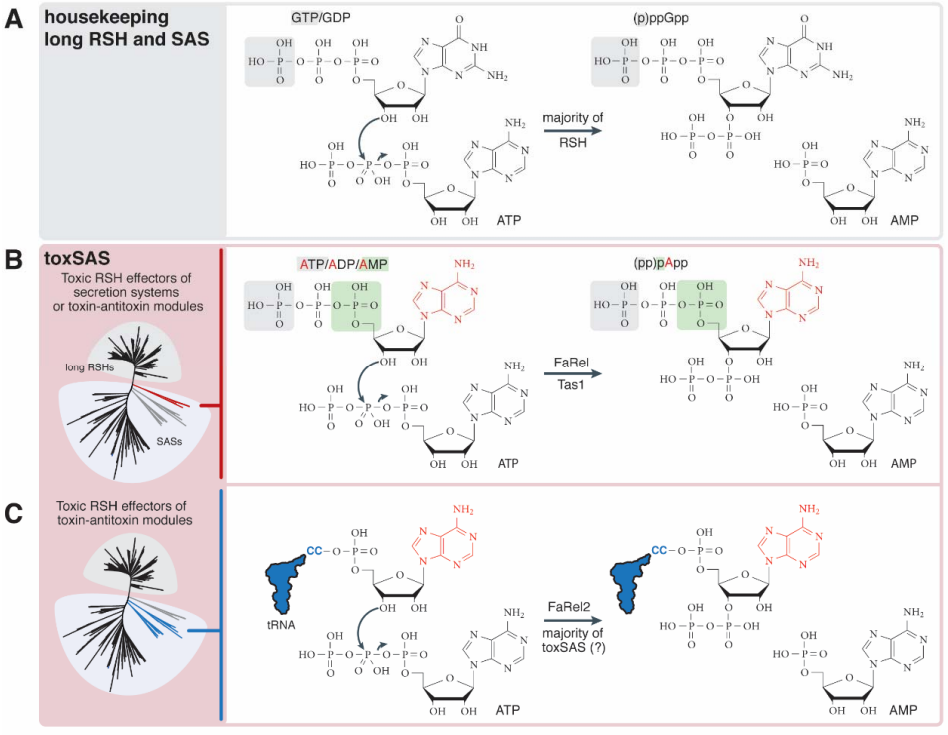

Figure 8. SYNTH substrate specificity of RSHs. (**A**) Housekeeping RSHs synthesize (p)ppGpp by transferring the pyrophosphate group of ATP onto the ribose of either GDP or GTP and degrade the alarmone by hydrolysing the nucleotide back to GDP or GTP. (**B**) Secretion system effector Tas1 and toxSAS FaRel to produce toxic (pp)pApp alarmones. (**C**) Representatives of the majority of toxSAS subfamilies specifically inhibit protein synthesis by transferring the pyrophosphate group of ATP onto the 3' ribose position tRNA's CCA end, producing PP-tRNA. Adopted from (T. Kurata et al., 2021).

2.3. Small Alarmone Hydrolases (SAH)

Bacterial Small Alarmone Hydrolases (SAH) are a diverse and widespread (Atkinson et al., 2011) – but relatively poorly characterised group of single domain RSHs. By analogy to long HD-competent RSHs SpoT and Rel, the hydrolytic activities of SAH were initially believed to be limited to hydrolysis of (p)ppGpp. When the only human SAH representative – MESH1 – was discovered, it was suggested that it acts to (p)ppGpp, despite the lack of evidence of the alarmone present in metazoa (Sun et al., 2010).

In parallel with the discovery of SYNTH-competent RSHs being able to synthesise (pp)pApp (Ahmad et al., 2019; Jimmy et al., 2020) and PP-tRNA (Kurata et al., 2021), researchers started probing potential other catalytic activities of SAH. *Cellulomonas marina* SAH antitoxin protein (ATfaRel) was shown to neutralise the toxic effect of (p)ppApp-producing (Jimmy et al., 2020) and PP-tRNA producing toxSAS (Kurata et al., 2021), same detoxifying effects were

observed for human MESH1 (Kurata et al., 2021). SAH from *Methylobacterium extorquens* (Ruwe et al., 2018) and *Pseudomonas aeruginosa* (Steinchen et al., 2021) were shown to have a hydrolase substrate reference towards (p)ppApp over (p)ppGpp. It has been proposed that MESH1 could also degrade NADPH to NADH (Ding, 2020). However, follow up studies did not support this claim (Potrykus et al., 2020), and the native substrate of MESH1 in metazoan remains unclear.

3. Toxin-antitoxin (TA) systems

First representatives of toxin -antitoxin (TA) systems were discovered in the early 80s (Gerdes et al., 1986; Ogura and Hiraga, 1983). The classical TA systems are bicistronic – i.e. comprised of two gene – operons, in which one gene encodes a protein toxin and the other encodes a protein or RNA antitoxin which neutralises the toxin, either directly or indirectly. Studies of TA systems have exploded in the last years, with numerous new TA families being discovered, their mechanisms of action being characterised, biological functions established and possible applications for biotechnology put forward. In the following sections I will cover classification of functional types of TAs, provide an overview of the mechanisms of toxicity used by different TAs, discuss the specificity and promiscuity of toxin neutralisation by class II (i.e. protein toxin neutralised by protein antitoxin) TA systems, and, finally, touch upon the emerging understanding of cellular TA functions, with a special focus on phage defence via abortive infection (Abi).

3.1. Classification of TA systems

In the recent years many new TA systems were discovered, and a classification of TAs into eight classes (I–VIII) was formulated (**Figure 9**) (Jurenas et al., 2022). Exceptions to the classical bicistronic architecture with protein toxin have been identified. While in classical TAs the two genes forming an operon, type I and type VIII systems are not organised as a single operon. While almost type I–VIII toxins are proteins, the newly discovered VIII type toxins are RNAs (Choi, 2018; Li, 2021). Below I will describe the eight classes of TA systems and give examples of these systems.

Class I TA systems are comprised of a protein-based toxin and antisense RNA-antitoxin which prevents toxin that ‘masks’ the Shine-Dalgarno (SD) element of the of the toxin-encoding mRNA and prevents its translation (Darfeuille, 2007; Fozo, 2008; Gerdes, 1988). Type II TAs – which are the focus of my research presented in this thesis – are made up of two protein-encoding genes transcribed as an operon, with the toxin being neutralised by the antitoxin through formation of an inert complex (Jurenas et al., 2019; Li, 2009; Tam, 1989). Type III TAs are comprised of a toxic protein and an antitoxin RNA that inhibits the protein toxin by forming a non-toxic RNA-protein complex (Fineran, 2009; Samson, 2013).

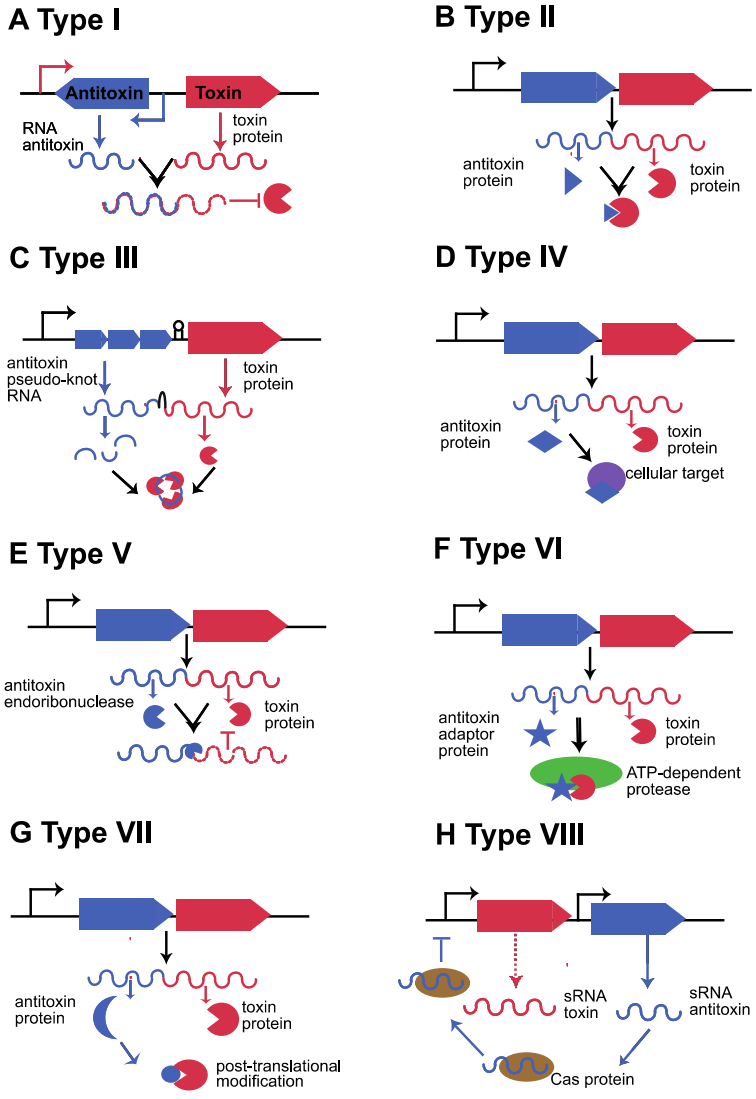

Figure 9. Classification of Toxin-Antitoxin systems. (**A**) Type I systems are composed of RNA antitoxin that binds toxin-encoding mRNA, thus repressing the protein toxin expression. (**B**) Type II systems are composed of protein antitoxin that directly binds and inhibits toxin protein. (**C**) Type III systems are composed of RNA antitoxins that bind and inhibit protein toxin. (**D**) Type IV systems have protein antitoxins that protect the toxin targets from toxin activity. (**E**) The type V system has an endoribonuclease antitoxin that degrades the transcript of the toxin. (**F**) The type VI system include an antitoxin protein that acts as an adaptor targeting the toxin for activation protease for degradation. (**G**) Type VII systems encode protein antitoxins that inactivate the toxin through post-translational modification. (**H**) Type VIII systems use RNA antitoxins that act as transcriptional repressors of expression of RNA toxin. Toxins are shown in red, antitoxins are shown in blue. Adopted form (Jurenas et al., 2022).

