DISSERTATIONES
TECHNOLOGIAE
UNIVERSITATIS
TARTUENSIS
51

TOOMAS METS

RNA fragmentation by MazF and MqsR toxins of *Escherichia coli*

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
51

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
51

TOOMAS METS

RNA fragmentation by MazF and MqsR toxins of *Escherichia coli*

UNIVERSITY OF TARTU
Press

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

This dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on March 28th, 2019 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

Supervisor: Niilo Kaldalu, PhD
Institute of Technology
Faculty of Science and Technology
University of Tartu
Estonia

Opponent: Prof. Gerhart Wagner. PhD
Department of Cell and Molecular Biology
Uppsala University
Sweden

Commencement: Auditorium 121, Nooruse 1, Tartu, at 10:15 on May 10th, 2019

Publication of this dissertation is granted by the Institute of Technology, Faculty of Science and Technology, University of Tartu

ISSN 2228-0855
ISBN 978-9949-03-005-7 (print)
ISBN 978-9949-03-006-4 (pdf)

Copyright: Toomas Mets, 2019

University of Tartu Press
www.tyk.ee

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS 7

ABRREVATIONS 8

INTRODUCTION 9

1. REVIEW OF THE LITERATURE 10
 1.1. Classes of toxin-antitoxin systems and their regulatory architecture 10
  1.1.1. Type I toxin-antitoxin systems 10
  1.1.2. Type II toxin-antitoxin systems 13
  1.1.3. Type III toxin-antitoxin systems 20
  1.1.4. Type IV toxin-antitoxin systems 21
  1.1.5. Type V toxin-antitoxin systems 21
  1.1.6. Type VI toxin-antitoxin systems 22
 1.2. Distribution of toxin-antitoxin systems 22
 1.3. Biological functions of toxin-antitoxin systems 23
  1.3.1. Stabilization of mobile genetic elements 24
  1.3.2. Anti-addiction 24
  1.3.3. Abortive infection 25
  1.3.4. Metabolic downregulation 25
  1.3.5. Programmed cell death 26
  1.3.6. Persisters 26
  1.3.7. Transcriptional regulation 28
  1.3.8. Translational regulation of specific genes 28
  1.3.9. Reprogramming of the translational system 29
  1.3.10. Bacterial virulence 30
 1.4. Recovery from toxin activity 31
 1.5. Network of toxin-antitoxin systems 32

2. AIMS OF THE STUDY 33

3. RESULTS AND DISCUSSION 34
 3.1. Transcription of toxin-antitoxin systems can be activated by expressing non-cognate toxins 34
 3.2. *E. coli* cells recover from 90 minute transient toxin expression 35
 3.3. MazF and MqsR of *E. coli* cleave precursor rRNA at several positions 36
 3.4. MazF- and MqsR-generated rRNA fragments are mainly found in aberrant subunits 39
 3.5. Expressing MazF and MqsR leads to major changes in the transcriptome 41
 3.6. mRNA cleavage by MazF and MqsR 43
 3.7. The proposed MazF regulon genes are cleaved in the coding sequence 46
 3.8. MazF regulon is cleaved by MazF without reading frame bias 47

3.9. MazF regulon is not preferentially translated during MazF induction 47
4. CONCLUSIONS 50
REFERENCES 51
SUMMARY IN ESTONIAN 63
ACKNOWLEDGMENTS 65
PUBLICATIONS 67
CURRICULUM VITAE 115
ELULOOKIRJELDUS 116

LIST OF ORIGINAL PUBLICATIONS

Publication I Kasari V, **Mets T**, Tenson T, and Kaldalu N. Transcriptional cross-activation between toxin-antitoxin systems of Escherichia coli. BMC Microbiol. 2013;13(1): 45.

Publication II **Mets T**, Lippus M, Schryer D, Liiv A, Kasari V, Paier A, Maiväli Ü, Remme J, Tenson T, and Kaldalu N. Toxins MazF and MqsR cleave Escherichia coli rRNA precursors at multiple sites. RNA Biol. 2017;14(1): 124–135.

Publication III **Mets T**, Kasvandik S, Saarma M, Maiväli Ü, Tenson T, and Kaldalu N. Fragmentation of Escherichia coli mRNA by MazF and MqsR. Biochimie. 2018;156: 79–91.

My contributions to the publications:

Publication I I performed and analysed the results of all growth resumption experiments with flow cytometry.

Publication II I performed all experiments and data analysis, except for preparation and sequencing of cDNA libraries and separating ribosomal fractions. I also participated in the writing of this paper.

Publication III I performed all experiments and data analysis, except for preparation and sequencing of cDNA libraries and proteomics. I wrote the first draft of the manuscript and participated in the following rewrites.

ABRREVATIONS

GFP	green fluorescent protein
ncRNA	non-coding RNA
ORF	open reading frame
PNK	polynucleotide kinase
RACE	rapid amplification of cDNA ends
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SILAC	stable isotope labeling with amino acids in cell culture
TA	toxin-antitoxin
TPM	transcripts per kilobase million
UTR	untranslated region

INTRODUCTION

Bacterial populations rarely encounter ideal growth conditions and spend most of their existence accommodating some kind of stress. Nutrient limitation, hazardous chemicals, and hostile neighbors are all part of bacterial life. Bacteria have evolved many stress response pathways to cope with different harsh environments. The genetic units termed toxin-antitoxin systems are often seen as parasitic and selfish, however, toxin-antitoxin systems also seem to be recruited into stress response pathways in some cases. These addictive modules encode for an autotoxic protein and an antitoxin that inhibits the activity of the toxin. Because antitoxins are much more unstable than toxins, the loss of the toxin-antitoxin module results in active toxins that inhibit cell growth. Different studies associate toxin-antitoxin systems with abortive bacteriophage infections, growth regulation during stress, antibiotic tolerant persister cells, and bacterial virulence. Thus, studying toxin-antitoxin systems is likely to be beneficial for understanding and combating bacterial infections.

The functions of toxin-antitoxin systems that are associated with stress response are mostly thought to be straightforward: they downregulate bacterial growth during stress. However, some toxin-antitoxin systems are reported to act in more subtle ways and regulate the expression of specific genes. An endoribonuclease toxin of *Escherichia coli*, MazF, is hypothesized to be a centerpiece of such a specific network. Recent publications claim that MazF completely reprograms the translational machinery of *E. coli*. MazF has been reported to remove a 43 nucleotide fragment from the 3’ end of 16S rRNA in mature ribosomes during various stresses [1]. It has been postulated that these modified ribosomes specifically translate 5′truncated mRNAs [1,2]. Upon activation, MazF is hypothesized to degrade the bulk of the transcripts, but cleave the mRNAs of around 300 genes only in 5’ UTR, which are then specifically translated by the truncated ribosomes [2]. We have seen the emergence of several large rRNA cleavage fragments in response to MazF and MqsR expression in *E. coli*, thus raising the possibility that these toxins also regulate growth via ribosome degradation. Alternatively, these toxins could only cleave precursor rRNAs and disturb ribosome biogenesis.

This dissertation largely focuses on studying the cleavage profiles of MazF and MqsR of *E. coli*. We mapped MazF and MqsR cleavage sites in rRNA and mRNA with an aim to clarify the function of these toxins. We also tested the intriguing hypothesis that toxins could activate non-cognate TA systems.

1. REVIEW OF THE LITERATURE

Bacterial toxin-antitoxin (TA) systems are small genetic modules that encode a toxic protein, which inhibits the growth of the bacteria producing it, and an antidote, which neutralizes the toxin [3–7]. Antitoxins are usually more labile than toxins and need to be constantly produced to keep the toxins at bay [6,8,9]. The toxins become free to act when the antitoxin to toxin ratio drops below a critical level. TA systems were first discovered as plasmid maintenance units [10,11], but soon afterwards many more were discovered in chromosomal DNA [12,13]. Currently, thousands of putative TA systems have been identified in a wide range of bacteria and archaea (http://bioinfo-mml.sjtu.edu.cn/TADB2/)[14]. Some bacteria have dozens upon dozens of TA system, e.g. *Mycobacterium tuberculosis* contains at least 88 of these small genetic modules [15]. Despite their abundance, the role of chromosomal TA systems in bacterial physiology remains elusive. Some hypotheses treat TA systems only as selfish genes, whose sole function is to propagate themselves [16]. On the other hand, several studies view TA systems playing a role in stress response, antibiotic tolerance, and virulence [7,17,18].

1.1. Classes of toxin-antitoxin systems and their regulatory architecture

TA systems are grouped into six types, based on the nature and inhibitory mechanism of the antitoxin (Figure 1) [19]. In types I and III, the antitoxins are RNAs, while the remaining TA systems all have protein antitoxins. Type I antitoxin is an antisense RNA complementary to the toxin mRNA, which obstructs the formation of the initiation complex and/or destabilizes the toxin transcript. Types II and III antitoxins inhibit toxins by direct interaction. Type IV antitoxins negate the activity of the toxin by stabilizing its target. The antitoxin of type V TA system cleaves toxin mRNA. Type VI antitoxin promotes the degradation of the toxin. An overview of the mechanisms and more well studied members of various types of TA systems is presented below.

1.1.1. Type I toxin-antitoxin systems

Type I antitoxins

Type I antitoxins are non-coding RNAs (ncRNA) that bind to toxin mRNAs and inhibit the translational initiation and/or promote mRNA degradation (Figure 1) [20–22]. Antitoxin RNAs are unstable and need to be constantly produced to obstruct the translation of toxin-encoding transcripts [8,22]. Most type I antitoxins have an overlap with toxin gene, but some, like IstR-1 of *tisB/istr1* system of *E. coli*, are located away from the toxin, and have more limited complementarity [22,23]. These two groups of antitoxins are classified as *cis*- or

trans-acting, respectively. In addition to antitoxin-mediated control, the toxin mRNAs are usually highly structured and often mask the Shine-Dalgarno sequence from ribosomes thereby avoiding toxin translation even without binding of the antitoxin [20,21]. Strong secondary structure also stabilizes the toxin mRNA making it more stable than the antitoxin RNA [24]. The unprocessed toxin mRNA has been demonstrated in several cases to be both untranslatable and inaccessible to antitoxins. Processing by nucleases is required to "activate" such mRNAs [20,21]. The toxin mRNAs of *cis*- and *trans*-acting antitoxins are activated differently: the former are processed in 3'-end and latter in 5'-end [20,21]. The regulation of toxin activity has been best studied for *hok*/*sok* TA system of *E. coli* R1 plasmid and *tisB*/*istR1* TA system of *E. coli* (reviewed by Berghoff and Wagner [21]). *hok/sok* represents a TA system with a *cis*-acting antitoxin, while *tisB/istR1* has a *trans*-acting antitoxin. Translation of *hok* is regulated by *mok* gene, which is located on the same transcript upstream of *hok*. It overlaps with *hok* and encodes a protein with an unknown function. RNase II and polyribonucleotide nucleotidyltransferase trim 39 nucleotides from the 3' end of *mok*/*hok* transcript, which induces structural rearrangements in toxin mRNA. The ribosomal binding site of *hok* gene remains sequestered, but *mok* becomes translatable. Synthesis of Mok opens up the ribosomal binding site of *hok*. The *sok* antitoxin inhibits the translation of *mok* and thus indirectly also shuts down the production of the toxin. RNase III cleaves the antitoxin/toxin mRNA duplex, thereby inactivating it [8]. *tisB* mRNA is cleaved by an unknown nuclease that removes 42 nucleotides from its 5' end. This exposes the ribosomal standby site and allows for ribosomal preloading. However, usually this is precluded because antitoxin *IstR1* masks the ribosomal standby site and RNase III cleaves the toxin mRNA/antitoxin complex [23].

Other strategies for stalling toxin translation have also been reported (reviewed by Masachis and Darfeuille [20]). *txpA* and *yonT* toxin transcripts have been speculated to have longer translational initiation times, caused by strong complementarity between their Shine-Dalgarno sequence and the 3' end of 16S rRNA [25,26]. Usage of rare start codons, such as GUG in *yonT*, is another feasible stalling mechanism. The ribosomal binding site in *yonT* mRNA is not masked [27], giving credibility to the existence of alternative initiation stalling mechanisms.

Type I toxins

Most type I toxins are short (less than 60 amino acids), hydrophobic proteins that contain a putative α-helical trans-membrane domain [22]. Despite the similarities, the mechanisms of action seem to differ. Hok [28] and TisB [29,30] toxins of *E. coli* form pores that result in depolarization of the cell membrane and decreased ATP production. Overexpression of such toxins leads to cell death, but it is likely that lethal doses of toxin are not reached under physiologically relevant conditions [22,29,31]. For example, Gerdes *et al.* argue that a feedback loop ensures safe levels of HokB in *E. coli* [31]. In short, membrane

bound RNase E constantly degrades the SokB antitoxin, but sufficient amounts of SokB remain in good growth conditions to neutralize the toxin. Stressful conditions increase *hokB* transcription beyond the level where existing SokB levels are able to handle. HokB then depolarizes the membrane and causes RNase E to detach, which decreases its activity and results in higher antitoxin levels [31]. A simpler loop may be enough: lower energy levels caused by toxins result in slower toxin translation.

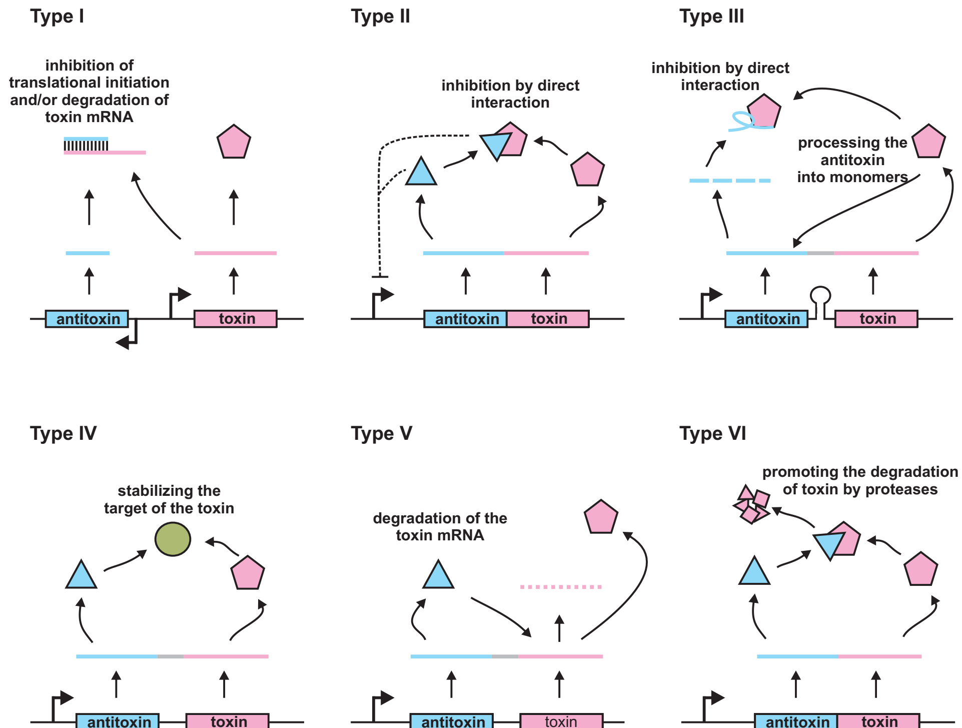

Figure 1. Different types of toxin-antitoxin systems. In type I TA systems the antitoxin is an antisense RNA which neutralizes toxins by binding to its mRNA and masking the ribosome binding site and/or promoting the degradation of toxin mRNA. Type II antitoxins are proteins which inactivate toxins by direct interaction. Type II antitoxins, as well as TA complexes, often also repress the toxin-antitoxin promoter. Type III toxins are RNAs which inhibit toxins by direct interaction. In type IV TA systems the antitoxin stabilizes the toxin target. Type V antitoxins are RNases which specifically degrade toxin mRNAs. Type VI TA systems are adapter proteins which promote the degradation of toxins.

The Fst/Ldr family of toxins cause abnormal nucleoid condensation (reviewed by Brielle et al. [22]). Overexpression of Fst toxin encoded by the RNAI/RNAII TA system of *Enterococcus faecalis* pAD1 plasmid leads to nucleoid condensation in *E. coli*, *B. subtilis*, *E. faecalis,* and *S. aureus* [32–35]. Nucleoid condensation, in turn, causes defects in cell division and growth [33]. Another member of the Fst/Ldr family, the LdrD toxin of *E. coli*, also causes nucleoid condensation while overexpressed in its parent organism [36]. On the other hand, transient production of its homolog, the LdrA of *E. coli*, was instead shown to inhibit ATP synthesis [37]. Thus, it is possible that this nucleoid condensation results from a damaged energy production system, and the toxins act much like Hok and TisB.

The BsrG toxin of SPβ prophage in *B. subtilis* disturbs the envelope biosynthesis when overexpressed and causes invagination of the cytoplasmic membrane. Prolonged BsrG production leads to altered cell morphology, abnormal cell division sites, and cell lysis [38].

Only two type I toxins, SymE and RalR, are known to localize outside of the membrane [22]. Both of these are cytosolic nucleases, although SymE cleaves RNA [39], and RalR DNA [40]. Upon overexpression, the SymE of *E. coli* strongly inhibits protein synthesis and the ability to form colonies [39]. Although SymE co-purifies with ribosomes, which suggests ribosome dependant activity, its expression also reduces the levels of non-coding RNAs [39]. Paradoxically, SymE belongs to the AbrB superfamily, which contains many type II antitoxins that inhibit endoribonuclease toxins [41]. In *E. coli* the *ralR/ralA* TA system forms part of the *rac* prophage. RalR overproduction results in filamentous cells and growth inhibition. *In vitro* experiments show strong non-specific endodesoxiribonuclease activity against both methylated and non-methylated DNA, and fail to detect RNase activity [40].

1.1.2. Type II toxin-antitoxin systems

Type II antitoxins

Type II TA systems are the most prevalent and best studied TA systems [6,19]. Type II antitoxins are small proteins that inactivate toxins by direct interaction (Figure 1) [6,19]. Their toxin and antitoxins form complexes where the antitoxin blocks the active site of the toxin or induces conformational changes that render the toxin inoperative (reviewed by Yamaguchi et al. [3] and by Chan et al. [42]). Also, in some ribosome dependant-ribonuclease toxins, the antitoxin has been shown to act by hindering the interactions between toxins and ribosomes [43,44]. Blocking the active sites seems to be the most common mechanism of toxin neutralization. In type II TA systems, the toxin and antitoxin genes usually form an operon where the antitoxin is located upstream. Still, there are several exceptions to this rule such as the *mqsRA, higBA,* and *rnlBA* TA systems of *E. coli* where the toxin gene precedes the antitoxin [45–48].

The different stability of toxin and antitoxin proteins is speculated to be the key to activating type II TA systems [6,7,19,42]. Most type II antitoxins have an unstructured toxin binding domain which makes them highly susceptible to degradation by proteases such as Lon, ClpXP, and/or ClpAP [3,6,7,19,49]. The antitoxins are stable in the toxin-antitoxin complex where the previously disordered toxin binding domain becomes ordered [3]. Toxins, on the other hand, are also stable in free form [6,19]. Assuming that TA complexes dissociate, the antitoxins need to be constantly produced to neutralize the toxins. Without an influx of new antitoxins, the toxins eventually outlive their inhibitors. By this theory, toxins are freed when protein production is slowed down, e.g. during various stresses. A more direct way to stop antitoxin production and free the toxins would be the loss of TA modules [16], for example, via the loss of a plasmid containing TA systems.

TA systems are hypothesized to avoid accidental toxin activity by ensuring an excess of antitoxins. One way to achieve this is thought to be through translational coupling. Because the genes of type II TA systems often overlap or are only few nucleotides apart [3,42], they are speculated to be translationally coupled [12]. Assuming translational coupling, the antitoxins, which are usually the first gene in the TA operon, are produced before the toxins. Such a mechanism would help to reduce stochastic increase in the toxin/antitoxin ratio. Prevalence of this strategy requires further study, however, because to our knowledge the *kis*/*kid* TA system on the R1 plasmid is currently the only TA system with experimental evidence that supports translational coupling [50]. A recent study that analyses ribosomal profiling and RNA sequencing data from ten type II TA systems within *E. coli* concluded that the translational initiation rate for most antitoxins is higher than for their cognate toxins [51]. The higher antitoxin production in two TA systems, *dinJ-yafQ,* and *yafNO*, is speculated to be secured at the transcriptional or post-transcriptional level, as many of the toxin transcripts are truncated [51]. Some type II TA systems with an upstream toxin gene, e.g. *rnlBA* of *E. coli* [52], have a separate promoter for antitoxin production located inside the transcript region that encodes the toxin. Such TA systems can produce a polycistronic TA transcript and also a transcript that contains only the antitoxin gene. The excess of antitoxin in the *rnlBA* of *E. coli* was reported to be controlled through higher levels of antitoxin mRNA [51]. Intriguingly, the translation rates of the RnlB toxin and RnlA antitoxin are similar, thus making differential transcriptional regulation likely the primary way of guaranteeing an excess of antitoxin in this system [51].

Antitoxins also act as the repressors of TA operons [6,19,42]. Generally, the C-terminal domain of the antitoxin binds to toxins and the N-terminal domain interacts with DNA [6,19,42]. Antitoxins bind to TA promoters also while in complex with the toxin [42]. The repression by the TA complex is usually stronger than by free antitoxin [42]. Such autorepression results in smaller metabolic burden for the bacteria, but still keeps a pool of toxins ready for activation [7]. Degradation of antitoxins leads to transcriptional derepression of the TA operon and consequently increases the amount of TA mRNA. After the stress is

relieved and translational activity increases, rapid antitoxin production can quickly abolish the toxin activity and repress the operon.

Some type II TA systems link the cellular toxin/antitoxin ratio with their promoter activity by a mechanism termed conditional cooperativity (reviewed by Harms et al. [7], by Page and Peti [19], and by Chan et al. [42]). The TA complexes of these TA systems have different stoichiometry based on the cellular toxin/antitoxin ratio. Complexes formed in an excess of antitoxin act as strong repressors, but as the toxin/antitoxin ratio increases, complexes with low affinity to the promoter start to form. For example, in the *relBE* system the RelE:RelB2 heterotrimers (i.e. 1:2 toxin/antitoxin ratio) bind to the promoter with strong affinity. However, a 1:1 toxin/antitoxin ratio leads mostly to the formation of RelE2:RelB2 heterotetramers which bind weakly to the promoter [19,42]. The benefits of conditional cooperativity are not clear, but several different models have been proposed, as summarised by Harms et al. [7]. It may be another form of insurance against accidental toxin activation: if the antitoxin levels drop because of intrinsic fluctuations, more TA transcripts will be synthesised and more antitoxins produced. Conditional cooperativity can also amplify the activation of TA systems during stress, as transcription gets derepressed sharply to produce more toxin-antitoxin mRNA. Finally, conditional cooperativity may generate bistability in the population and act as a sharp switch from a growing to a dormant bacterial population.

Several type II TA systems consist of three components (reviewed by Chan et al. [42]) and these extra proteins are involved in either transcriptional autorepression of the TA operon or inactivation of toxins. In the ω-ε-ζ TA of *Streptococcus pyogenes*, the ω protein acts as the sole repressor of the toxin-antitoxin operon [53], while in the *paaRAE* TA of *E. coli* O157:H7 the extra component PaaR enhances the repression [54]. PasC of *pasABC* TA from *Acidithiobacillus ferroxidans* strengthens the ability of the antitoxin to inactivate the toxin [55].

Type II toxins

Type II toxins have a diverse set of targets and mechanisms of action (Figure 2) [6,7,56]. Toxins attack translation, replication or cell wall synthesis. Still, most type II toxins inhibit protein production [6,7,56]. More prominent examples of mechanisms by which type II toxins inhibit the cell growth are presented below.

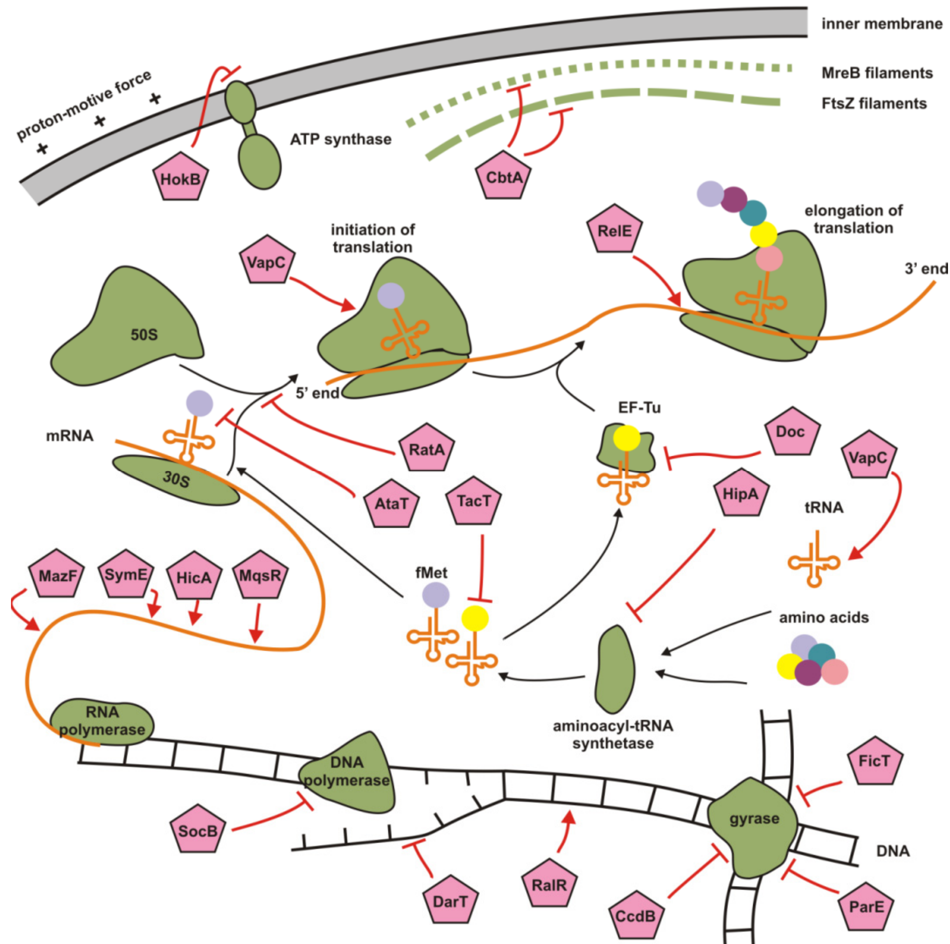

Figure 2. Targets of selected toxins. Red lines connect toxins with their molecular targets or show the molecular function, which is being inhibited. Destructive actions of toxins are shown with arrow head ends and inhibitory and/or corruptive actions of toxins with blunt ends. Based on Harms et al. 2018 [7].

Endoribonuclease toxins of type II TA systems

The most common mechanism of action among type II toxins (and toxins of all TA systems in general) is endoribonuclease activity. Some toxins bind to ribosomes and act on mRNAs during translation. Others cleave RNA independently, usually having specificity to certain sequences [5–7]. Ribosome independent endoribonucleases mostly cleave single stranded unprotected RNA. They have been classically viewed as degraders of mRNA [3], however, recent studies demonstrate their ability to cleave the rRNA, tRNA, and ncRNA as well [5].