Type IV is comprised of proteinaceous toxin and antitoxin with the toxic effects being counteracted via antitoxin-mediated modification of toxin's cellular targets (Jankevicius et al., 2016; Jimmy et al., 2020; Masuda et al., 2012). Type V is comprised of an RNase antitoxin which degrades the toxin-coding mRNA (Jurenas et al., 2022). In Type VI proteinaceous antitoxin counteracts the proteinaceous toxin by acting like adaptor for ATP dependent proteases which specifically degrade the protein toxin (Aakre et al., 2013). The VII class is similar to VI, but instead of acting via toxin degradation, inactivation of the toxin is mediated by its posttranslational modification (Songailiene, 2020; Yu, 2020). Finally, the most recent type to be discovered is type VIII type, which is unique in containing an RNA toxin (Choi, 2018; Li, 2021). In type VIII an RNA antitoxin represses the expression of toxic RNA by acting as antisense or by acting as a Cas-dependent transcriptional repressor.

Finally, some toxins are neutralised by two synergistically acting antitoxins acting as class II and class IV. One example is FaRel toxSAS which is neutralised by a class II antitoxin that forms a binary complexed with FaRel and a class IV SAH antitoxin that neutralises FaRel by degrading the toxic alarmone (p)ppApp (Jimmy et al., 2020). In the following chapters I will focus on type II TAs since these systems were the focus of my research.

3.2. Molecular mechanisms of controlled growth inhibition employed by TA systems

Toxic effectors act on a variety of cellular targets, with several aspects of bacterial physiology being targeted more commonly than the others (**Figure 10**). Below I will describe most common growth inhibition strategies employed by TA toxins.

Many TA toxins target replication either by damaging DNA directly or compromising replication machinery (Aakre et al., 2013; Jankevicius et al., 2016; Jurenas et al., 2022). One of such TAs is chromosomally encoded type IV DarTG system, with the DarT toxin catalysing ADP-ribosylation of thymine residues is single stranded DNA (ssDNA). This modification leads to compromised DNA replication and induction of SOS DNA damage response. The antitoxin DarG neutralizes the toxin's activity by glycohydrolisation of ADP-ribose (ADPr) moiety, thus removing the modification from thymine residues (Jankevicius et al., 2016). SocB VI type toxin of the SocAB TA system interrupts the replication elongation by direct binging to DnaN, resulting in the replication fork collapse, and, similarly to DarT, this toxic effect also induces the SOS response (Aakre et al., 2013). ATP-depended proteases ClpX and ClpP (ClpXP) are essential for neutralisation of SocB toxin (LeRoux, 2022; Texier, 2021), since SocA acts as a proteolytic adaptor antitoxin which helps ClpXP to degrade the SocB. Two more examples of toxin-antitoxin systems that inhibit replication are CcdB and ParE (toxic effectors of type II TAs CcdA/CcdB and ParD, respectively) which inhibit replication by binging to DNA gyrase (Yuan J., 2010).

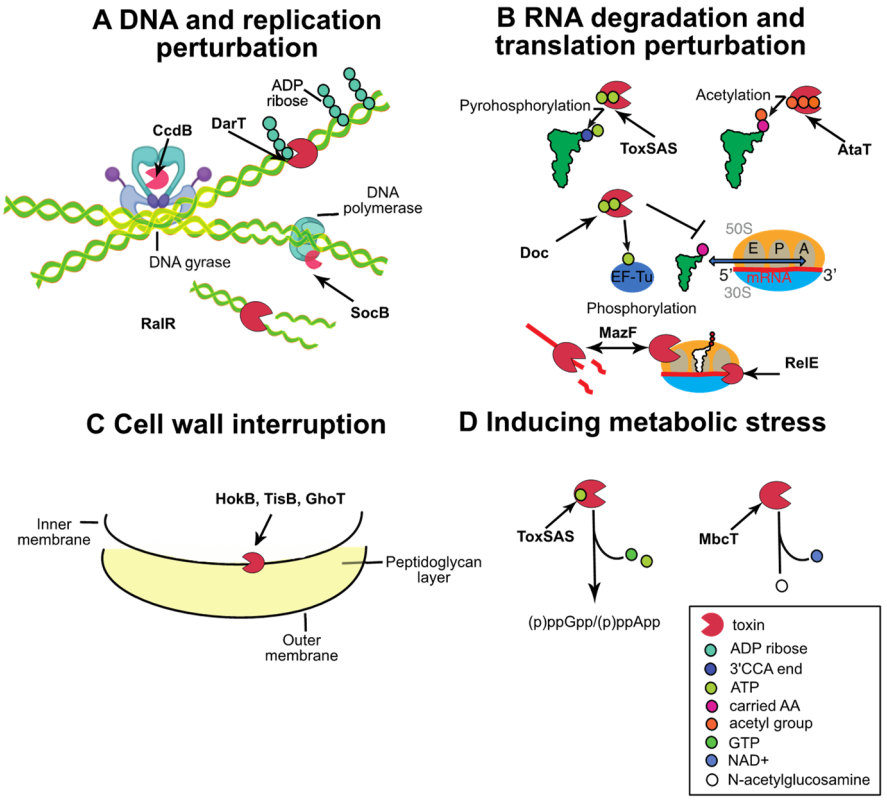

Figure 10. Diversity of molecular targets of toxic effectors of TA systems. (**A**) DNA is directly targeted by the DNase RalR and the ADP-ribosyltransferase DarT. The replication machinery is targeted by SocB which binds to the β-sliding clamp subunit of DNA polymerase III. DNA gyrase is inhibited by direct binding of CcdB. (**B**) Translation is targeted by a numerous toxins acting at every level of protein synthesis: toxSAS inhibit tRNA aminoacylation by pyrophosphorylation of the 3' ribose position tRNA's CCA end. AtaT toxins acetylate the amino acid residue of charged tRNAs. Doc phosphorylates elongation factor Tu (EF-Tu) and prevents the aminoacyl-tRNA delivery. MazF RNase toxins degrade free mRNAs and ribosomal RNAs. RelE RNase toxins cleave mRNA in the ribosomal A site. (**C**) TisB, HokB and GhoT are small peptides that form pores in the inner membrane to compromise its integrity. (**D**) ToxSAS toxins synthesizing the alarmone (p)ppApp to the drop cellular levels of GTP and ATP. The MbcT toxin degrades NAD+. Adapted from (Jurenas et al., 2022).

Another important target of TA toxins is translational machinery (Jurenas et al., 2022). Most of the toxins targeting translation are RNases (Harms et al., 2018; Jurenas et al., 2017). A classical example is RelE toxin of the RelBE type II system – a ribosome-depended enzyme that cleaves the mRNA in the ribosomal A site (Pedersen K., 2003). Another example is MazF of the MasEF system which degrades free RNAs such as mRNA and rRNA precursors (Culviner, 2018; Mets, 2017). GNAT domain-containing toxins inactivate aminoacyl-tRNAs by

acetylation of the amino acid moiety, with prime examples being AtaT toxin of the AtaR-AtaT system, that specifically modifies initiator fMet-tRNAi^{fMet} (Cheverton A. M., 2016; Jurenas et al., 2017). The HipA toxin phosphorylates aminoacyl-tRNA synthetases to inhibit tRNA charging (i.e. aminoacylation) (Kaspy et al., 2013). Finally, as it was demonstrated in our laboratory, many of toxSAS toxins pyrophosphorylate 3' CCA end of deacylated tRNA thus precluding its charging with amino acids (Jimmy et al., 2020; Kurata et al., 2021).

The third major TA target is cell envelope, which is targeted by many type I TA toxins. A classic example is type I HokB toxin which causes membrane depolarization and disrupts the proton motive force upon insertion into the inner membrane. Type V GhoT toxin also induces to cell lysis, with GhoS antitoxin rescuing the cell by digesting *ghoT* transcripts (Wang et al., 2012).

Finally, several TA toxins abrogate the growth by inducing an acute metabolic stress by either producing toxic small metabolites or degrading essential housekeeping metabolites. Toxicity of FaRel toxSAS is mediated by production of alarmone nucleotide (p)ppApp (Jimmy et al., 2020). Type II MbcT toxin which depletes NAD^+ a central electron carrier essential for redox reactions (Freire, 2019).

3.3. Specificity and promiscuity of toxin neutralization amongst class II TA systems

Bacterial species encode numerous TA systems in their genomes, and often bacteria encode several paralogous TAs that are similar at sequence and levels (Leplae et al., 2011). However, naturally occurring paralogues of TA systems do not exabit cross-talk, i.e. antitoxins are specific for cognate toxins and do not cross-neutralise non-cognate toxins (Aakre et al., 2015). However, though directed evolution – i.e. by applying rounds of selection to mutagenized libraries expressing variants of antitoxins – is possible to change neutralisation specificity, as shown for ParD-ParE TA proteins (Aakre et al., 2015). Furthermore, through addition selection rounds it is possible to narrow the specificity spectrum, so that the evolved antitoxin cannot neutralise the original cognate toxin (Aakre et al., 2015). This evolutionary plasticity of TA pairs is referred to promiscuity (**Figure 11A**). A more extreme case of antitoxin promiscuity regarding neutralised toxins– hyperpromiscuity – is provided by members the Panacea (PanA) antitoxin family (Kurata et al., 2022). In this case, members of Panacea-containing antitoxins can neutralise diverse toxins that do not share either common fold or sequence homology **(Figure 11B**).