Ribosome-dependent endoribonucleases

Most RelE family (RelE, YoeB, YafQ, and HigB toxins) and YafO family toxins cleave mRNA in a ribosome-dependent manner (reviewed by Yamaguchi and Inouye [57]). RelE of *E. coli* binds to the ribosomal A site where it interacts with 16S rRNA and cleaves codons between the second and third nucleotide [58,59]. The UAG stop codon is the main target of RelE, but other codons are also cleaved [58]. YoeB of *E. coli*, binds to the 50S subunit in ribosomal A site and cleaves primarily right after the start codon [60]. HigB of *Proteus vulgaris* and YafQ of *E. coli* cleave preferentially at AAA lysine codon [61,62]. YafO of *E. coli* binds to the 50S subunit and cleaves mRNA 11-13 nucleotides downstream of the start codon [63]. The GraT toxin of *Pseudomonas putida's graTA* system is a HigB homolog [64], which cleaves codons with an adenine at the second position [44]. One of the main effects of GraT is the inhibition of ribosomal biogenesis [65,66]. The overexpression of several ribosome-dependent ribonuclease toxins have been demonstrated to effectively shut down translation and lead to growth inhibition [57].

Ribosome-independent endoribonucleases

MazF of *E. coli* was the first toxin shown to have sequence specific ribonuclease activity [67]. MazF cleaves the ˇACA sequence, whereˇ indicates the cleavage position, leaving 3'-cyclic P and 5'-OH ends [2,68]. Recently a context bias for nucleotides surrounding ACA was reported, opening up the possibility of the preferred recognition sequence being longer [69]. Crystallography experiments show that the nucleotides at both sides of ACA sequences have to be single stranded to interact with MazF [70]. Initially, MazF of *E. coli* was thought to only degrade mRNA, however, recent studies show that it is also capable of cleaving rRNA [1,69,71]. Vesper et al. report cleavage of 16S rRNA at 3'-end in ribosomes [1]. Culviner and Laub on the other hand detected widespread cleavage in rRNA precursors [69].

The MazF family has many members across the bacterial and archaeal phyla. The MazF cleavage recognition sequences vary from three to seven bases [5]. MazF of *Haloquadratum walsbyi* currently holds the record for the longest verified cleavage recognition sequence with seven nucleotide UUˇACUCA [72]. Specific seven nucleotide sequence has a one in 16,384 chance of random occurrence. Only 7% of open reading frames (ORF-s) in *H. walsbyi* contain the UUACUCA sequence [72]. Such toxins must target transcripts of vital importance to inhibit growth. rRNA would satisfy this requirement, but has not been shown to be cleaved by MazF of *H. walsbyi* [72].

Two of nine *Mycobacterium tuberculosis's* MazF family members, MazF-mt3 and MazF-mt6, have been shown to cleave rRNA [73,74]. Both toxins cleave a conserved UUCCU sequence in 23S rRNA at helix 70 [73,74], which is essential for ribosome assembly and interactions with the ribosome recycling factor and tRNA [5]. Cleavage by MazF-mt3 was initially detected in *E. coli* after transient toxin expression using RNA sequencing and verified for 23S rRNA of *M. tuberculosis* using an *in vitro* cleavage assay with total RNA [74].

Ectopic expression of MazF-mt6 results in the cleavage of 23S rRNA in *M. smegmatis* and *E. coli*, the cleavage of 23S rRNA in *M. tuberculosis* was demonstrated using an *in vitro* total RNA cleavage assay [73]. MazF-mt6 was reported to cleave 23S rRNA in the 50S ribosomal subunits of *E. coli* using a cell-free translation system (70S was dissociated by a low Mg^{2+} concentration), however, the experiments did not detect cleavage in 70S subunits [74]. MazF-mt3 also cleaves, in addition to 23S rRNA, the anti-Shine-Dalgarno sequence of *E. coli* 16S rRNA, however, the cleavage seems to mostly happen in precursor rRNA as evidenced from very low *in vivo* cleavage of rRNA in assembled 70S ribosomes [74].

MazF-mt9 of *M. tuberculosis* is currently the only MazF family toxin known to cleave tRNAs. MazF-mt9 cleaves the D-loop of $tRNA^{Pro14}$ and the anticodon loop of $tRNA^{Lys43}$. Both sites have the UUˇU recognition sequence in a single stranded form [75]. tRNA is also the substrate for several VapC family toxins which act as metal ion dependent PIN (PilT N-terminus) domain ribonucleases [76]. VapC toxins have high substrate specificity and several of them recognize structure beyond the nucleotide sequence [77–80]. VapC toxins of *Shigella flexneri 2a*, *Salmonella enterica* and *Leptospira interrogan* cleave the initiator $tRNA^{fmet}$ in the anticodon loop [77,78]. *M. tuberculosis* has 48 *vapBC* TA systems and several of these toxins have been demonstrated to cleave tRNA in the anticodon loop [79,80]. VapC-mt20 and VapC-mt26 of *M. tuberculosis* were shown to cleave Sarcin-Ricin loop of 23S rRNA, cleavage by VapC-mt26 was demonstrated in 70S ribosomes using an *in vitro* cleavage assay [80,81].

Although most of the RelE family toxins are ribosome-dependant endoribonucleases, some of them, including MqsR and YhaV, act independently of the ribosome [5]. MqsR of *E. coli* cleaves GˇCN sequences, with GˇCU and GˇCA being primary recognition motives [47,82]. *In vitro* RNA digestion experiments have shown that YhaV of *E. coli* cleaves both mRNA and rRNA [83]. Recognition sequence for YhaV has not been identified.

The cleavage of regulatory non-coding RNAs are less studied. MazF of *E. coli* cleaves ACA in a central loop of 6S RNA [69] and the HicA toxin of *E. coli* cleaves tmRNA at two AˇAAC sites [84]. Ribosome-independent endoribonucleases can, in principle, cleave all ncRNA which have a recognition sequence in single stranded portion of the RNA, or the necessary folds for toxins that cleave structured RNA.

In conclusion, some ribosome-independent endoribonuclease toxins can cleave a wide variety of unstructured transcripts while others target only highly specific structures [5]. The cleavage of mRNA, rRNA, and/or tRNA can all lead to growth inhibition.

Toxic kinases

HipA of *E. coli's hipBA* system inactivates glutamyl-tRNA synthetase by phosphorylation of serine 239 [85,86]. The uncharged $tRNA^{Glu}$ pool stalls the translation and induces the stringent response [85,86]. PhD toxin of phage P1 phosphorylates translation elongation factor Tu 1 (EF-Tu) at Thr^{382}, which consequently loses its ability to bind tRNAs [87]. PezT of *Streptococcus pneumo-*

niae, a member of the Zeta toxin family, inhibits peptidoglycan synthesis by phosphorylating uridine diphosphate-N-acetylglucosamine. MurA, an essential protein for peptidoglycan synthesis, is inhibited by phosphorylated uridine diphosphate-N-acetylglucosamine. Overexpression of PezT in fast-growing *E. coli* culture leads to cell lysis [88].

Inhibitors of ribosome association
RatA toxin of *E. coli* inhibits translation by binding to 50S subunits and interfering with 70S ribosome association [89]. RatA only inhibits the formation of new 70S, but is not capable of dissociating existing 70S ribosomes. Overproduction of RatA inhibits growth and translation less efficiently than ribonuclease toxins. Although genomic organisation suggests a TA system, the hypothetical antitoxin has not been observed to inactivate the toxin or bind to it.

Acetyltransferases of aminoacyl tRNAs
A subset of toxins that are homologous to Gcn5N-acetyltransferases inactivate charged tRNAs by acetylation [90,91]. TacT of *Salmonella typhimurium* acetylates primary amino groups of amino acids on charged tRNAs. Amino acids with acetylated amine groups likely do not form peptide bonds and lead to translational shut down. The specificity of TacT of *S. typhimurium* is unknown, however, authors speculate it to be broad [90]. Overexpressing TacT of *S. typhimurium* during lag phase prolongs it by several hours, yet growth remains unaffected if TacT is expressed in exponentially growing culture [90]. This indicates that TacT of *S. typhimurium* does not act as a general growth inhibitor, and may instead have evolved to strengthen growth stasis. In contrast with TacT, the acetyltransferase toxin AtaT of *E. coli* O157:H7 is highly specific to charged initiator $tRNA^{fMet}$ and inhibits the formation of the initiation complex [91]. Overexpression of AtaT effectively inhibits the colony formation ability of *E. coli* [91].

DNA replication inhibitors
CcdB of F plasmid and ParE of RK2 plasmid inhibit DNA replication by corrupting DNA gyrase. As with quinolone antibiotics, CcdB and ParE freeze the gyrase in an open complex with cleaved DNA, denying the re-ligation step [92–94]. This results in double stranded DNA breaks, filamentous cells, inhibited cell growth, and eventually cell death [93,95,96]. Although similar in action, CcdB and ParE do not share structural similarity and likely interact with gyrase through different mechanisms. This is supported by the finding that CcdB resistance mutation in *gyrA* does not protect against ParE of RK-2 plasmid or ParE2 of *Vibrio cholera* [95,97].

The toxins of the FicT family inhibit replication more mildly, without inducing double stranded breaks in the DNA [98]. FicT toxins inactivate DNA gyrase and topoisomerase IV by AMPylating their ATP binding domains. Different FicT toxins from various organisms have been shown to inactivate the gyrase and topoisomerase IV of *E. coli*. Overexpression of FicT leads to filaments and growth inhibition due to DNA knotting, catenation, and relaxation.

DNA-targeting toxins
DarT of *Thermus aquaticus* ADP-ribosylates single stranded DNA [99]. Bromodeoxyuridine incorporation assay shows that DNA replication in *E. coli* becomes inhibited while overexpressing DarT. The antitoxin DarG seems to mainly counteract the toxin with its de-ADP-ribosylation activity, however, inhibition of toxin by direct interaction also seems to occur [99]. It is thus debatable if *darTG* belongs to type II TA or constitutes a new class, where the antitoxin acts by reversing toxin-generated modifications.

1.1.3. Type III toxin-antitoxin systems

Toxins of type III TA systems are inhibited by RNA antitoxins, but unlike type I, the RNA interacts directly with the toxic protein (Figure 1)(reviewed by Goeders et al. [9]). Based on toxin sequence similarity, the type III TA systems fall into three sub families: *toxIN*, *cptIN,* and *tenpIN* [100]. Most information about type III TA comes from studying the *toxIN* sub family [9]. As with type II modules, the toxin and antitoxin genes are located in the same bicistronic operon, where the antitoxin gene precedes the toxin. A rho-independent terminator is situated between the antitoxin and toxin gene, possibly ensuring a high antitoxin to toxin ratio. The excess of antitoxin is also promoted at post-transcriptional levels. The antitoxin gene encodes an ncRNA composed of short repeats, which is cut to monomers by the cognate toxin. These monomers inhibit the toxin by binding to their active sites. Thus, a single antitoxin transcript results in several toxin-neutralizing RNAs. The number of repeats varies, however, their length tends to be similar within one toxin sub-family [9,100]. Similar to type I and II TA systems, the type III antitoxins are less stable than their cognate toxins [101].

Antitoxin RNA monomers form a central pseudoknot structure and bind toxins with flanking tails and parts of the pseudoknot [9]. The crystal structures of *toxIN* family members that have been resolved so far reveal heterohexameric toxin-antitoxin complexes [102,103]. The products of *cptIN* of *Eubacterium rectale,* on the other hand, form heterotetrameric toxin-antitoxin complexes [104]. A recent report proposes that only a small portion of ToxN toxins stay in heterohexameric complexes and these toxins are mostly engaged in processing the antitoxin precursors [105].

Type III toxins are sequence-specific endoribonucleases that are structurally similar to MazF family toxins [9]. ToxN family members preferentially cleave adenine-rich sequences: ToxN of *Pectobacterium atrosepticum* plasmid pECA1039 cleaves at AAˇAU, ToxN of *Bacillus thuringiensis* plasmid pAW63 cleaves at AˇAAAA and AbiQ of *Lactococcus lactis* plasmid pSRQ900 cleaves at AˇAAA [103,106]. Overexpressing toxins in the ToxN family leads to growth inhibition [101,103,106]. As with most type II endoribonuclease toxins, mRNA is speculated to be the main target for type III toxins [9].

1.1.4. Type IV toxin-antitoxin systems

Antitoxins of type IV TA systems are proteins that negate the activity of the toxin by stabilizing the target (Figure 1). There is no direct contact between the toxins and antitoxins of type IV TA systems. The gene organization of type IV TA systems is the classical toxin-antitoxin bicistronic operon with an upstream antitoxin gene [107]. *cbeA/cbtA* of *E. coli* is the best studied type IV TA system [107–109]. The CbtA toxin inhibits cell division by binding to cytoskeletal proteins MreB and FtsZ and preventing their polymerization [109]. Cells become lemon shaped in bacterial cultures where CbtA is overexpressed. *In vitro*, CbtA inhibits the GTPase activity of FtsZ, yet MreB retains its ATPase activity [109]. A recent genetic study reports that CbtA binds to the H6/H7 loop of GTP-binding N-terminal domain of FtsZ and to the flat surface of MreB, which is required for the formation of double filaments [110]. CbeA antitoxin binds to MreB and FtsZ and enhances their filament bundling [108]. Toxins of two *cbtA/cbeA* homolog systems, YkfI and YpjF of *E. coli,* also inhibit growth and induce lemon-shaped cells when overexpressed. Prolonged expression of these toxins leads to cell lysis. Both toxins interact with FtsZ, but only YpjF has been shown to interact with MreB [110,111].

abiEi/abiEii of *Lactococcus lactis* and its homologs are also hypothesized to act as type IV TA systems [112]. The hypothetical toxin AbiEii is a GTP-binding nucleotidyltransferase with unknown working mechanism and target. Overexpressing AbiEii toxin of *L. lactis* or *Streptococcus agalactiae* in *E. coli* results in an impaired ability to form colonies, whereas no growth inhibition is seen when they are co-expressed with cognate AbiEi protein. Similar to type II, the bicistronic TA operon is repressed by the AbiEi antitoxin. These modules are speculated to belong to type IV TA systems because no interaction between the toxin and antitoxin has thus far been detected.

1.1.5. Type V toxin-antitoxin systems

In Type V TA systems the antitoxin is RNase which degrades the toxin mRNA (Figure 1). Currently, only one representative of type V is known: the *ghoST* of *E. coli*. The genes are again located in a bicistronic operon. The GhoT toxin is a small transmembrane protein similar to the *Hok* family of type I toxins [113]. It forms multimers that act as transmembrane pores [114]. Overexpression of GhoT results in membrane damage that leads to cell lysis. An *in vitro* cleavage assay showed the GhoS antitoxin to be an endoribonuclease that preferentially cleaves the GhoT portion of *ghoST* mRNA. RT-qPCR revealed that in stationary phase the *ghoS* mRNA is ~20 times more stable than that of *ghoT* [113]. This implies that in stationary phase the GhoT toxin is strictly controlled by the antitoxin. Overexpression of GhoS does not inhibit cell growth, meaning that its RNase activity is highly specific. Also, GhoS does not regulate the *ghoST* promoter [113].

1.1.6. Type VI toxin-antitoxin systems

socAB of *Caulobacter crescentus* is so far the sole studied representative of type VI TA systems (Figure 1) [115]. *socA* and *socB* form a bicistronic operon where the antitoxin gene is located upstream. *socB* was discovered during a screen for genes that make ClpXP protease essential in the *Caulobacter*. Overexpressing SocB with a modified C-terminus, that makes it unable to interact with ClpX, leads to growth inhibition, cell filamentation, and the SOS response. SocB inhibits the elongation of replication by binding to β sliding clamp (DnaN) and blocking its interactions with DNA Polymerase III. SocB toxins seem to have high target specificity because SocB of *C. crescentus* is not toxic within *E. coli*. SocA antagonizes the toxicity of SocB by promoting its degradation by the ClpXP protease. *In vitro* cleavage experiments indicate that SocA acts as an adapter that brings SocB close to the ClpX pore. According to bioinformatic analysis, *socAB* homologs are found only in α-proteobacteria.

1.2. Distribution of toxin-antitoxin systems

Many *in silico* methods have been developed to identify new TA systems (excellent overview by Lobato-Márquez et al.[18], two recent studies by Xie Yet al. [14], and Coray et al. [116]). In general, TA systems are abundant in chromosomes and plasmids of bacteria and archaea [12–14,116,117]. Chromosomal TA systems often locate in mobile regions such as super-integrons and prophages [12,118,119]. The repertoire of TA systems differs between closely related strains indicating the high genetic mobility of these elements [16]. Some confined studies report higher TA counts in pathogenic bacterial strains compared to their non-virulent relatives (reviewed by Lobato-Márquez et al. [18]).

The prevalence of TA systems is biased towards type II, which make up the lion's share of all TA loci. They are widely distributed, potentially due to their susceptibility for horizontal transfer. Type III TA systems also seem to be prone to horizontal transfer and are found in many different phyla [9,100,116]. Type I TA loci generally have a narrow phylogenetic distribution [116,120] and are rarely found on plasmids [116]. As expected, type I toxin families found on plasmids have a wider distribution [116]. However, the prevalence and diversity of type I TA systems may actually be higher than current studies show because searching for type I TA systems is difficult due to their short toxin and highly variable antitoxin sequences. The putative type IV *abiEi/abiEii* TA systems seem to be ubiquitous in bacterial and archaeal genomes [112]. As already mentioned, homologs of the type VI TA system *socAB* have only been detected in α-proteobacteria. To the best of our knowledge, the distribution of type IV *cbtA/cbeA* and type V *ghoST* TA systems have not been studied.

1.3. Biological functions of toxin-antitoxin systems

Because many TA systems are prevalent in the mobilome, are prone to horizontal transfer, and stabilize plasmids and labile genomic regions, researchers have speculated that they primarily act as selfish genetic units. By this theory, TA systems are addictive modules that propagate themselves on the expense of the host as they help to maintain the genetic elements carrying their genes (Figure 3A) [16]. Such selfish elements should be easily inactivated by a single mutation in the toxin gene. Many toxins have indeed shown to be inactive in their host (several *hok* family members in *E. coli* K-12 [121]) or close relatives of the host (half of the repertoire of *M. tuberculosis* toxins did not inhibit the growth of *Mycobacterium smegmatis* [15]). On the other hand, there are abundant chromosomal toxins capable of growth inhibition and several TA systems are associated with different phenotypes. For example, deletion of chromosomal TA systems have resulted in increased susceptibility to bacteriophages [9], decreased antibiotic tolerance [7], or lower virulence [18]. It is likely that many TA systems indeed only act as selfish entities, yet some seem to be integrated into cellular networks or are beneficial to the cell on their own merit (Figure 3). Still, the biological function of most TA systems remains elusive, possibly due to their redundancy, as several TA systems may carry the same function. Here, we discuss the suggested roles of TA systems in bacterial physiology.

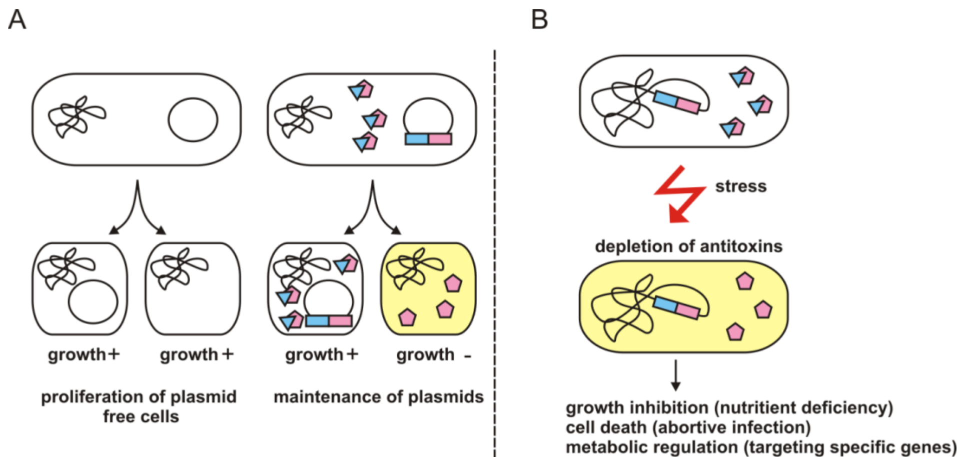

Figure 3. Speculated functions of toxin-antitoxin systems. (A) The selfish module model represented by plasmid addiction. This model sees TA systems as selfish entities which propagate themselves and mobile genetic elements carrying them. Toxins, being more stable than antitoxins, become active in daughter cells that have lost the TA systems. The growth of these cells gets shut down or they get killed, giving growth advantage to bacteria inheriting the TA systems. (B) Stress response model. Some TA systems are hypothesized to be integrated in cellular stress response networks. Antitoxin pool gets depleted during stress while the more stable toxins persist. Freed toxins help cells to cope with stressful conditions. Common hypotheses for helpful toxin activity include growth inhibition to accommodate with stressful conditions, altruistic cell death

during bacteriophage infection and regulation of specific genes. Antitoxin genes and proteins are depicted blue, toxin genes and proteins pink. TA systems are depicted as type II modules where toxins are inactivated by direct interactions with antitoxin.

1.3.1. Stabilization of mobile genetic elements

Several plasmid-borne TA systems have been shown to protect against plasmid loss. The antitoxins need to be constantly produced to counter toxins. When a plasmid is lost, the antitoxin pool depletes quickly and toxins become free to inhibit cell growth. Therefore, daughter cells without the plasmid have their growth shut down or are killed [16]. Propagation of the R1 plasmid by the type I *hok/sok* TA system [10] and the F-plasmid by the type II *ccdAB* TA system [11] are the best studied examples of plasmid stabilization. Type III and IV TA systems have also been shown to stabilize plasmids. The type III TA systems *toxIN* of *Pectobacterium atrosepticum* and *cptIN*$_{Er}$ of *Eubacterium rectale* have been shown to stabilize a model plasmid in *E. coli* strain W3110 and ToxIN of *Bacillus thuringiensis* was observed to maintain a model plasmid in *B. subtilis* YB886 [103]. The hypothetical type IV TA system *abiEi/abiEii* of *Streptococcus agalactiae* was shown to stabilize plasmids within *E. coli* DH5α [112].

TA systems can also promote plasmid maintenance through more sophisticated pathways. For example, a drop in copy number of R1 plasmid results in the activation of the Kid toxin of *kis/kid* TA system, likely due to the shortage of new antitoxins [122]. Kid is a ribosome-independent endoribonuclease toxin that recognizes the UUACU sequence. One of the targets of Kid is an antisense RNA that represses the production of R1 plasmid replication protein RepA, whereas RepA itself remains untouched by the toxin. Thus, Kid enhances plasmid replication by derepressing RepA production and halting cell growth until the number of R1 plasmids increases [122].

Cooper et al. [123] argue that having TA systems in plasmids is mainly beneficial for plasmid-plasmid competition. They show that the *parDE* system helps to out-compete plasmids without the TA system. Thus, when plasmids are not compatible, TA systems can help to ensure the prevalence of its carrier [123].

Several TA systems have been shown to stabilize labile regions of chromosomal DNA (reviewed by Van Melderen [17] and by Cambray et al. [118]). Still, not all TA systems can stabilize mobile genetic elements as some tested chromosomal TA systems fail to secure plasmid maintenance (reviewed by Harms et al. [7], and by Van Melderen and Saavedra De Bast [16]).

1.3.2. Anti-addiction

Chromosomal TA systems can guard against mobile genetic elements that are stabilized by homologous TA systems. The antitoxins produced by chromosomal TA systems can, in some cases, neutralize homologous plasmid borne toxins and thus allow for plasmid loss. For example, *ccdAB* of *Erwinia chry-*

santhemi was shown to cure *E. coli* MG1655 from F plasmid-derived *ccdAB* addiction [124]. Interestingly, *ccdBA* from *E. coli* O157:H7 does not protect against F plasmid addiction [125]. Mobilome stabilizing TA systems are probably under constant evolutionary pressure for their toxins to be uniquely antagonized by cognate antitoxins.

1.3.3. Abortive infection

Abortive infection is the altruistic suicide of bacteria during viral attack to stop the spread of the infection in the population [7]. Many TA systems activate during bacteriophage infection and halt the advancement of the phage. It is unknown how viral infections trigger TA systems, but it is likely that the changes in translation or transcription unbalance the toxin/antitoxin ratio and free the toxins. *hok/sok* of *E. coli* K-12 R1 plasmid strongly supresses, and *mazEF* of *E. coli* K-12 partially supresses, T4 infection [126,127]. Members of the *toxIN* and *tenpIN* TA families abort infections by a variety of bacteriophages (reviewed by Goeders et al. [9]). The hypothesized type IV TA system *abiEi/abiEii* of *Lactococcus lactis* has been shown to provide protection against the φ712 phage [112,128].

The importance of TA systems in combating bacteriophages is supported by the existence of numerous phage borne mechanisms that neutralize the TA systems. The Dmd protein encoded by the T4 bacteriophage acts as antitoxin for the RnlA toxin of *E. coli* K-12 and LsoA toxin of *E. coli* O157:H7 plasmid pOSAK1 and counters their toxicity during infection (reviewed by Otsuka [129]). Upon the deletion of the *dmd* gene, the *lsoAB* and *rnlBA* TA systems effectively suppress T4 infection [48,130]. T4 also encodes for the Alt protein, which adds an ADP-ribosyl group to the MazF of *E. coli*. ADP-ribosylated MazF has lower endoribonuclease activity *in vitro*. However, deleting *alt* does not affect the virulence of T4, indicating that other inhibitors of MazF might be involved [126]. The Gp4.5 protein of bacteriophage T7 inhibits the activity of Lon protease in *E. coli* K-12 and thus protects protein antitoxins from degradation [117]. A screen was performed to identify φTE phage mutants that can escape abortive infection by the *toxIN* TA system of *Pectobacterium atrosepticum*. All escape mutants had modifications in the φTE gene that encodes for RNA with motifs similar to type III antitoxins repeats [128]. Most escape mutants had expanded antitoxin motifs that were capable of neutralizing the toxin. One escape mutant obtained the *toxI* antitoxin through recombination between antitoxin repeats and the bacterial antitoxin gene [131]. Thus, some TA systems seem to be part of the arms race between phages and bacteria.

1.3.4. Metabolic downregulation

The increase in transcription of many TA systems during various stresses and the occurrence of stress specific regulatory elements upstream of several TA operons, have led to the hypothesis that TA systems regulate growth in stressful

conditions [17,57]. For example, amino acid and glucose starvation induce transcription of the *mazEF*, *relBE*, *mqsRA*, *yafNO,* and *higBA* TA systems in *E. coli* [47,132–134] and several type I toxins and type II TA systems in *E. coli* are preceded by LexA binding sites [7]. The ability of TA systems to downregulate metabolism may help to preserve resources under harsh conditions. This hypothesis remains controversial because several studies fail to see any disadvantages in growth for TA system deletion strains during various stresses (reviewed by Van Melderen [17]). For example, deleting five TA systems from *E. coli* K-12 did not affect the growth of the mutant during amino acid starvation, nutritional downshift, rifampin treatment, stationary phase, and acidic stress [135]. Neither did the absence of five TA systems affect recovery from these stresses [135].