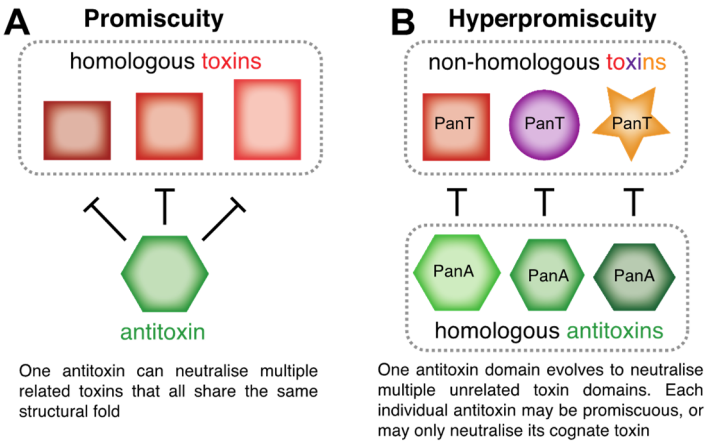

Figure 11. Antitoxin promiscuity and hyperpromiscuity. (**A**) A promiscuous antitoxin has relaxed neutralization specificity toward its target toxin and can neutralize a range of related toxins which all share the same structural fold. (**B**) A hyperpromiscuous antitoxin domain, as exemplified by Panacea, can evolve to neutralize unrelated toxins that do not share neither structural fold nor mechanism of toxic action (Kurata et al., 2022).

3.4. Biological functions of TA systems

The first biological function of TA systems to be discovered is the F plasmid addiction mediated by CcdAB TA pair (Gerdes et al., 1986; Ogura and Hiraga, 1983) (**Figure 12**). Plasmid addiction mediated by so-called post segregational killing (PSK) is a result of an interplay between a short-lived proteolytically unstable antitoxin combined with proteolytically stable toxin (Jensen and Gerdes, 1995; Van Melderen et al., 1994). If the bacterium loses the TA-encoding plasmid, the antitoxin is degraded over time, resulting in free toxin which, in turn, kills the cell. By eliminating the cells that have lost the plasmid from the population, PSK causes bacterial 'addiction' to the TA-encoding plasmid. In addition to CcdAB, other PSK loci include Hok/Sok system and Kis/Kid system encoded on plasmid R1, as well as ParDE system encoded on plasmid RK2 (Roberts, 1994; Tsuchimoto, 1988).

The next biological function of TA systems to be proposed is their role as metabolic 'breaks' that are engaged upon stress (Christensen and Gerdes, 2003; Ronneau, 2019). However, this stress response function of TAs is a matter of controversy. It was shown that while different stresses do induce the transcriptional activation of type II TA systems in *E. coli*, this does not result in generation of free toxins that would, in turn, repress the growth and throttle the metabolism (LeRoux, 2020). Antibiotic challenge is one type of stress, and one of the mechanisms how bacteria survive this stress is formation of a small sub-population antibiotic tolerant bacteria within a genetically identical population – formation of so-called persisters cells (Kaldalu N., 2020). High levels of persistence were detected in an *E. coli* strain that carried a mutation in the *hipAB* TA system, the

so-called *hipA7* allele (*hip* stands for 'high incidence of persistence') (Korch et al., 2003). This observation suggested that TAs could act as the drivers of persistence. However, knockout of *hipAB* locus does not have effect on the persister frequency (Korch et al., 2003). The Kenn Gerdes lab pursued this direction further and created an *E. coli* strain in which ten TA loci were genetically ablated, the Δ10 TA *E. coli* – and, indeed, this strain appeared to have much lower persistence levels, seemingly supporting the key role of TAs as the drivers of persistence (al., 2018; Maisonneuve and Gerdes, 2014). However, later it has been discovered that the Δ10 strain (and several related strains lacking TA modules) were infected with Φ80 and λ bacteriophages, which explained the phenotype (Goormaghtigh et al., 2018; Harms et al., 2017). Currently the possible role of TAs in persistence is unclear.

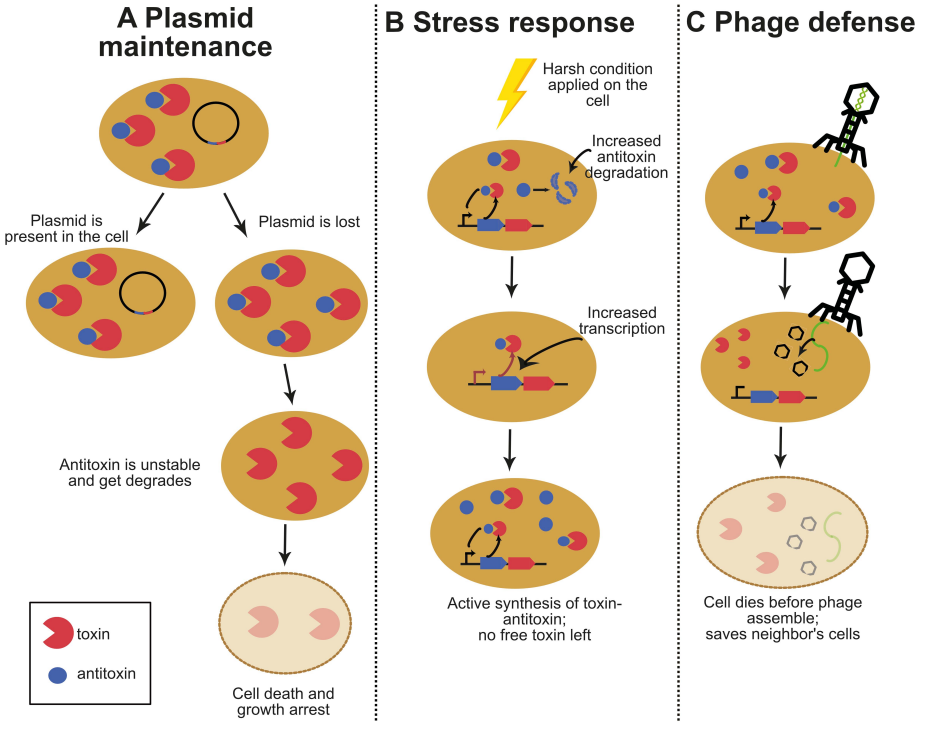

Figure 12. Biological functions of TAs. Three major categories of proposed functions for TA systems are (**A**) plasmid maintenance, (**B**) stress response and (**C**) phage defence. (**A**) Loss of a TA-encoding plasmid can lead to degradation of the antitoxin, leading to free toxin in the cell, which then prevents growth of a cell that is lacking the plasmid. This ensures cellular addiction to the TA-encoding plasmid. (**B**) In response to abiotic stress antitoxins can be degraded which leads to transcriptional induction of TA modules. This can lead to TA-mediated toxicity slowing down bacterial growth and promoting stress tolerance. (**C**) For some TA systems, phage infection was shown lead to release of active toxin. This results in infected cell effectively committing suicide and limiting the phage spread in bacterial population. Adapted from (LeRoux, 2022).

Finally, the function that gained a lot of attention in the recent years is the role of TAs as inducible toxic effectors that mediate anti-phage defence via abortive infection (Abi) mechanisms (LeRoux, 2022; Lopatina, 2020; Maikova, 2018). TA-mediated Abi relies on bacterial cell sensing the invading phage, activating the TA-mediated cytotoxicity and 'committing suicide' in order to halt the phage spread through bacterial population (LeRoux, 2022; Wein, 2022). The first TA system that was suggested to act as an anti-phage system is the Type II RnlAB system with RnlA RNase effector toxin degrading phage (and host) RNA in response to infection (Otsuka, 2005). Similarly, to RnlAB, type III ToxIN abortive infection TA system acts via release of the toxN RNase (Guegler and Laub, 2021). It was shown that mycobacterial prophage Phrann that encodes a gp29:gp30 toxSAS TA pair renders its bacterial host resistant from co-infection by other temperate phages such as Tweety, suggesting that toxSAS TAs could act as phage defence systems (Dedrick et al., 2017). Recent experiments with CapRel^{SJ46} toxSAS system have directly established the role of this TA in phage defence by demonstrating that this fused TA (i.e. a TA in which toxin and antitoxin constitute one polypeptide) directly binds phage major capsid protein, which, in turn, results in toxSAS activation and abortive infection (Zhang et al., 2022).

AIMS OF THE STUDY

My PhD studies focused on several mechanisms employed to supress cellular metabolism, with a specific focus on translation inhibition by toxins and ribosome hibernation factors. Specifically, the aims were:

- To uncover the mechanisms of toxSAS-mediated growth inhibition (Paper I)
- To uncover the mechanisms of PanT-mediated growth inhibition (Paper II)
- To exploit the genetic ablation of ribosome inhibition though dimerization in order to develop more active lysate-based bacterial and yeast *in vitro* translation systems (Paper III)

MATERIALS AND METHODS

All materials and methods are described in publications I, II and III. The most important methods are specified bellow.

1. Toxicity neutralization assay

Toxicity neutralization assays were performed in papers I and II.

LA plate with 1% glucose and 25 μg/ml chloramphenicol and 100 μg/ml carbenicillin containing *E. coli* BW25113 with toxin plasmid and/or antitoxin plasmid and plates with empty vector plasmid control (pBAD33 for toxin and pKK223-3 for antitoxin) were grown overnight at 37 °C.

Next day a single colony was inoculated into 2 ml of LB medium contain 100 μg/ml carbenicillin, 25 μg/ml chloramphenicol and 1% glucose. The cultures were incubated in the liquid Luria Broth (LB) medium at 37 °C for 16 hours at 180 rpm.