1.3.5. Programmed cell death

MazF of *E. coli* was reported by the Engelberg-Kulka's group to be a part of altruistic cell death pathway triggered by extra cellular death factor EDF (reviewed by Kumar and Engelberg-Kulka [136], and by Engelberg-Kulka et al. [137]). Various stressful conditions (amino acid starvation, oxidative stress, treatment with transcription and translation inhibiting antibiotics and induction of DNA damage) are suggested to cause MazF-mediated cell death in majority of the population to provide nutrients for the few surviving cells. However, these data are highly controversial because other groups have not seen MazF-mediated cell death neither under physiological conditions nor while overexpressing the toxin [134,135,138,139]. These discrepancies may arise from the strains used: the authors of the MazF-mediated programmed cell death hypothesis conducted their experiments with an *E. coli* strain deficient in *relA*, and their *mazEF* deletion strain was also deficient in *mazG* [135]. At the same time, other authors used strains where these genes remained intact. However, Tsilibaris et al. also failed to see MazF-mediated cell death when exposing the same *relA* deficient strain used by Engelberg-Kulka's group to conditions that were reported to activate the programmed cell death [135].

In *Myxococcus xanthus*, MazF was found to cause altruistic cell death during fruiting body formation [140]. However, the exact role of MazF in the process remains unclear because the effect of MazF is dependent on a mutation in the membrane secretin *pilQ gene* [141,142]. We conclude that there is currently no solid evidence to consider *mazF* as a gene involved in programmed cell death.

1.3.6. Persisters

The metabolic downshift caused by TA systems is thought to play a role in generating persister cells [7]. Persisters are a dormant subpopulation of bacteria that are tolerant to multiple antibiotics (reviewed by Page and Peti [19], and Lewis [143]). After the antibiotic treatment ends, persisters resuscitate and restore the population. The exact mechanisms which make persisters insensitive

to antibiotics are as of yet not clear, however, their low metabolism is thought to be an important factor because most antibiotics only corrupt active processes. Persisters make up a tiny fraction of the population in exponentially growing culture ($\sim10^{-6}$ in *E. coli*), however, their numbers increase drastically in stationary phase (up to $\sim10^{-2}$ in *E. coli*). Occurrence of persisters under favourable growth conditions has been hypothesized to be a bet hedging strategy at the population level: a few bacteria are prepared for potential abrupt changes in the environment at the cost of lower fitness in the current environment. The tolerance to antibiotics correlates with cell growth rate [144,145], so it is expected that the number of persisters increases under growth limiting conditions.

A mutant of *E. coli's* HipA toxin, *hipA7*, was the first persistence increasing genotype discovered [146]. Activation of HipA leads to the stringent response which may induce persister generation [147]. On the other hand, deletion of *hipBA* in *E. coli* does not affect persistence [132,148]. TA systems and persisters were further linked by reports that the *E. coli* and *M. tuberculosis* cells that survive antibiotic treatment have high transcript levels of several TA genes [132,149,150]. Also, the overexpression of toxins increases persistence, however, so does the overproduction of other cellular proteins with growth inhibiting capabilities [132,151,152].

Recently, the results of research by Kenn Gerdes's group that associated persister formation with TA systems were retracted. The authors initially saw that step by step deletion of ten TA systems of *E. coli* gradually decreases the number of ampicillin and ciprofloxacin tolerant cells [153]. Further study revealed that the phenotype was caused by contamination by the ϕ80 bacteriophage [154]. Gerdes's group also claimed that induction of *relBE* and *yefM/yoeB* TA systems in *E. coli* correlates with antibiotic tolerant cells [155]. They based their theory on a microfluidics experiments with green fluorescent protein (GFP) tagged TA system genes. These results have been challenged by Goormaghtigh et al. [156] who demonstrated that GFP is not a suitable reporter for these experiments due to its weak signal that stays below the level of bacterial autofluorescence. No correlation between the induction of *relBE* and *yefM/yoeB* TA systems and persister cells was detected when using *mScarlet-I* based reporters [156].

There are some TA systems that have been directly linked with peristers. For example, TisB toxin of *E. coli* provides strong supporting evidence for being involved in the formation of persisters [152]. Deletion of *tisB* reduces the amount of ciprofloxacin tolerant cells by 10- to 100-fold [152]. DNA damage caused by ciprofloxacin triggers the production of TisB as the expression of toxins depends on the SOS response [152]. Also, type II TA *pasTI* plays a part in *E. coli* CFT073 strain persister generation, but not in MG1655. *ΔpasTI* CFT073 has a 100-fold reduced ampicillin and ciprofloxacin tolerant cell count *in vitro* and reduced ability to infect mice kidneys [157]. Another *in vivo* experiment, this time with *Salmonella Typhimurium,* shows persistence being reduced in bacteria colonizing mice macrophages in several single TA deletion mutants [158]. In summary, persister formation appears to not be the major function for most chromosomal

TA systems, as initially speculated. Nevertheless, some TA systems seem to contribute to the generation of antibiotic tolerant cells.

1.3.7. Transcriptional regulation

When discussing the activities of TA systems, the effects of toxins are usually addressed, however, in some cases the antitoxins also affect the phenotype of bacteria. Some type II antitoxins interact with promoters beyond their own operon. The MqsA antitoxin of *E. coli* K-12 *mqsRA* TA system represses the expression of several genes, most prominently the sigma factor *rpoS* and curli regulator *csgD* [159,160]. MqsA is thought to regulate biofilm formation by controlling these two genes. Degradation of MqsA by Lon under stressful conditions such as oxidative stress, frees *rpoS* and *csgD* from inhibition and promotes biofilm formation [159]. The DinJ antitoxin of *E. coli* K-12 *dinJ/yafQ* TA system represses the *csgE* gene, which is a positive modulator of RpoS translation [161]. However, deleting the *dinJ/yafQ* system does not affect growth during various stresses [135]. HipB antitoxin of *E. coli* K-12 *hipBA* was shown to bind to promoters of *relA*, *eutH* and *fadH* genes, and negatively regulate their expression [162]. Bioinformatics analysis predicts that 33 genes are being regulated by HipB [162]. *S. aureus*'s *savRS* TA system was reported to repress virulence genes *hla* and *efb* [163]. These examples show that bacteria are able to utilize type II antitoxins in their regulatory circuits. Antitoxins may act as stress sensing units that allow for quick derepression of stress response genes due to their labile nature. However, more data are needed to verify whether these interactions also result in beneficial phenotypes.

1.3.8. Translational regulation of specific genes

Endoribonuclease toxins with long recognition sequences have been hypothesized to regulate the translation of specific set of genes because five to seven nucleotide cleavage recognition sequences are likely absent or inaccessible in most genes [5]. Yet, to the best of our knowledge, there are no proven examples of this hypothesis. Still, there are interesting cases waiting for further study. The previously described MazF toxin of *Haloquadratum walsbyi* that recognizes a seven nucleotide cleavage site is inactive in the high salinity growth environment, but cleaves RNA effectively at lower salinity [72]. *H. walsbyi* floats on saturated salt water and uses light to produce a proton gradient via bacteriorhodopsin for ATP production. In case of fresh water influx, *H. walsbyi* is speculated to lose its ability to float on salt water and ATP production decreases. As MazF is active at lower salt levels, the decrease in salinity could activate MazF. The transcriptional activator of rhodopsin has three MazF recognition sequences, thus making regulation of rhodopsin expression a potential role for MazF of *H. walsbyi* [72].

1.3.9. Reprogramming of the translational system

A series of recent publications claim that the MazF toxin completely reprograms the translation of *E. coli* MC4100 (a K-12 derivate) [1,2,71]. MazF was shown to cleave 43 nucleotides from the 3' end of 16S rRNA under various stressful conditions [1]. During amino acid starvation induced by serine hydroxamate, 80% of the 16S were estimated to be cut in wild type, but not in a *ΔmazEF* mutant strain. Ribosomes with truncated 16S were termed as stress ribosomes and were reported to specifically translate leaderless mRNAs *in vitro* [1]. A later study stated that cleavage anywhere in the 5' untranslated region (UTR) is sufficient for stress ribosomes to form initiation complexes *in vitro* [2]. RNA sequencing of a MazF overexpression culture identified 330 genes whose mRNAs were cut in 5' UTR by the toxin in polysome fraction and were termed to constitute MazF regulon. Consequently, MazF was hypothesized to become active during stress and inactivate most mRNAs through ORF cleavage, but at the same time ensure the translation of stress genes [2].

RtcB was reported to religate the 43 nucleotide fragment back to 16S and restore the original mature ribosomes [71]. The cleaved 43 nucleotide fragment was reported to be stable and co-migrate with 30S subunit fraction, and thus implying a direct interaction. *In vivo* re-ligation was allegedly shown during recovery from serine hydroxamate induced amino acid starvation. Such a system would elegantly preserve resources otherwise wasted on truncated 30S degradation and re-synthesis, and allow for faster recovery from stress.

A recent study by Culviner & Laub failed to find any evidence for such an elaborate stress response mechanism for MazF of *E. coli* K-12 [69]. RNA sequencing conducted with a short term MazF overexpression culture showed extensive cleavage in coding regions across all the transcriptome, but identified only 41 genes with truncated 5' UTR. Most of the 5' UTR cleaved genes also had strong cleavage sites within ORF. Cleavage in the coding region was verified by northern blot for several model transcripts of MazF regulon. This implies that the 5' UTR cleaved genes can't be translated into complete products. MazF expression also reduced the ribosome density at the 3' end of transcripts, indicating that MazF generated cleavage halts translation. Ribosome profiling did not show increased translation for 5' truncated transcripts. MazF-dependant generation of stress ribosomes was also challenged. An hour long overproduction of MazF generated an insignificant amount of 43 nucleotide 16S 3' end fragment. Also, pulse labelling experiments showed that MazF attacks freshly synthesized rRNA precursors, and not the rRNA in mature pre-existing ribosomes. Thus, it is unlikely that MazF reprograms the translational machinery of *E. coli*.

1.3.10. Bacterial virulence

TA systems seem to promote virulence in several pathogenic bacteria, summarised in a recent review by Lobato-M´arquez et al. [18]. Intriguingly, there are several pathogens whose virulence is reduced upon TA deletion(s) [157,164–168]. As pathogens encounter several dangers while colonizing the host, all previously presented hypothetical stress coping mechanisms would probably benefit virulence. The stabilization of virulence plasmids or pathogenicity islands plays an important role in virulence, exemplified by pAD1 plasmid stabilization by RNAI/RNAII in *Enterococcus faecalis* [33,169] and SXT element stabilization by *mosAT* in *Vibrio cholerae* [170]. Control of biofilm formation, as demonstrated by the *mqsRA* system of *E. coli* K-12 [159,160], is of vital importance for pathogenicity of many bacteria [18]. Deletion of *relBE-1, relBE-4,* and *relBE-7* TA in *Vibrio cholerae* seem to influence biofilm formation via an unknown mechanism [167]. Although it is puzzling why deleting *relBE-2*, which is identical to *relBE-7,* has no phenotype [167].

TA system deletions in uropathogenic *E. coli,* non-typeable *Haemophilus influenza, Salmonella Typhimurium, Vibrio cholera,* and *Mycobacterium tuberculosis* were shown to decrease survival in *ex vivo* and/or *in vivo* infection models [18]. In *Neisseria gonorrhoeae*, the transposon insertion in *fitAB* TA system increases the intracellular proliferation of the bacteria in a model cell culture, implying that *fitAB* plays a role in growth regulation [171]. An increase in virulence and proliferation was seen after deletion of the sRNA antitoxin *ef0408-0409* regulating type I toxin *ef0409 of Enterococcus faecalis* [172]. The hyper-virulence may be due to uncontrolled toxin activation or the sRNA might regulate some virulence-associated gene(s).

Two pathogens are speculated to have weaponized TA systems by secreting the toxin to lyse host cells [18]. *Staphylococcus aureus*es *sprG1* locus encodes for two type I toxins, whose expression is post-transcriptionally controlled by SprF1 antitoxin RNA [173]. Both of these toxins have been found in the extracellular space and shown to lyse erythrocytes and bacteria [173]. Still, no *in vivo* lysis or virulence data following the deletion of *sprG1* has been presented. The toxins of *chpIK* and *mazEF* TA systems of *Leptospira interrogans* were reported to be found in macrophages during *ex vivo* infection [174]. One major concern is that this experiment lacked negative controls where the TA systems were deleted.

These results are only the first steps in our understating of the roles TA systems play in virulence. One has to be extra careful when interpreting transposon mutagenesis and single TA system component deletion experiments because these may easily give raise to artefacts of gene regulation. There are almost no data that explain the mechanisms whereby TA systems promote virulence and much work is required to understand how they function.

1.4. Recovery from toxin activity

Considering that TA systems potentially regulate growth and virulence, recovery from toxin activity is an important step of TA regulation. The first step in recovery is neutralizing the toxins. As described previously, many type II antitoxins have higher translation rates than their cognate toxins or a portion of the toxin mRNAs are truncated. This ensures that after the stress ends, the antitoxins are synthesized in excess of the toxins and quickly neutralize the free toxins. As type II antitoxins also repress the TA promoter, their shortage results in a high number of TA transcripts. Recovery from toxin activity is faster due to many antitoxin templates provided by the uninhibited TA operon. Rapidly increasing the amount of antitoxin inhibits the toxins and represses the TA promoter [7,56]. Notably, the majority of toxins with a hypothesized role in stress tolerance or virulence belong to type II. Several type I toxins are controlled by external regulators, for example LexA regulates the transcription of TisB [152], and thus toxin production can be shut down by varying their regulator concentrations.

Several antitoxins also promote recovery by reversing the corruption done by toxins. CcdA dislodges CcdB from gyrase, thus restoring its activity [175]. DarG antitoxin removes the ADP-ribose groups, added by the toxin DarT, from the single stranded DNA [99]. Normal cellular regulatory mechanisms also promote the recovery: tmRNA rescues halted ribosomes from mRNAs cleaved by ribonuclease toxins and peptidyl-tRNA hydrolase recycles aminoacyl-tRNAs acetylated by TacT toxin (reviewed by Hall et al. [56]).

Some toxins regulate their own toxicity, which can be interpreted as insurance to avoid lethal damage. For example, MazF of *E. coli* K-12 cleaves its own mRNA [176]. HipA toxin, which acts as kinase, inactivates itself by autophosphorylation at position Ser^{150} [177]. The majority of HipA and its main target, glutamyl-tRNA synthetase, molecules are phosphorylated in HipA overexpression experiments [178]. Effective autophosphorylation should ensure safe levels of active HipA and allow for quicker recovery from damage caused by the toxin. Also, as mentioned before, the membrane damage caused by HokB of *E. coli* K-12 is hypothesized to reduce the activity of RNase E, a degrader of SokB antitoxin [31]. Thus, toxins can regulate their own toxicity by inactivating themselves or by stabilizing their antitoxins.

In principle, all the mechanisms which are hypothesized to help the recovery from toxin damage can be seen as protection from accidental activation. For example, if the toxin amount of a plasmid stabilizing TA system increases stochastically, it would not instantly cause major harm to the cell.

1.5. Network of toxin-antitoxin systems

Considering the abundance of TA systems in bacteria, some overlap in function is expected and TA systems may be integrated into networks. For example, many type II TA systems are thought to be switched on through the degradation of antitoxins by the same proteases [5]. TA systems preceded by LexA boxes in *E. coli* K-12 could all see increased transcription in case of DNA damage [7]. It has been hypothesized that TA systems able to induce abortive infection could respond to the same viral signals and activate in bulk [9]. In some cases, a toxin directly regulates the production of other toxins. For instance, overexpression of MqsR in *E. coli* results in the cleavage of *ghoS* antitoxin mRNA, and the production of GhoT toxin [179]. On the other hand, keeping redundant genes in bacteria wastes resources and when TA systems indeed activate under exactly the same conditions without synergy, some of them are expected to become non-functional or lost from the genome.

Many bacteria contain homologous TA systems, which raises the question whether the components of different TA systems interact. Studies that have tried to clarify this issue have mostly focused on type II TA systems. They have found that generally non-cognate proteins do not interact (reviewed by Goeders and Van Melderen [6]). Even components of highly homologous systems are rarely seen to form complexes. Cross-interactions may disturb the TA balance and cause unwanted toxin production, and thus be under negative selection. Nonetheless, there are several examples of TA cross-interactions in the same bacterium. Components of different *relBE* systems of *M. tuberculosis* were shown to form complexes in *E. coli* and bind to promoters *in vitro* [180]. Interactions were also seen between various components of *mazE-vapC* and *mazEF* systems of *M. tuberculosis* in pull-down experiments conducted in *E. coli* [181]. The function of these interactions remains unclear.

2. AIMS OF THE STUDY

Endoribonuclease toxins of *E. coli* have been classically viewed as growth inhibitors that act by cleaving the bulk of the mRNA [3]. Recently, the MazF toxin of *E. coli* was proposed to be a centrepiece of an elaborate stress response system, capable of reprogramming translation [1,2]. According to this theory, MazF alters the translational preference of ribosomes by removing the anti-Shine-Dalgarno sequence from 16S rRNA. These truncated ribosomes are reported to specifically translate 5' UTR-truncated transcripts of stress-related genes, which are also generated by MazF. We saw in our preliminary experiments that overexpression of MazF or MqsR toxin results in large fragments of rRNA. This result contradicts with the hypothesis of MazF-mediated translational reprogramming. The fragmentation of rRNA by MazF and MqsR implies that these toxins may either cleave rRNA at different sites in mature and functional ribosomes or, alternatively, cleave only the unstructured precursor rRNA. Thus, we aimed to study the rRNA cleavage following the overexpression of MazF and MqsR to examine which of these hypotheses holds true and to test whether MazF generated stress ribosomes really do exist. We also studied mRNA cleavage by MazF to clarify whether MazF generates a specific pool of stress-related transcripts with truncated 5' UTR-s or it attacks mRNAs without bias.

It has been speculated that several TA systems contribute to growth inhibition under the same stressful conditions and in antibiotic tolerant cells [47,132–134]. We tested the possibility of a TA network where transcription of one or more TA systems is induced by the production of non-cognate toxins. Finally, if chromosomal TA systems are indeed involved in growth regulation, cells should be able to recover from their expression. Thus, we were interested in whether bacteria can resume growth after being exposed to toxin overexpression.

In summary, the specific goals of this research were:

1) to study the rRNA cleavage by MazF and MqsR in *E. coli* (publication II)
2) to map and analyse mRNA cleavage patterns generated by MazF and MqsR in *E. coli* (publication III)
3) to study how *E. coli* can recover from the overexpression of toxins (publication I, III)
4) to study if toxins can activate transcription of non-cognate type II TA operons (publication I, III)

3. RESULTS AND DISCUSSION

3.1. Transcription of toxin-antitoxin systems can be activated by expressing non-cognate toxins

Bacteria usually have many TA systems, several of which are transcriptionally triggered under the same conditions. For example, in *E. coli* the *relBE, dinJ/ yafQ* and *mazEF* have higher expression levels in dormant cells that survive lysis by ampicillin and *mazEF*, *relBE*, *mqsRA*, *yafNO,* and *higBA* have increased transcript levels during glucose and amino acid starvation [47,132–134]. The operons of most type II TA systems are repressed by their TA complexes and/or antitoxins [6,19,42]. Thus, the transcriptional activation of TA operons could indicate a drop in antitoxins and the liberation of toxins. To study the possibility of an interconnected network of TA systems, we tested whether the transcription of type II TA operons can also be activated by non-cognate type II toxins. Northern blot analysis revealed that overexpressing MazF, MqsR, HicA, or HipA toxins in *E. coli* BW25113 leads to transcriptional activation of the *relBEF* operon within an hour (publication I; Figure 1). Also, ectopic RelE production results in activation of the *mazEF*, *mqsRA*, *hicAB*, *dinJ-yafQ, yefM-yoeB*, and *prlF-yhaV* TA systems (publication I; Figure 2). Transcriptional cross-activation is not a universal phenomenon between all TA systems because only the production of YafQ, RelE, and HipA, but not MazF and HicA, increase the *mqsRA* transcript level in *E. coli* (data not shown). We also studied the transcriptional activation of TA systems during mupirocin-induced amino acid starvation. We used northern blot analysis to measure the relative RNA levels of *mazEF* and *mqsRA* in *E. coli* BW25113 and BW25113Δ*relBEF* strains. The transcription of both systems increased in the wild type strain, but only *mqsRA* was activated in the *relBEF* knock-out (publication I; Figure 3). This shows that transcriptional cross-activation also happens under physiologically relevant conditions.

Degradation of antitoxins by proteases is commonly considered to be the main reason for TA system activation [6,7,19,42]. We tested whether the transcriptional activation of the *relBEF* operon by non-cognate toxin expression is affected in a protease deficient *E. coli* strain. To our surprise, cross-activation also happened in a triple protease knockout strain that lacked the genes that express the proteases Lon, ClpPX, and HslVU (publication I; Figure 4). Although Lon and ClpPX are commonly associated with antitoxin degradation [49], it seems that other proteases of *E. coli* also effectively eliminate antitoxins. Because new antitoxins are not synthesised during the translational arrest induced by the overexpressed toxins, and existing ones are rapidly degraded, TA operons become derepressed. This model contradicts with our experiments, which show that only some toxins activate the transcription of *mqsRA* (data not shown) and that *relBEF* is required for transcriptional upregulation of *mazEF* during amino acid starvation (publication I; Figure 3). It is possible that, under

some of our conditions, the activation of TA systems was also controlled at the post-transcriptional level: all of the toxins we tested, except for HipA, were ribonucleases that effectively cleave mRNAs of many non-cognate TA systems (publication I; Figure 1, Figure 2). Because the cleavage specificity of endoribonucleases differs, some TA transcripts may be left untouched by the non-cognate toxin and enough antitoxin gets produced to repress the TA operon. All experiments described until now were performed by Villu Kasari.

We also looked for transcriptional cross-activation of TA systems in random primed paired-end RNA sequencing data that was obtained from bacteria challenged with overexpression of MazF or MqsR for two hours. We again saw that type II toxins can activate non-cognate type II TA systems (publication III; Figure 1A, B, E, Data Set S2). The relative transcript levels of the type II antitoxins *higA, relB, and rnlB,* and the type II toxins *relE and hipA* displayed at least a five-fold increase in response to both MazF and MqsR overexpression (publication III; Data Set S2). MqsR production led to additional increase in the levels of the type II antitoxin genes *prlF* and *hipB,* and the type II toxin genes *yoeB*, *higB,* and *chpB*. Again, several type II TA systems remained uninduced, which indicates some specificity of cross-activation. We also observed an increase in the transcript levels of many type I TA system genes following MazF or MqsR production. Transcription of the type IV antitoxin *yafW* was induced by both toxins and the type IV toxin *ykfI* by MazF (publication III; Data Set S2). The transcriptional increase of type I and type IV TA genes in response to MazF and MqsR cannot be caused by the proteolysis of antitoxins because type I antitoxins are RNAs and the antitoxins of type IV TA systems with upregulated mRNA levels have not been shown to repress their TA operons. Interestingly, all the upregulated type I and type IV TA systems localize in prophages. Thus, their expression may be the result of prophage activation during translational inhibition that was induced by endoribonuclease toxins.

3.2. *E. coli* cells recover from 90 minute transient toxin expression

Overproduction of toxins is a standard method of studying their effects on cellular growth. The downside of this approach is that very high toxin levels may be lethal to the cell and may not represent normal physiology following toxin expression. Therefore, we were interested if *E. coli* can recover from overproduction of the toxins that we used in most of these experiments. We monitored the recovery of single cells from transient toxin production using GFP dilution. GFP was synthesized in growing cells for 2.5h; then the medium was changed to stop the production of GFP and induce the synthesis of toxins. Toxins were then produced for 90 min before transferring cells to a growth medium without the inductor. Flow cytometry was used to measure the dilution of GFP: the more the cells divide, the weaker their GFP signal is. All of the bacteria recovered from 90 min overexpression of MazF, MqsR, RelE, and

HipA toxin (publication I; Figure S6). The regrowth following RelE and HipA expression was rather uniform. MazF and MqsR production generated heterogeneity: a subpopulation of cells began growing later. Our results show that a few hours of RelE, HipA, MazF and MqsR expression is not lethal to *E. coli,* although they may strongly inhibit the growth of part of the population.

3.3. MazF and MqsR of *E. coli* cleave precursor rRNA at several positions

Both ribosome-dependent and -independent ribonuclease toxins of *E. coli* are considered to act by degrading mRNA [3]. As an exception, rRNA was recently shown to be targeted by the MazF toxin [1]. MazF reportedly generates specialised stress ribosomes by removing a 43 nucleotide fragment from the 3' end of the 16S rRNA [1]. The ribosomal fraction with 3' trimmed 16S rRNA was extracted from MazF expression culture and shown to specifically translate leaderless mRNAs *in vitro* [1]. This ribosomal fraction has also been reported to form initiation complexes with mRNAs that have truncated 5' UTR [2]. All mRNAs that were translated *in vitro* corresponded to MazF cleavage products. The 43 nucleotide fragment of 16S rRNA was reported to stay connected with the stress ribosome and be religated to restore normal ribosomes after stress ends [71].

We observed much larger rRNA cleavage products while doing quality checks for RNA extracted from the MazF overexpression experiments in *E. coli*. These fragments could result from the activity of MazF and/or other endoribonucleases triggered due to MazF. Because rRNA degradation might be an overlooked mechanism by which toxins regulate growth in *E. coli*, we decided to further study the rRNA fragmentation by toxins. We analysed the integrity of rRNA following the overexpression of three ribosome independent ribonuclease toxins using northern blot analysis. Production of MazF and MqsR resulted in the fragmentation of 16S and 23S rRNA (publication II; Figure 1B, C), however, HicA left the rRNA intact (data not shown). Both MazF and MqsR expression generated distinct rRNA fragmentation patterns that indicate unique cleavage sites or activation of different ribonucleases. While overexpressing MazF, we also saw the appearance of a 16S rRNAs 3'-end fragment corresponding to 43 nucleotides, but its intensity on northern blot was low compared to most other cleavage products (publication II; Figure 1C).

To further study the rRNA fragmentation in response to expression of MazF and MqsR, we mapped the cleavage sites in two hour MazF and MqsR induction cultures using a modified version of differential sequencing developed by the Woychik group [73,182]. A long, two hour, expression time was used to maximize cleavage fragment accumulation. The cleavage site mapping method takes advantage of the differently modified RNA ends: the unprocessed transcripts have 5'-PPP and 3'-OH ends whereas most cellular processive RNases produce 5'-P and 3'-OH ends [183], and MazF generates 5'-OH and

2’3’-cyclic-P ends [68]. The RNA ends generated by MqsR were unknown prior to this study, but we assumed them to be the same as for MazF. *E. coli* lacks 5’-to-3’ exoribonucleases, thus making the 5’ portion of the fragments much more reliable for identifying cleavage sites. An adapter required for PCR amplification and sequencing was ligated to the 5’-P ends of the fragments. Thus, the 5’-P fragment ends correspond to the 5’ ends of mapped reads. To detect toxin cleavage sites, the 5’-OH ends were converted to 5’-P ends using the T4 polynucleotide kinase (PNK). Comparing untreated libraries with PNK treated libraries allowed us to reliably identify the cleavage sites. We compiled the reads of each library to a single composite rRNA because most of the reads mapped perfectly to all of the seven rRNA genes. We counted the number of 5’ read ends mapping to each composite rRNA position. Positions that displayed at least a 30-fold difference in 5’ end counts between untreated and PNK treated libraries that were located at a corresponding toxin recognition sequence (ˇACA for MazF and GˇCN for MqsR) were considered as primary toxin cleavage sites. We set a high threshold for the read count differences to ensure identification of strong cleavage sites. Also, the background noise in PNK treated samples was higher compared with the untreated samples, which was likely due to the non-specific activity of endoribonuclease RNase I [183], which also leaves 5’-OH ends after cleavage. Thus, we needed a higher threshold that was clearly above the background noise that resulted from the random cleavage events by RNase I. Our assumption that MqsR generates the same RNA ends as MazF turned out to be true, because PNK treatment of MqsR expression cultures resulted in strong read stacks that specifically mapped to the GˇCN recognition sequence.