Next, the overnight culture was diluted to an OD_{600} of 1.0 with LB medium. 90 μl of LB medium was added into wells of sterile 96-well Falcon plate. Bacterial cultures were diluted serially (10^{-1} to 10^{-8}-fold) by taking 10 μl from the well on the immediate left and transferring it to the next well (pipette tips was changed between dilutions).5 μl of the dilutions were spotted onto desired non-induction (1% glucose and 25 mg/ml chloramphenicol and 100 mg/ml carbenicillin) and induction plates (0,2% arabinose, 1mM IPTG and 25 μg/ml chloramphenicol and 100 μg/ml carbenicillin)using a multi-channel pipette. Plates were incubated at 37 °C for 23 hours. The results were recorded by counting CFU or making pictures of the plates.

2. Metabolic labelling

Metabolic labelling assays were performed in papers I and II.

BW25113 *E. coli* wild-type strain carrying pKK223-3 and toxin plasmid was streaked on LA plate supplemented with 1% glucose, 25 μg/ml chloramphenicol and 100 μg/ml carbenicillin.

Three colonies were used to inoculate individual tubes containing 2 ml of MOPS liquid medium with 1% glucose, 25 μg/ml chloramphenicol and 100 μg/ml carbenicillin. The tubes were incubated with shaking (180 rpm) at 37 °C overnight (16–18 hours).

Next day the overnight precultures were diluted to OD_{600} of 0.05 in 15 ml MOPS liquid medium with 0.5% glycerol supplemented with 19 AA at final concentration 25 μg/ml (methionine excluded from the medium) plus 25 μg/ml chloramphenicol and 100 μg/ml carbenicillin. The culture was grown at 37 °C, 200 rpm until OD_{600} of 0.2–0.3.

While the culture was growing, 10 µl of 4.3 µCi ^{35}S-methionine, 0.65 µCi ^{3}H-uridine or 2 µCi ^{3}H-thymidine were added into autoclaved 1.5 ml tubes, kept on ice or at 4 °C in the fridge. Separately 200 µl aliquots of 50% TCA were prepared in 1.5 ml Eppendorf tubes and kept on ice or at 4 °C. Tubes with isotopes were prewarmed at 37 °C for 10 minutes before metabolic labelling.

When OD_{600} of the bacterial culture reached to 0.2–0.3, 1ml of culture was transferred into prewarmed tube containing isotope solution and kept at 37 °C for 8 minutes. Immediately after that 20% of arabinose was added at final concentration 0.2%. Time points were taken after 2, 5, 10, 15 minutes. In parallel, the OD_{600} of the culture was measured. The labelled solution was added to ice cold 1.5 ml tube containing 200 µl of 50% TCA. The mixtures were kept on ice.

After that filtration system was set up. The GF/C filter was in the beginning washed with 1–2 ml of 5% TCA. Later TCA-stopped bacterial culture was added and washed 2 times with 5 ml of pre-chilled 5% TCA, and 2 times with 5 ml of pre-chilled 95% ethanol. The filters were put into scintillation vials and dried for at least two hours or overnight at room temperature. After this 5 ml of scintillation cocktail was added to the vials. The vials were put on the orbital shaker for 15 minutes at room temperature and then subjected to scintillation counting using TRI-CARB 4910TR 100 V scintillation counter (PerkinElmer).

3. *In vitro* transcription-translation assays

Transcription-translation assays using cell-free lysates were performed in paper III.

3.1. Preparation of cell-free translational extracts

Cell-free translational lysates were prepared in paper III.

The *Bacillus subtilis* 168 WT and Δ*hpf* mutant were plated on LA plate without antibiotics. Overnight cultures were started by inoculation single colony into 50 ml liquid LB. The culture was grown for 16 hours, at 37 °C, 180 rpm. Next day, the preculture was diluted to OD_{600} of 0.05 in 800 ml of LB. The cells grew at 37 °C, 180 rpm for 5 hours to OD_{600} 1.8–2.2. Bacterial cells were collected by centrifugation at 10 000g for 3 minutes at 4 °C in a Beckman JLA-10.500 rotor. The cell pellets were washed twice with 100 ml and 50 ml of ice-cold 1x 12S lysis buffer (10 mM Tris-acetate pH 8.2, 60 mM $C_5H_8KNO_4$, 14 mM $Mg(C_2H_3O_2)_2$, 0.5 mM PMSF, 1 mM DTT). After each washing step cells were pelleted by centrifugation at 10 000 g for 3 minutes at 4 °C in a Beckman JLA-10.500 rotor. The final centrifugation was done in 50 ml Falcon tubes at 3220 g for 30 minutes at 4 °C (Eppendorf Centrifuge 5810/5810 R). The cells were weighted using Delta Range balance Mettler, PM4600 (4–5 g was usually obtained). At this step cells can be zip-frozen in liquid nitrogen and stored at –80 °C until further use. After that cell pellets were resuspended in 12 ml of 1x12S lysis buffer and lysed by Pressure Cell/Homogenizer at 1.5 bar, 1 pass.

The cell debris was pelleted at 16 000 g for 10 minutes using Beckman JA-25.50 rotor. The supernatant was collected and desalted using 5 ml Zebra Spin Desalting Columns (ThermoFisher) and equilibrated with 1x 12S lysis buffer. The OD_{260} was measured, and lysates were diluted if needed to equal optical density.

The *Saccharomyces cerevisiae* MBS and MJY1079 strains were plated on YPD agar plate without antibiotics. Overnight cultures were started by inoculation of a single colony into 50 ml of YPD liquid medium and incubated at 30 °C, 140 rpm for 24 hours. Next day, preculture were diluted to OD_{600} of 0.001 in 800 ml of YPD. The cells grew at 30 °C, 140 rpm for 12–18 hours to an OD_{600} of 4–7. After that the cells were harvested by centrifugation at 7000 g for 10 minutes at 4 °C in a Beckman JLA-10.500 rotor. The pellets were washed twice with 1x Sarnow A buffer (30 mM HEPES pH 7.6, 100 mM KCH_3COO, 2 mM $Mg(C_2H_3O_2)_2$, 0.5 mM PMSF, 1 mM DTT). Cell pellets were centrifuged under the same condition after each washing step with 100 ml and 50 ml of ice-cold buffer. The final centrifugation was done in 50 ml Falcon tubes with 20 ml of 1x Sarnow A buffer at 3220g for 30 minutes at 4 °C (Eppendorf Centrifuge 5810/5810 R). At this step cells can be zip-frozen in liquid nitrogen and stored at –80 °C until further use. After that cell pellets were resuspended in 1/10 volume (w/v) of 1x Sarnow A buffer. The yeast pellets were lysed in liquid nitrogen in mortar for 20 minutes. The obtained lysates were collected in 50 ml Falcon tubes and left on ice for 5 hours to melt. The cell debris was removed by centrifugation at 4000 g for 30 minutes at 4 °C (Eppendorf Centrifuge 5810/5810 R). The supernatant was transferred to new Eppendorf tubes and centrifuged at 21 000 g for 10 minutes at 4 °C (Heraeus Fresco 21 Microcentrifuge, ThermoFisher). The supernatant was desalted with 5 ml Zebra Spin Desalting Columns (ThermoFisher). The lysates were aliquoted and quickly frozen in liquid nitrogen and stored at –80 °C.

3.2. Cell-free transcription-translation reactions and luciferase assay

For *B. subtills* lysates the total volume of a reaction was 30 µl, which included 50% v/v of lysate and 50% v/v of buffer. The final reaction conditions were: 5–15 mM $Mg(C_2H_3O_2)_2$, 2 mM of each amino acid (excluding methionine), 60 mM HEPES pH 8.2, 4.8 mM ATP, 3.4 mM GTP, CTP and UTP, 0.068 mM folinic acid, 36% (w/v) PEG-8000, 60 mM glucose, 2 µg/ml plasmid pIVEX2.3MCs FFluc, 2 mM DTT, 1 mg/ml T7 RNAP, 20 mM K2HPO4 pH 7.22, 90 mM K-glutamate, 80 mM NH4CH3CO2. The total volume (30 µl) of the cell-free reaction was aliquoted into 1.5 ml Eppendorf Safe-Lock Tubes. The lysates were mixed well by pipetting and incubated at 37 °C on Eppendorf Thermomixer with shaking (500 rpm) for 1 hour.

For *S. cerevisiae* lysates, the total volume of the reaction was 30 µl as well (50% v/v of lysate and 50% v/v of buffer). The final reaction conditions were: 1–3 mM $Mg(C_2H_3O_2)_2$, 0,025 mM each amino acid (excluding methionine),

1 mM 20x S-J buffer (200 mM HEPES pH 7.4, 2M KCH_3COO, 20 mM DTT), 3 mM ATP, 0.25 mM GTP, 25 M phosphocreatine, 0.3 μ/μl rRNasin, 0.04 mg/ml creatine phosphokinase, 0.005 mg/ml capped T7-FFLuc-A30 mRNA. Before using the mRNA, it was unfolded at 70 °C for 7 minutes and refolded by cooling on ice. The cell-free reaction mixture was aliquoted into 1.5 ml Eppendorf Safe-Lock Tubes, mixed well by pipetting and incubated at 25 °C on Eppendorf Thermomixer with shaking (500 rpm) for 30 or 60 minutes.

Luciferase Assay was used for both bacterial and yeast lysates to determine the influence of ribosome dimerization on the translation process. A Steady-Glo Luciferase Assay (Promega) was conducted following the manufacturer's instructions. Luciferase assay reagent was aliquoted (50 μl) into 1.5 ml Eppendorf tubes and kept in the dark at room temperature. Then, 10 μl of translation reaction was added and luminescence recorded immediately using a GloMax 20/20 Luminometer (Lus-0-Inj Promega program).

RESULTS AND DISSCUSSION

I. toxSAS FaRel2, PhRel, PhRel2 and CapRel pyrophosphorylate tRNA CCA end to abrogate the tRNA aminoacylation (Paper I)

Out of the five subfamilies of toxSAS enzymes which act as toxic effectors of TA modules, the mechanism of toxicity of only one – the (p)ppApp-producing FaRel – was characterised in detail in the original report (Jimmy et al., 2020). Therefore, I have set out to uncover the molecular mechanism of toxicity of the four unexplored toxSAS representatives: PhRel2 identified in *Bacillus subtilis* la1a, FaRel2 identified in *Coprobacillus* sp. D7, PhRel (Gp29) identified in *Mycobacterium* phage Phrann and CapRel identified in *Mycobacterium tuberculosis* AB308.

First, I performed the HPLC analysis of bacterial nucleotide pools (Varik et al., 2017). Surprisingly, expression of none of the TA systems under study produced (p)ppGpp or (pp)pApp nucleotides, indicating that their mechanism of toxicity is profoundly different from that of FaRel (**Figure 13A**).

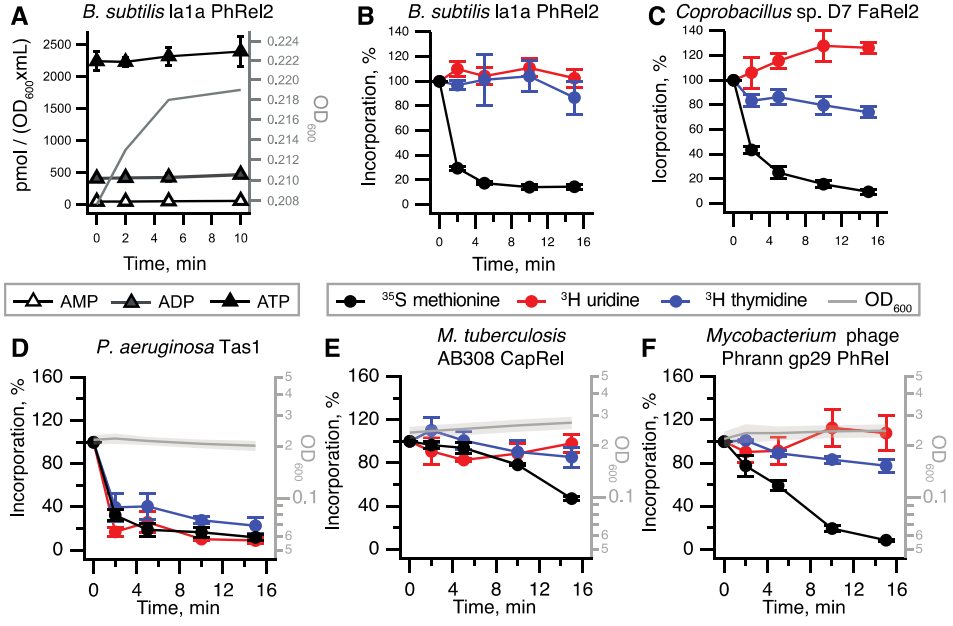

Figure 13. Inhibition of protein synthesis is an evolutionary widespread mechanism of toxSAS-mediated growth arrest. (**A**) The expression of *B. subtilis* la1a PhRel2 does not perturb the adenosine nucleotide pools, and (pp)pApp is not detectable upon expression of the toxin. (**B-F**) Metabolic labelling assays following incorporation of ^{35}S-methionine (black traces), ^{3}H-uridine (red traces), and ^{3}H-thymidine (blue traces). Expression of *B. subtilis* la1a PhRel2 (**B**), *Coprobacillus* sp. D7 FaRel2 (**C**), *P. aeruginosa* Tas1 SS effector (**D**), *M. tuberculosis* AB308 CapRel (**E**) and *Mycobacterium* phage Phrann PhRel Gp29 (**F**) from the pBAD33-based constructs was induced with 0.2% L-arabinose. Adapted from (Kurata et al., 2021).

Next, I used metabolic labelling approach to uncover which of the biosynthetic processes is inhibited by toxSASs (Jimmy et al., 2020). I followed the incorporation of ^{35}S methionine into proteins, ^{3}H uridine into RNA and ^{3}H thymidine into DNA. In stark contrast to FaRel (Jimmy et al., 2020) – and (pp)pApp-producing Tas1 (**Figure 13D**) – the rest of toxSAS inhibit translation, as signified by compromised ^{35}S methionine incorporation (**Figure 13**).

The next question we asked was as to how the translation-targeting toxSAS inhibit protein synthesis on molecular level. The hypothesis was that just as FaRel could switch its substrate specificity from recognising adenosine to guanosine in NT/DPs, the translation-targeting toxSAS could further change their substrate specificity and recognise the A residue of tRNA CCA end instead of that of free AT/DP nucleotide. This turned out to be exactly the case. We could detect that when radioactive ATP was present then FaRel2 was incubated with initiator $tRNA_i^{fMet}$ and ^{32}P-labelled ATP (**Figure 14B**).

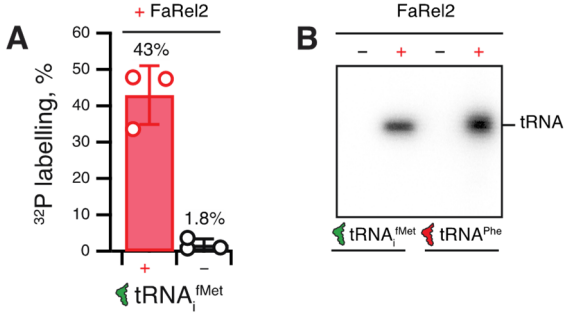

Figure 14. *Coprobacillus* sp. D7 FaRel2 pyrophosphorylates the tRNA 3'CCA end to inhibit tRNA aminoacylation. A reconstituted ^{32}P transfer reaction using 50 nM FaRel2, 100 µM γ-^{32}P-ATP, and either 5 µM $tRNA_i^{fMet}$ or 5 µM $tRNA^{Phe}$ as a substrate. ^{32}P transfer was either quantified via scintillation counting (**A**) or visualised using a Phosphoimager (**B**). Adapted from (Kurata et al., 2021).

Final validation of the chemical nature of CCA modification installed by toxSAS came from the lab of our collaborator, Tsutomu Suzuki. Using LC-MS system to analyse the RNA fragments generated from 3' terminal regions of tRNA CCA, the Suzuki lab directly established that the 3'CCA end of tRNA is pyrophosphorylated by *Coprobacillus* sp. FaRel2 (see (Kurata et al., 2021) for detail).

Next, I tested if the cellular toxicity of tRNA-modifying FaRel2 could be neutralized by SAH enzymes: human SAH enzyme Mesh1 and *C. marina* ATfaRel (**Figure 15**). After co-expression of human MESH1 or *C. marina* ATfaRel counteracted the growth inhibition by FaRel2, albite did not fully. This indicates that the SAH tested can convert pyrophosphorylated tRNA back to deacylated tRNA, i.e., catalyse RNA modification – a type of reaction that was never reported for SAH before. Complementary biochemical experiments using ^{32}P-labelled tRNA-PP and SAH that performed by my co-author Tatsuaki Kurata directly supported my microbiological experiments (see (Kurata et al., 2021) for detail).

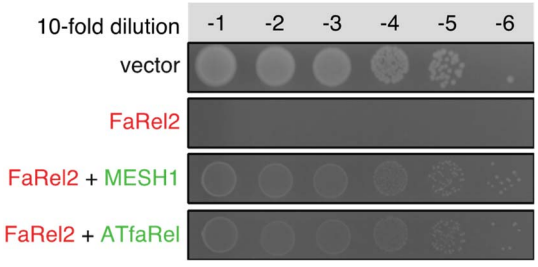

Figure 15. Co-expression of SAH enzymes *C. marina* ATfaRel and *H. sapiens* MESH1 counteracts FaRel2-mediated toxicity. Co-expression of either human MESH1 or *C. marina* ATfaRel SAH enzymes counteracts the toxicity of *Coprobacillus* sp. D7 FaRel2 tRNA-modifying toxSAS. The SAH enzymes and FaRel2 toxSAS were expressed from pMG25 and pMR33 derivative plasmids, respectively. Adapted from (Kurata et al., 2021).

Inhibition of translation though modification of tRNA is a recurring theme in TA-mediated toxicity, with notable examples being GCN5-related N-acetyl-transferase (GNAT)-like toxins AtaT (Cheverton A. M., 2016; Jurenas et al., 2017), VapC PIN-like domain nucleases (Gobert et al., 2019; Winther and Gerdes, 2011) and DUF1814-family nucleotidyltransferase-like toxins MenT1 adding pyrimidines (C or U) to tRNA 3'CCA end (Cai, 2020). Our discovery adds one more example to this common theme. It also rises the question as to why amongst the three steps of the central dogma – translation, transcription, replication – it is the translation that is commonly targeted by TAs. To the best of my knowledge no TAs targeting transcription has been identified to date.

II. Panacea: a hyperpromiscuous antitoxin protein domain involved in neutralization of diverse toxin domains (Paper II)

FlaGs-guided bioinformatic analysis (Saha et al., 2021) of the antitoxin that neutralises *B. subtilis* Ia1a PhRel2 toxSAS uncovered that i) the antitoxin contains a domain of unknown function (DUF) DUF4065 ii) DUF4065 is can be found in TA-like arraignments with numerous unrelated toxins (Kurata et al., 2022). New TA pairs with DUF4065-containint antitoxins were found to be widely distributed in bacteria, archaea and bacteriophages. Given the wide distribution DUF4065 and its ability to neutralise unrelated toxins, we renamed it to Panacea after Greek goddess of universal remedy, with Panacea-containing toxins referred to as PanA's and their toxins as PanT's.