We also mapped the 3’-ends of cleaved fragments to find out whether the truncated transcripts originate from mature ribosomes or precursor rRNA. Although the 3’ ends of cleavage fragments are very unstable, they provide a great way to differentiate between cleavage in mature ribosomes and precursors. Because ribosomes are highly structured and rRNA is covered with proteins, the exoribonucleases cannot process 3’ ends very far away from cleavage sites. The close proximity of 5’ and upstream 3’ cleaved ends would imply cleavage within ribosomes. The 3’ ends of fragments were mapped using poly(A) tailing. A poly(T)-adapter hybrid was used for cDNA first strand synthesis to ensure that the following amplifications and sequencing products would always contain reads with poly(A) tails. 3’ ends of fragments were traced to the beginning of the poly(A) tails. Because poly(A) tail synthesis requires 3’-OH groups, we generated a second library to also allow for identification of unprocessed toxin cleavage sites. The 2’-3’-cyclic-P ends were converted to 3’-OH ends using T4 PNK and toxin cleavage sites were identified by comparing 3’ end counts per position in treated and untreated libraries. These cDNA libraries were prepared and sequenced by vertis Biotechnologie AG.

These methods may encounter technical limitations in methylated regions of RNA, like some sites in mature rRNA, because methyl groups likely block the cDNA synthesis by reverse transcriptase. This needs to be taken into account when searching for the truncated 3’ ends of 16S rRNA in the hypothetical stress

ribosomes: the upstream region of the $^{\vee 1500}$ACA site, which is speculated to be cleaved by MazF to generate specialized ribosomes, is heavily methylated in mature ribosomes. In addition, toxin cleavage products have different stability and one has to be extra careful while evaluating the degree of cleavage on the basis of toxin generated 5’ and 3’ ends. Read counts do not necessarily reflect a toxins’ preference for a cleavage site, but instead may show the stability of the cleavage products.

RNA sequencing of 5’ ends revealed several primary MazF and MqsR cleavage sites in both 16S and 23S rRNA (publication II; Figure 2, Figure S5). These sites are at least partially double stranded in mature ribosomes or/and located deep inside the subunits (publication II; Figure 3). Thus, the sites are inaccessible to toxins in mature ribosomes and these cleavages most probably occurred on the unstructured rRNA precursors. We detected MazF cleavage also at the $^{\vee 1500}$ACA site in 16S rRNA (publication II; Figure 2), which is the position reported to be cleaved in mature subunits to generate stress ribosomes [1]. In our experiment, the cleavage cannot originate from mature ribosomes. We used 100 nucleotide reads, which are too long to detect the 43 nucleotide 3’ end fragment of 16S rRNA. MazF also cleaved a 5’-precursor of 16S rRNA at position $^{\vee -46}$ACA. Because we performed sequencing in only one replicate, we verified several cleavage sites in total RNA using primer extension (publication II; Figure S12, Figure S13). Note that following the overexpression of MazF or MqsR, we also detected several sites with high 5’ end read counts that were independent of PNK treatment and did not map to toxin cleavage sequences (publication II; Figure S10). These sites are likely cleaved by ribonucleases involved in the cleanup of rRNA precursors that were damaged by toxins and cannot be packed into mature ribosomes. In addition, cleavage by toxins may induce rRNA refolding and open up attackable sites for other ribonucleases.

Our 3’ sequencing data support our initial suspicion that MazF and MqsR mainly target precursor rRNA. In most cleavage sites identified by 5’ sequencing, we did not detect 3’ ends in a nearby upstream region, which indicates extensive trimming that is not possible in highly structured mature ribosomes. As an exception, 3’ ends matching prominent MazF cleavage sites in the decoding center at positions $^{\vee 1394}$ACA and $^{\vee 1396}$ACA appeared only after PNK treatment (publication II; Figure S10). The decoding center of mature ribosomes should be inaccessible to toxins, so the reason why both 3’ and 5’ fragments are stable in these cleavage sites currently remains unclear. We failed to detect the 3’ end of 16S rRNA at position $^{\vee 1500}$ACA that would correspond to the truncated stress ribosome using RNA sequencing. We also checked for 16S rRNAs 3’ ends generated by MazF in total RNA using modified 3’-RACE (Rapid Amplification of cDNA Ends). Total RNA was treated with PNK and subjected to poly(A) tailing. First strand cDNA was synthesized using poly(T) primer, followed by PCR amplification, fragment separation by gel electrophoresis, and sequencing. Amplification products of total RNA samples not treated with PNK were used as a control. Again, we identified MazF generated 2’3’-cyclic ends at

the decoding center (publication II; Figure 5A, Figure S14B), but not at the ˇ^{1500}ACA cleavage site.

We also applied 3’ RACE to total RNA extracted under various conditions where the stress ribosome was reported to be formed. We hoped to see 3’ ends generated by MazF cleavage at ˇ^{1500}ACA in stationary phase, chloramphenicol-treated, and mupirocin induced amino acid starvation cultures. However, 3’ RACE failed to detect MazF generated 3’ ends under any of these conditions (data not shown). We used northern blot analysis to search for the 43 nucleotide fragment cleaved from the 3’-end of 16S rRNA under the same stressful conditions, but failed to detect it. The fragment did not appear even when using an exoribonuclease deficient strain where cleavage fragments accumulate (publication II; Figure S16B). Note, that our experiments do not completely rule out the possibility of stress ribosomes because our 3’ end identification methods are biased towards detecting precursors. Nevertheless, our results do not support the hypothesis of MazF-generated stress ribosomes.

3.4. MazF- and MqsR-generated rRNA fragments are mainly found in aberrant subunits

RNA sequencing and northern blot data show that overexpressing MazF or MqsR for two hours results in accumulation of precursor rRNA ends (publication II; Figure 4A, Figure S11B). A simple explanation would be that there are just not enough ribosomal proteins to correctly pack and process rRNAs due to the action of toxins that strongly inhibit translation. Formation of aberrant ribosomal subunits has been seen in response to several protein synthesis inhibiting antibiotics [184,185]. We analyzed ribosomal RNA fractions from sucrose gradients of two hour toxin expression cultures (prepared by Anton Paier and Aivar Liiv) and observed aberrant subunits also in both of our toxin induction experiments (publication II; Figure 4B). Northern blot analysis showed that the irregular particles contained the majority of fragmented RNA (publication II; Figure 4C). In an MqsR expression culture we also saw the accumulation of 16S rRNA precursors in aberrant subunits (publication II; Figure S11A). The rRNA fragmentation patterns matched with the corresponding toxin fragmentation patterns in total RNA, thus indicating that we see toxin cleavage and not artefacts of gradient preparation. The heavy fragmentation of aberrant subunits implies that the defects might result from toxin cleaved precursor rRNA that cannot be packed into normal subunits.

We also tested whether RNA from 70S contains any toxin cleavage sites using more sensitive techniques. We failed to see 5’ ends at major toxin cleavage sites using primer extension (publication II; Figure S12, Figure S13), but did detect MazF cleavage in 16S rRNA using 3’-RACE and northern blot hybridization from 6% PAGE in 7M urea (publication II; Figure 5). 3’-RACE identified MazF generated 3’ ends at MazF cleavage sites in positions ˇ^{1394}ACA and ˇ^{1396}ACA (publication II; Figure 5A, Figure S14B). The fragments detected

by northern blot analysis correspond to downstream cleavage products of these positions in both mature and precursor 16S rRNA. We propose that the fragment originates from unprocessed rRNA because we also detected precursor rRNAs in the 70S fraction. Primer extension allowed us to identify 5’ ends of 23S precursor in MazF and MqsR expression cultures (publication II; Figure S11B) and 3’ RACE revealed the 3’ end of 16S precursor in MazF expression culture (publication II; Figure 5A, Figure S14A). The final steps of rRNA processing occur in translating ribosomes and are not required for the formation of 70S ribosomes [186–188], so one can expect to find precursor rRNAs in the 70S fraction.

We did not detect 16S 3’ ends that correspond to cleavage at $^{\vee 1500}$ACA in the 70S fraction in MazF expression culture, which, again, might be due to heavy methylation of the region. On the other hand, these ends should be detectable in precursor rRNA of total RNA samples. Their absence leads us to believe that this cleavage is rare, at least in precursors, and only a sensitive northern blot can detect its products. We did not see the 43 nucleotide fragment that migrates together with the 70S ribosome fraction as was previously reported (publication II; Figure 5B). Thus, our experiments do not provide evidence for the proposed MazF generated stress ribosome hypothesis.

Since the release of our paper, Culviner and Laub have published another study that tackles the issue of MazF generated stress ribosomes and rRNA cleavage by MazF [69]. They detected very little 43 nucleotide fragments of the 16S 3’ end after an hour-long MazF production period [69]. Also, they demonstrated that MazF only cleaves freshly synthesized rRNA by using pulse labelling experiments [69]. Thus, their data agrees with our result that MazF does not generate specialized stress ribosomes.

This work reminds one of the importance of employing diverse techniques while studying rRNA cleavage because not all rRNA fragments originate from correctly processed and packed mature ribosomes. Our data indicate that, in addition to mRNA, MazF and MqsR also extensively cleave precursor rRNAs. This could halt ribosome biogenesis under stressful conditions where toxins are speculated to be active (Figure 4). Rapid ribosome synthesis under harsh conditions would be a waste of resources and endoribonuclease toxins could be one of the mechanisms that help to shut it down. Cleaved and then processed rRNA fragments can be utilized elsewhere. Alternatively, the degradation of rRNA precursors is yet another way to inhibit growth. Either way, downscaling translational machinery seems be one of the main roles of MazF and MqsR toxins.

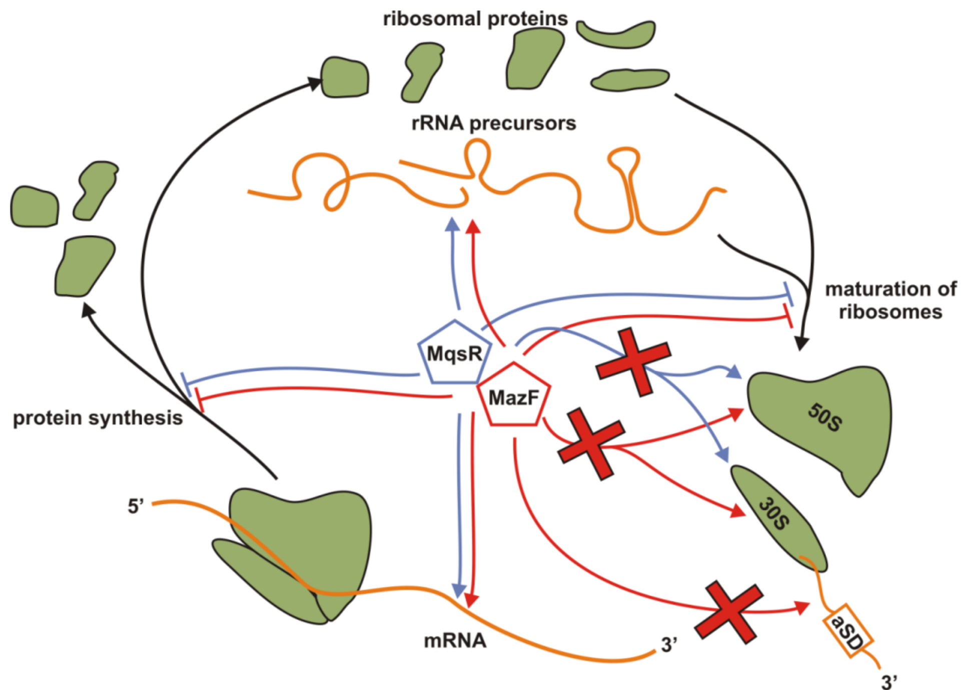

Figure 4. Our model of MazF and MqsR activity. MazF and MqsR degrade unstructured RNA, like mRNA and rRNA precursors, but cannot attack highly structured mature ribosomes. MazF does not generate specialised ribosomes. Red lines ending with arrow heads show molecular targets of MazF and MqsR, and red lines with blunt ends show molecular processes inhibited by MazF and MqsR. aSD means anti-Shine-Dalgarno sequence.

3.5. Expressing MazF and MqsR leads to major changes in the transcriptome

During various stresses, MazF is hypothesized to generate a pool of 330 transcripts (the co-called MazF regulon) cleaved only in 5' UTR, which are specifically translated by the MazF-generated stress ribosomes. By this model, MazF would shut down the translation of the majority of the genes, while promoting the translation of some essential stress-related genes [2]. Regardless of the existence of MazF-generated stress ribosomes, the reported pool of 330 MazF-truncated stress specific transcripts offers an intriguing opportunity for biased translation during stress. This contradicts with the initial hypothesis, which sees MazF acting only as a growth inhibitor that cleaves unstructured mRNA [67]. How the transcripts of the MazF regulon are protected from cleavage in the coding region has not been explicitly made clear, but authors who described the MazF regulon seem to imply that cleavage sites become masked by the translating stress ribosomes [189]. To test whether MazF cleaves mRNAs selectively or without bias we analysed differential and random primed RNA

sequencing data obtained from *E. coli* cultures where MazF was overexpressed for two hours. In addition, we studied mRNA cleavage in response to MqsR expression, to analyse if another endoribonuclease toxin can also generate a toxin-specific set of truncated mRNAs.