The toxins in these pairs were predicted to be mRNA interferases (RNases) (Christensen and Gerdes, 2003; Yamaguchi et al., 2009), members of Fic/Doc toxin family (Castro-Roa, 2013), membrane disruptors (Verstraeten et al., 2015), enzymes involved in nucleotide modification (Bordes, 2011) and toxSASs (Jimmy et al., 2020) (**Figure 16A**). For experimental validation of PanTAs systems in neutralization assays were selected nine TA pairs that are sampling all of these diverse predicted mechanisms of toxicity (**Figure 16B**).

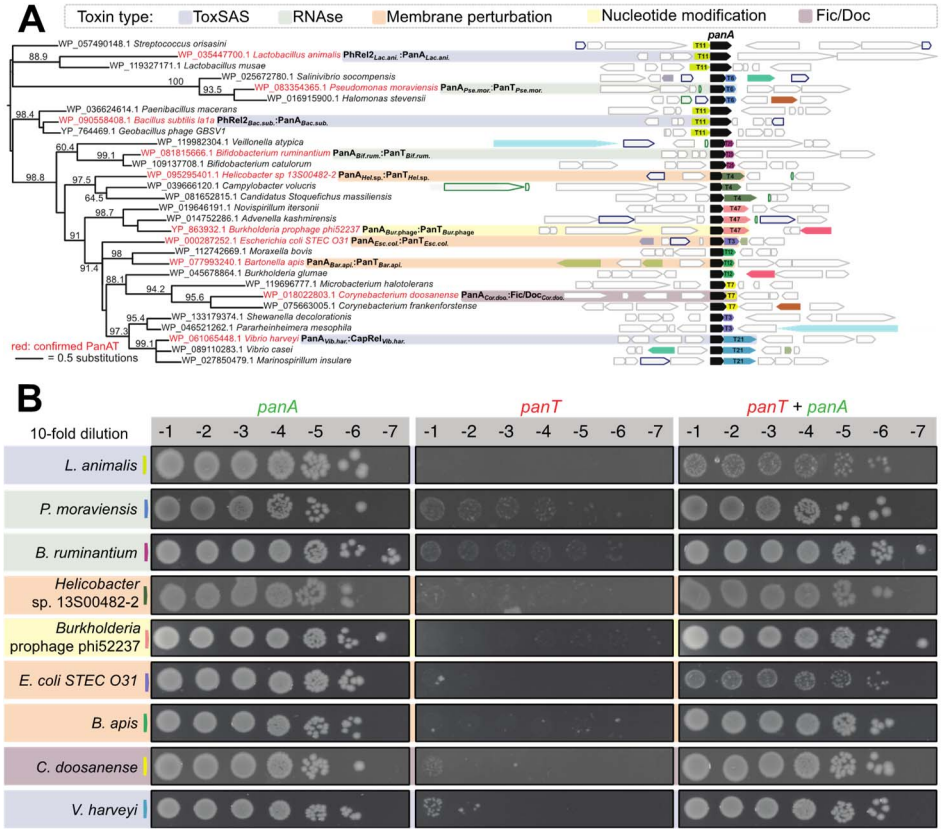

Figure 16. PanA antitoxins neutralise evolutionarily diverse TA toxins. (**A**) The maximum likelihood tree of PanA sequences, annotated with conserved gene neighborhoods generated with FlaGs. (**B**) Validation of panAT TA pairs by toxicity neutralization assays. Overnight cultures of *E. coli* strains transformed with pBAD33 and pKK223-3 vectors or derivatives expressing putative *panT* toxins and *panA* antitoxins, correspondingly, were adjusted to OD_{600} 1.0, serially diluted, and spotted on LB medium supplemented with appropriate antibiotics and inducers (0.2% arabinose for *panT* induction and 1 mM IPTG for *panA* induction). Adapted from (Kurata et al., 2022).

To uncover the mode of action of PanTs, I used the metabolic labelling assay which was already described in the previous chapter (Paper I, (Kurata et al., 2021)).

As expected for predicted translation inhibitors, toxSASs *L. animalis* $PhRel2_{Lac.ani.}$ and toxSAS *V. harveyi* $CapRel_{Vib.\ har.}$, RNases $PanT_{Pse.\ mor.}$ as well as $PanT_{Bif.\ rum.}$ and Fic/Doc toxin *C. doosanense* $Fic/Doc_{Cor.\ doo.}$ all specifically inhibited incorporation of radiolabelled ^{35}S methionine, thus establishing translation as one of the primary targets of PanTs (**Figure 17**).

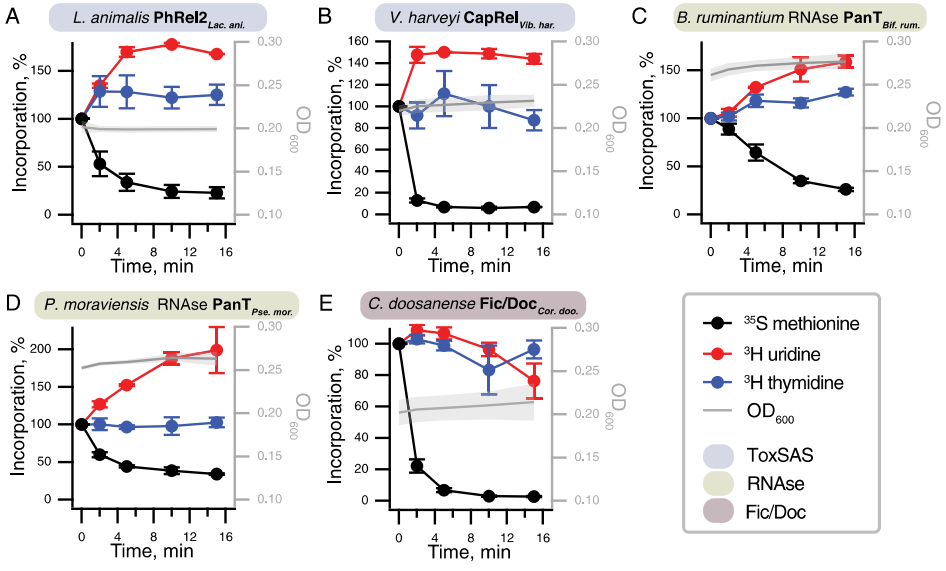

Figure 17. **Protein synthesis is a major target of PanT toxins**. Metabolic-labeling assays following the incorporation of ^{35}S methionine (black traces), ^{3}H uridine (red), and ^{3}H thymidine (blue) upon expression of translation-inhibiting PanT representatives: (**A**) *L. animalis* PhRel2 and (**B**) *V. harveyi* CapRel tox-SAS; putative RNases (**C**) PanT$_{\text{Bif. rum.}}$ and (**D**) PanT$_{\text{Pse. mor.}}$ and (**E**) *C. doosanense* Fic/Doc$_{\text{Cor. doo.}}$ toxin. Expression of PanTs in *E. coli* BW25113 was induced with 0.2% L-arabinose. Adapted from (Kurata et al., 2022).

Next, I performed metabolic labelling with predicted membrane disrupting toxins. For this kind of toxins, one would expect that all the three probed metabolic processes – translation, transcription and replication – would be affected simultaneously and to the same extent. Results obtained with PanT$_{Esc.col.}$ (**Figure 18A**), PanT$_{Bar.api.}$ (**Figure 18C**), and PanT$_{Hel.sp.}$ (**Figure 18C**) are in good agreement with this prediction. In the case of *E. coli* STEC PanT$_{Esc.col}$ toxin and *Helicobacter* sp. PanT$_{Hel.sp}$ (**Figure 18A–C**) abrogation of incorporation of radiolabelled precursors happens immediately after toxin induction. In the case of *B. apis* PanT$_{Bar.api.}$ toxin the inhibition of replication, transcription and translation is more gradual, and at 15 min time point after induction of toxin the decrease in incorporation efficiency was over 80%.

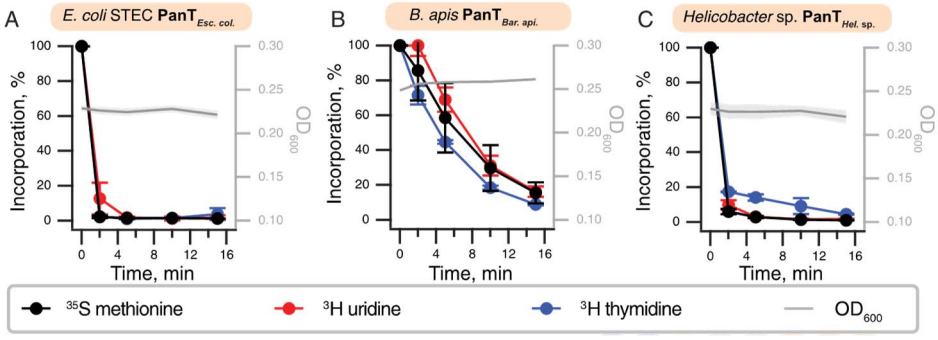

Figure 18. Membrane-targeting PanT's simultaneously abrogate translation, transcription and replication. Metabolic labelling assays with wild-type *E. coli* BW25113 expressing (**A**) PanT$_{Esc.\ col.}$, (**B**) PanT$_{Bar.\ api.}$, or (**C**) PanT$_{Hel.\ sp.}$ PanT toxins. Adapted from (Kurata et al., 2022).

I have obtained rather unexpected results with *Burkholderia* prophage PanT$_{Bur.phage}$ toxin that was predicted to be a nucleotide-modifying enzyme. Metabolic labelling data revealed that this toxin inhibits transcription, with weaker effects on translation and even weaker on replication (**Figure 19A**). HPLC-based analysis of nucleotide pools revealed that PanT$_{Bur.phage}$ induction *E. coli* resulted in depletion of GTP and production of ppGpp (**Figure 19C**). One possibility is that the toxin itself produced (p)ppGpp, and the other is that it activates the alarmone production by RelA. To discriminate between these two possibilities, I have repeated the HPLC analysis using a *relA* knockout *E. coli* strain (**Figure 19B**). In this strain no accumulation of (p)ppGpp was detected, suggesting that PanT$_{Bur.phage}$ activates RelA. To test if activation of RelA is crucial for PanT$_{Bur.phage}$-mediated toxicity, I assessed the growth inhibition PanT$_{Bur.phage}$ as well as performed the metabolic labelling assays in a *relA* knockout *E. coli* strain (**Figure 19B**). Since PanT$_{Bur.phage}$ inhibited the growth of this strain just as it did in the case of wild type and exhibited the same pattern in metabolic labelling assays, I concluded that activation of RelA-mediated stringent response is not crucial for PanT$_{Bur.phage}$ mechanism of action.

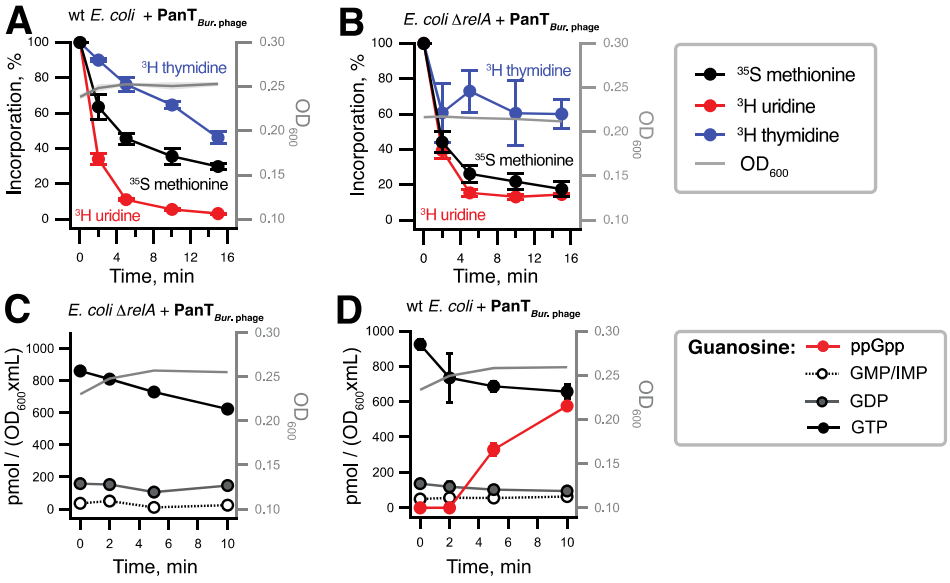

Figure 19. Expression of PanT$_{Bur.\ phage}$ toxin encoded in *Burkholderia* prophage phi52237 compromises transcription and translation and induces the RelA-mediated stringent response. (**A** and **B**) Metabolic labelling assays using either wild-type (**A**) or Δ*relA* (**B**) *E. coli* BW25113 expressing PanT$_{Bur.\ phage}$ toxin. (**C** and **D**) HPLC analysis of guanosine nucleotide pools in either wild-type (**C**) or Δ*relA* (**D**) *E. coli* BW25113 expressing PanT $_{Bur.\ phage}$ toxin. Cell cultures were grown in defined minimal MOPS medium supplemented with 0.5% glycerol at 37 °C with vigorous aeration. Expression of PanT $_{Bur.\ phage}$ toxin was induced with 0.2% L-arabinose at the OD_{600} 0.2. Intracellular nucleotides are expressed in pmol per OD_{600}×mL as per inset. Error bars indicate the SE of the arithmetic mean of three biological replicates. Adapted from (Kurata et al., 2022).

Finally, I performed cross-neutralization assay to test if non-cognate PanA can neutralize of non-cognate PanT (**Figure 20A**). The rationale for this experiment is that if PanAs can swap partners in the course of evolution, they might be intrinsically promiscuous, i.e. able to neutralise non-cognate toxins. However, the experiments resealed that PanAs are specific to cognate toxins, suggesting that promiscuity requires mutational adaptation of the PanA to its new PanT partners. Indeed, through directed evolution experiments, my college Toomas Mets could select evolved PanA$_{Vib.\ har.}$ version that was able to neutralise non-cognate PanT PhRel2$_{Bac.\ sub.}$ (Kurata et al., 2022).

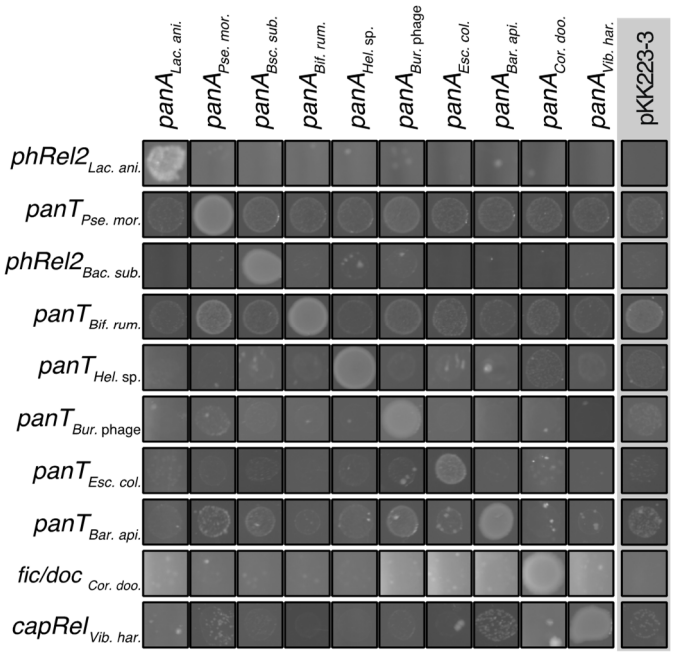

Figure 20. PanA antitoxins are highly specific for cognate PanT toxins. Exhaustive cross-neutralization testing was used to probe the specificity of PanA antitoxins toward PanT toxins. The overnight cultures of *E. coli* strains transformed with pBAD33 and pKK223-3 vectors or derivatives there of expressing toxin and PanA antitoxins was adjusted to 1.0, cultures serially diluted from 10^1-to10^6-fold and spotted on LB agar medium supplemented with appropriate antibiotics as well as inducers (0.2% arabinose for toxin induction and 1 mM IPTG for induction PanA variants); 10^1-fold dilution is shown. Adapted from (Kurata et al., 2022).

Our study brought the Panacea domain to the spotlight. Many questions remained unanswered by our initial study: is Panacea neutralising the toxins directly or does it merely serve as a scaffold, a platform, with Panacea-associated domains doing the job? Is Panacea unique – or are there other hyperpromiscuous antitoxin domains? Systematic application of FlaGs in an iterative mode can answer the latter question, and the former question requires structural studies. Prediction of PanTA structures by AlfaFold2 (Jumper et al., 2021; Jumper and Hassabis, 2022) can generate insights that can then be validated experimentally.

III. Elimination of ribosome inactivating factors improves the efficiency of protein production in yeast and B. subtilis in vitro translational lysates (Paper III)

Efficiency of lysate-based *in vitro* translation systems depends on the activity of the individual components. Since ribosomal dimerization throttles the translational capacity of the cell by decreasing the concentration of active ribosomes, we reasoned that genetic ablation of the system via disruption of the genes involved in ribosomal dimerization could increase the activity of lysates made from genetically engineered strains. To probe this hypothesis, I chouse to work with two experimental lysate systems. One bacterial, made from *B. subtilis* lacking Hpf factor essential for the 100S formation, and one yeast, made from *Saccharomyces cerevisiae* lacking Stm1 protein that plays a similar role to *B. subtilis* Hpf (Balagopal V., 2011; Ben-Shem A., 2011). The efficiency of translation in lysates was by using a model mRNA encoding luciferase and measuring the luminescence, reported in relative luminescence units (RLU).

For the first set of experiments, I used wild-type *B. subtilis* 168 as well as an isogenic knockout strain in which *hpf* gene was genetically disrupted. The polysome profile of the cells shows that in the case of the wild type-strain the 100S peak is clearly visible (**Figure 21A**), in the Δ*hpf* mutant 100S peak is lacking (**Figure 21B**).

To systematically test the effect of HPF loss on the efficiency of transcription-translation system, I performed two titrations of the main constitutes of the *in vitro* lysate system. Firstly, I optimized the ratio between the compound mix (contains amino acids, ATP and other small molecules that support the biochemical reactions) and cell-free lysate (**Figure 21D**). The highest activity was observed at the 1:1 ratio of the compound mix to cell-free lysate, and the Δ*hpf* lysate was clearly more active. Next, I needed to detect the optimal concentration of magnesium ions since it is crucial for transcription-translation system (Zaher H. S., 2014). Working at the optimal ratio between the compound mix to cell-free lysate, I titrated the final concentration of Mg^{2+} from 7 to 22 mM (**Figure 21E**). The *hpf* knockout lysate was more active than WT lysate, and the optimal activity was observed at 12 mM Mg^{2+}.

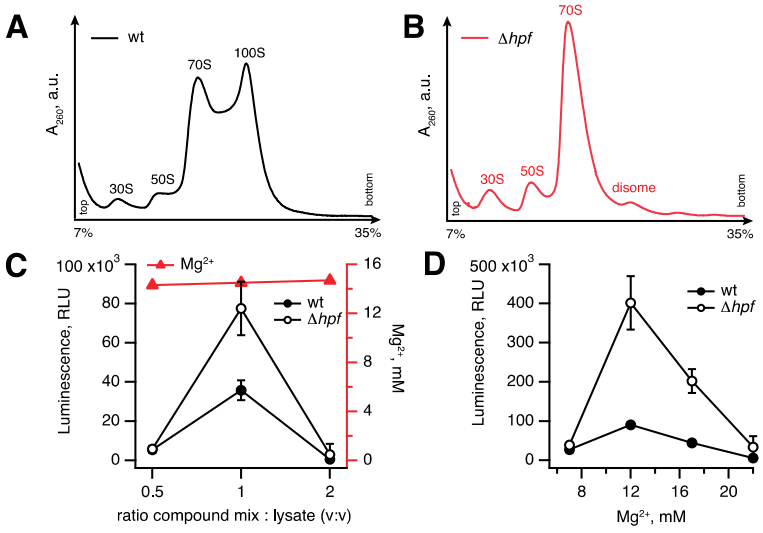

Figure 21. Elimination of HPF improves the efficiency of *B. subtilis* coupled transcription-translation system. Polysome profile analysis of translational lysates prepared from wild type (**A**) and Δ*hpf* (**B**) lysates demonstrates strictly HPF-dependent 100S ribosomes formation. (**D**) A cell-free translation system was assembled by combining the compound mix with the cell-free extract. The efficiency of translation was quantified by the activity of the firefly luciferase using the Steady-Glo Luciferase Assay (Promega). Titrations of the compound mix to cell-free extract ratio and magnesium ion concentration (**E**) in the cell-free translation system. Luminescence readings were taken after incubation for 1 hour at 37 °C. Error bars indicate the standard error of the geometric mean of biological replicates, i.e. independently prepared cell-free extracts (n≥3). Adapted from (Brodiazhenko T., 2018).

Finally, I performed a similar set of experiments with *S. cerevisiae* cell extracts prepared from wild type and *stm1*Δ *S. cerevisiae*. Again, I first optimised the ratio between the compound mix and lysate and then optimised the concentration of Mg^{2+} ions. In the case of *S. cerevisiae* extracts the activity increased with increased compound mixture concentration, and at 2-fold excess of the compound mixture over the lysate the activity of the *stm1*Δ system was higher than the of the wild type one (**Figure 22A**). Keeping the 2-fold excess of the compound mixture over the lysate, I have titrated the Mg^{2+}. The maximum activity was observed at 4.5 mM Mg^{2+}, with the *stm1*Δ *S. cerevisiae* lysate robustly outperforming the wild-type lysate throughout the whole Mg^{2+} titration range (**Figure 22B**).

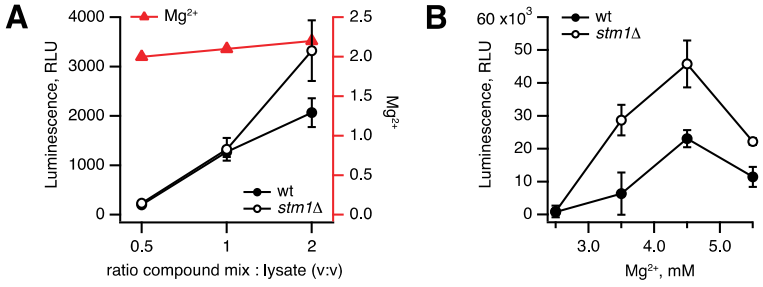

Figure 22. Elimination of Stm1 improves the efficiency of *S. cerevisiae* translation system. A cell-free translation system was assembled by combining the compound mix with the cell-free extract. The efficiency of translation was quantified by the activity of firefly luciferase using the Steady-Glo Luciferase Assay (Promega). Titrations of the compound mix to cell-free extract ratio (**B**) and magnesium ion concentration (**C**) in the cell-free translation system. Luminescence readings were taken after incubation for 1 hour at 25 °C. Error bars indicate the standard error of the geometric mean of biological replicates, i.e., independently prepared cell-free extracts (n≥3). Adapted from (Brodiazhenko T., 2018).

Our study serves as a first step towards developing even more active lysates. The strains with ablated ribosome dimerization can be further engineered by, for instance, expression of rare tRNAs, deleting components of protein degradation machinery.

CONCLUSIONS

The main conclusions of the work presented in this thesis are:

i) Enzymatic activities of RSH enzymes are not limited to production and degradation of (p)ppGpp. Members of toxSAS RSH PhRel2, FaRel2, PhRel and CapRel subfamilies catalyse pyrophosphorylation of tRNA 3'CCA end, and members of FaRel family catalyse synthesis of (pp)pApp. Members of SAH subfamily MESH1 and ATfaRel catalyse removal of the pyrophosphate from PP-tRNA and degradation of (pp)pApp.

ii) Antitoxins containing common Panacea (PanA) domain neutralize diverse toxin families (PanT). PanA-mediated PanT neutralisation is highly specific for cognate toxin-antitoxin pair.

iii) Genetic elimination of ribosome dimerization factors in Firmicute bacterium *B. subtilis* (*hfp*) and yeast *S. cerevisiae* (*stm1*) strains is a promising strategy for producing more active *in vitro* translation lysates. Loss of *hfp* and *stm1* fully eliminates the formation of translationally inactive ribosomal dimers in bacteria and yeast, respectively. Mg^{2+} concentration and the ratio between the lysate and the compound mix was identified as the key parameters that should be optimised for achieving the maximal activity of the lysate.

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SUMMARY IN ESTONIAN

RelA-SpoT valguperekonna ensüümid kui Toksiin-Antitoksiin süsteemide osalised

Nagu kõik elusorganismid, tunnetavad bakterid keskkonda ja reageerivad suurele hulgale erinevatele stressidele, kohandades vastavalt oma füsioloogiat.

Üks peamisi stressivastuseid on poomisvastus. Poomisvastus vahendab bakterite kohanemist toitainete vähesusega, samuti vastust abiootilisele keskkonnastressidele nagu näiteks kuumašokk. Rohkem kui kuus aastakümmet tagasi avastati, et häirenukleotiidid ppGpp ja pppGpp – ühiselt viidatud kui (p)ppGpp – ehk maagilised laigud tekivad *Escherichia coli* rakkudes vastusena aminohapete vähesusele. Poomisvastuse esimene füsioloogiline roll, mis tuvastati, oli stabiilse RNA (rRNA ja tRNA) sünteesi pärssimine, mis on kooskõlastatud aminohapete biosünteesi ja stressitaluvusega seotud geenide ekspressiooni indutseerimisega. Aastakümneid kestnud uuringud on aga näidanud, et lisaks transkriptsioonile on (p)ppGpp sihtmärkideks ka mitmed muud rakus toimuvad protsessid, nagu translatsioon, ribosoomide kokkupanek, antibiootikumiresistentsus ja virulentsus.

Veel üks oluline bakterite regulatsioonisüsteem põhineb toksiini – antitoksiin (TA) süsteemidel. Esimesed toksiini-antitoksiin (TA) süsteemide esindajad avastati 80ndate alguses. Klassikalised TA süsteemid on bitsistroonilised – st koosnevad kahest geenist – operonist, milles üks geen kodeerib valgulist toksiini ja teine antitoksiini, valku või RNAd, mis toksiini kas otseselt või kaudselt neutraliseerib. TA-süsteemide uuringud on viimastel aastatel plahvatuslikult kasvanud, avastatud on arvukalt uusi TA perekondi, iseloomustatud nende toimemehhanisme, iseloomustatud bioloogilisi funktsioone ja pakutud välja võimalikke rakendusi biotehnoloogias. Enim iseloomustatud funktsioonid hõlmavad plasmiidi säilitamist, kaitset bakteriofaagide vastu ja rakufüsioloogia reguleerimist.

Käesolevas uuringus kirjeldati RSH perekonna ensüümide uusi aktiivsusi ja toksiinide neutraliseerimise spetsiifilisust PanA antitoksiini perekonna liikmete poolt.

Lisaks eelpool kirjeldatud protsessidele toimub stressi ajal ribosoomide dimerisatsioon. See stressivastus on kasulik rakkudele ellujäämiseks, kuid võib lüsaatide kasutamise korral biotehnoloogias olla probleemiks kuna vähendab rakuvabade translatsioonisüsteemide aktiivsust. Seetõttu uuriti ribosoomi dimeriseerumise eest vastutavate valkude eemaldamise mõju rakulüsaatide aktiivsusele.

Leiti, et RSH ensüümide ensümaatiline aktiivsus ei piirdu (p)ppGpp tootmise ja lagunemisega. ToxSAS RSH PhRel2, FaRel2, PhRel ja CapRel alamperekondade liikmed katalüüsivad tRNA 3'CCA otsa pürofosforüülimist ja FaRel perekonna liikmed katalüüsivad (pp)pApp sünteesi. SAH alamperekonna liikmed MESH1 ja ATfaRel katalüüsivad pürofosfaadi eemaldamist PP-tRNA-st ja (pp)pApp lagunemist.

Ühist PanA domeeni sisaldavad antitoksiinid neutraliseerivad erinevaid toksiine. PanA-vahendatud toksiinide neutraliseerimine on toksiini osas siiski spetsiifiline.

Ribosoomi dimerisatsioonifaktorite geneetiline elimineerimine bakteri *B. subtilis* (hfp) ja pärmi *S. cerevisiae* (stm1) tüvedes on paljulubav strateegia aktiivsemate rakuvabade translatsioonilüsaatide tootmiseks. Reaktsiooni optimeerimisel on oluline panna tähele Mg^{2+} ja muude komponentide kontsentratsioone ja omavahelisi suhteid.

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TEADUSTEGEVUS

Minu teadustöö keskendub bakterite stressivastuse mehhanismide uurimisele. Täpsemalt uurin ma poomisvastuse keskse regulatoorse molekuli (p)ppGpp sünteesi. Lisaks uurin ma bakteriaalseid toksiin-antitoksiin süsteeme, mille mehhanismid on seotud poomisvastusega

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KÄSIKIRJAD

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