The random primed paired-end RNA sequencing libraries we used for mapping the cleavage sites also contain information about relative transcript levels. Thus, we first studied whether the expression of MazF or MqsR results in transcriptional upregulation of a specific set of stress response genes, e.g. the hypothesized MazF regulon. cDNA libraries were again prepared and sequenced by Vertis Biotechnologie AG. These cDNA libraries were originally designed for detecting ligation of different toxin cleaved fragments by RNA ligase RtcB and thus they are composed of reads with abnormal, 300 nucleotide, length. Despite that, the libraries were applicable for transcript level determination. Overexpression of MazF or MqsR both led to major changes in relative transcript levels (publication III; Figure 1A). Significant shifts in mRNA abundance have also been previously described in short term MazF expression experiments [2,190]. Many stress response genes, e.g. *rpoS*, *ada,* and the *psp* operon, displayed an increase in transcript levels while ribosomal protein and flagellar genes showed a reduction (publication III; Figure 1A). This is in line with previous reports that show that MqsR induction reduces the transcript levels of motility and ribosomal protein genes [191]. The transcript levels of the proposed MazF regulon genes did not have a directional shift in MazF expression culture (publication III; Figure 1D). Thus, these genes do not seem to be induced at the transcriptional level.

We verified increased transcriptional expression levels in toxin cultures for *rpoS, relE/hokD*, *higA* and decreased levels for *rpmB* and *ptsH* using RT-qPCR (publication III; Figure S3). Because toxins alter the transcriptional levels of most genes, we used a spike-in culture of *Pseudomonas aeruginosa* PAO1 as a source for reference transcripts (*oprL*, *cheZ,* and *PA3340*). The spike-in culture also allowed us to normalize the RNA content to the biomass. We expected that a two hour toxin production regime would significantly reduce the total mRNA levels, however, to our surprise, the amount of mRNA remained the same. Normal RNA levels in bacterial cultures where endoribonuclease toxins are overexpressed could be explained by a recent study by the Brynildsens group. They describe futile cycling of RNA in MazF expression cultures where energy is being depleted due to continuous RNA synthesis [192]. They hypothesize that an end product is rarely produced from mRNAs due to constant degradation by MazF. This leads to a deficiency in transcriptional regulators, which in turn results in uncontrolled transcription that lasts until the cells are depleted of energy. Broken feedback loops that drive this futile cycle could explain why the RNA levels remain the same in our toxin expression experiments.

Transcript levels do not necessarily measure the promoter activity under conditions where endoribonucleases are overexpressed: transcript level values could represent a graveyard of old RNA cleavage fragments. We wanted to know how well the promoter activities correlate with transcript levels after two

hours of toxin overproduction. The primary transcripts are short-lived (around 3 min half-life on average) [193], and detecting them in our toxin-affected sample would equate to freshly synthesized RNA. We used differential sequencing of 5'-PPP ends to assess the synthesis of new RNA. Its principle is the same as for detecting MazF and MqsR cleavage sites: the adapter can be only ligated to the 5'-P ends of fragments, and 5'-PPP ends need to be converted to 5'-P ends using tobacco acid pyrophosphatase to be detectable during sequencing. Comparing the 5' end counts per position between treated and untreated libraries revealed transcriptional start sites. We detected 1228 transcriptional start sites of which 964 are within five nucleotides of a previously described *E. coli* transcriptional start site (publication III; Figure 1B, Table S5) [194]. The majority of these sites appeared in MazF and MqsR expression cultures.

The primary transcript abundancies poorly correlate with transcript levels (the r^2 for MqsR was 0.45 and the r^2 for MazF was 0.17), which can be explained by extensive RNA fragmentation (publication III; Figure 1C). Cleavage near the 5' ends of transcripts renders them undetectable in differential sequencing because of the 100 nucleotide read length. Also, upstream cleavage fragments are unstable in general due to the action of 3'-to-5' exoribonucleases within *E. coli* and are degraded more rapidly. High transcript levels can still be detected when downstream fragments remain stable because relative RNA level quantifying methods, such as the TPM (Transcripts Per Kilobase Million) method we used [195], calculate transcript abundances based on the number of reads mapped against ORF. Indeed, inspecting read count density profiles revealed the accumulation of downstream cleavage fragments (selected examples in publication III; Figure 2). *yjdM* is a good example of the discrepancy between TPM and primary transcript levels in toxin expression cultures. The *yjdM* transcript is cleaved near the 5' end by MazF and MqsR (publication III; Figure 2C) and shows decreased primary transcript levels following toxin expression (data not shown). Transcript levels, on the other hand, remain stable. In conclusion, interpreting RNA sequencing data of endoribonuclease expression experiments is not straightforward. Usually, changes in mRNA levels are expected to cause matching changes in protein expression. However, due to extensive mRNA cleavage and broken feedback loops, changes in mRNA quantity do not lead to corresponding changes in protein levels. We showed that almost all translational activity is arrested during MazF expression using mass spectrometry (discussed in chapter 3.9).

3.6. mRNA cleavage by MazF and MqsR

We used the accumulating 5' ends of MazF- or MqsR-generated RNA fragments to map corresponding toxin cleavage sites across the entire transcriptome. Both our differential and random primed paired-end sequencing libraries could be used to identify cleavage sites in mRNAs. Despite the fact that our differential sequencing libraries were designed to detect rRNA cleavage, plenty

of reads (120 000 – 330 000 reads per library) mapped outside of rRNA operons and we used these to find mRNA cleavage sites (publication III; Table S2). Lower read counts made us change the cleavage site parameters to allow for cleavage site identification in weakly expressed transcripts. We calculated 5'-OH/5'-P end ratios across the entire transcriptome and excluded genomic positions where the ratio was below five in toxin expression culture. Next, we searched for sites with enriched 5'-OH/5'-P end ratios in toxin culture (5'-OH/5'-P end ratio in toxin culture had to be five times higher than in control). 95% of the positions detected this way mapped to corresponding toxin recognition sequence and we consider these to be cleavage sites.

The random primed paired-end sequencing library fragments were generated by sonication, which disrupts RNA at random sites. We noticed the accumulation of 5' read stacks while looking at coverage profiles of toxin expression culture (selected examples in publication III; Figure 2). Closer investigation revealed that many of these 5' end stacks map to a toxin recognition sequence and are likely not artefacts of sonication, but represent stable toxin cleavage products. 90% of the 5'-end stacks that were present in MazF culture but absent in the control experiment appeared at toxin recognition sequences. We regard these positions as MazF cleavage sites. For MqsR, only 50% of the toxin culture specific 5'-end stacks mapped to recognition sequences. We considered this ratio to be too low and mapped MqsR cleavage sites using only differential sequencing.

For both of our sequencing approaches we used RNA from bacterial cultures with rather long two hour toxin expression times. This allowed for the accumulation of stable cleavage fragments and increased the probability of detecting cleavage sites in transcripts with low expression levels.

Both MazF and MqsR extensively cleave mRNAs, with MazF cleavage detected in 56% and MqsR cleavage in 30% of open reading frames (publication III; Table 1, Table S3, Data Set S1). MazF attacked 13.4% (publication III; Table 2) and MqsR 1.5% (publication III; Table S4) of all possible recognition sequences located in the ORFs. These toxins likely target many more transcripts and cleavage sites because genes with very low expression levels under these conditions were undetectable. We did indeed see a bias towards identifying cleavage sites in highly expressed genes (publication III; Figure 3A, B). Also, the 100 and 300 nucleotide long reads used in our experiment limit the detection of shorter cleavage fragments. Sequencing experiments with higher read counts and shorter reads are required to reveal all potential MazF and MqsR cleavage sites. In addition, we determined MazF cleavage sites in *E. coli* culture that had been recovering from toxin expression for 30 min. Because we did not subject the recovery culture to differential sequencing, our cleavage site analysis is based only on random primed paired-end data. We see that the mRNA in 30 min regrowth culture is still highly fragmented (publication III; Table S3), which indicates that the cells have not yet recovered from the toxin damage.

Random primed libraries allow us to assess the cleavage depth by comparing the 5' end stacks of fragments that map to cleavage sites to the total coverage at

that position. We note that different stabilities of the cleavage fragments distort this analysis. Still, it is fairly safe to assume that if the 5' end count of a position exceeds the number of overlapping reads, the position is effectively being cleaved. Many highly expressed transcripts have several strong and/or dozens of weaker cleavage sites. For example, MazF cleaves the vast majority of *relE* transcripts at a single site (publication III; Figure 2A) and MqsR cleaves *fusA* at 41 positions (publication III; Data Set S1). These transcripts are likely not translated into proteins. We conclude that an increase in TPM levels during endoribonuclease toxin expression does not necessarily equate to increased protein production or higher levels of functional ncRNAs. High transcript levels are often the result of accumulating cleavage fragments. The transcript level profile we observe is a mixture of newly synthesized RNA and cleavage fragments awaiting cleanup.

We used primer extension to verify major MazF cleavage sites in *relE*, *rpsA*, *lon,* and *higA* transcripts and the MqsR cleavage site in *rpmC* transcript (publication III; Figure S4). In the study, which identified the MazF regulon, the toxin was expressed for only 15 min, which is considerably shorter than our two hour experiment. We checked whether the cleavage in *lon*, *rpsA,* and *relE* transcripts also persists using shorter MazF induction times (publication III; Figure S5). *rpsA* and *lon* ORF-s were both cleaved after 10 min of MazF expression. Primer extension was not able to detect cleavage in *relE* after either 10 or 20 min of MazF expression. Still, we saw massive *relBEF* fragmentation in 15 min MazF expression culture using northern blot analysis (publication I; Figure 1). *relBEF* transcript levels seem to be too low in short-term MazF expression cultures to be detectable with primer extension.

Massive cleavage site data allowed us to elaborate on the preferred recognition sequences of MazF and MqsR. Culviner and Laub propose that MazF of *E. coli* favours a seven nucleotide cleavage sequence, where ACA is at the center [69]. This is in line with a recently resolved structure showing that MazF interacts with the upstream and downstream nucleotide around ACA; all of the nucleotides need to be unpaired for MazF to bind [70]. We observed a slight context bias only at the flanking nucleotides: C is underrepresented at the upstream and G at the downstream position, while A is the most frequent nucleotide at both of these positons (publication III; Figure 3C). The discrepancies between our sequence logos can be explained by the different set of cleavage sites and nucleotide probabilities at surrounding positions. We used an equiprobable background while Culviner and Laub used the nucleotide bias surrounding all ACA sites in the coding region. As reported previously, MqsR preferentially cleaves at GˇCU (publication III; Figure 3C, Table S4). GˇCC was the second most abundant recognition sequence and GˇCA the third, and cleavage at GˇCG occurred rarely.

3.7. The proposed MazF regulon genes are cleaved in the coding sequence

We looked for 5' truncated mRNAs in MazF expression culture using the same parameters as the authors who reported the MazF regulon: 5' UTR was defined as the area of 101 nucleotides upstream of the start codon and any overlap with preceding genes was ignored. Cleavage in the proposed MazF regulon was significantly overrepresented with as 58% of the transcripts of the putative regulon were truncated in 5' UTR compared with only 16% of all genes (publication III; Table 1). However, most of the MazF regulon mRNAs were also cleaved in ORF. The authors of the original publication focused only on 5' UTR cleavage and missed strong cleavage sites inside the ORFs [2]. We see another set of transcripts cleaved only in the 5' UTR, which is composed of 40 of the proposed MazF regulon transcripts and 123 other transcripts. Theoretically, this set of transcripts could form an alternative MazF regulon, seen only under our tested conditions. We find this speculation to be highly unlikely because of the 163 transcripts where we detected cleavage sites only in 5' UTR do not show any statistically significant functional enrichment. The alternative MazF regulon is a collection of shorter transcripts for which our technical limitations (long reads) prevent the detection of in-gene cleavage sites (data not shown). In addition, shorter transcripts contain fewer ACA sites, so selection of smaller mRNAs while searching for uncleaved transcripts is to be expected. MqsR cleaved 48 mRNAs only in 5' UTR. Most of these transcripts did not belong to the proposed MazF regulon nor were they a part of the 163 genes cleaved in only 5' UTR by MazF. As with MazF, the MqsR-generated transcript pool, for which we see cleavage only in 5' UTR, contains mainly short mRNAs (data not shown).

Culviner and Laub also studied the cleavage of mRNAs in response to MazF expression and published their results a few months before us [69]. Their RNA sequencing experiments revealed 41 transcripts that were cleaved in 5' UTR, but most of these also contained cleavage in ORF [69]. Thus, their data agrees with our results.

One could argue that we unfairly disregarded the MazF regulon by looking purely at the number of cleavage sites. Abundant translatable 5' truncated mRNAs can still exist if the cleavage in ORF is minor and in 5' UTR extensive. The MazF regulon transcripts have on average deeper 5' UTR cleavage sites than the rest of the transcriptome, however, coding sequences in both groups are cleaved to a similar degree (publication III; Figure S6A, B). Therefore, most of the MazF regulon transcripts are unlikely to be translated into complete proteins.

3.8. MazF regulon is cleaved by MazF without reading frame bias

Authors of the MazF regulon hypothesis propose that MazF binds to truncated ribosomes and performs frame-dependent quality checks [189]. Stress ribosome bound MazF is hypothesized to only cleave in-frame ACA sites, thus generating pressure to remove this codon from the MazF regulon. This mechanism would benefit the cell by excluding frame shifts while translating essential stress genes under harmful conditions. The authors base their hypothesis on a few MazF regulon genes without in-frame ACA sites, and GFP mutant reporters that produce a weaker signal after insertion of in-frame ACA sites. We studied the plausibility of frame specific cleavage using our transcriptome-wide cleavage data. We see abundant out of frame cleavage in MazF regulon transcripts and in the rest of the transcriptome (publication III; Table 2). MazF targets a larger percentage of ACA triplets, both in and out of frame, in the proposed MazF regulon compared with the rest of the transcriptome. We separately checked for out of frame cleavage in the seven genes (*efp, deoC, soxS, rbfA, ahpC, rpsA,* and *groEL*) used as examples of in-frame ACA deficit. mRNAs of six of them have out of frame cleavage in our analysis which implies that MazF does not facilitate their expression.

Isabella Moll's group also proposed that Thr codon usage is altered in the MazF regulon due to a lack of in-frame ACA. We analysed the Thr codon usage of the proposed MazF regulon and over the entire genome. The ACA/all Thr codon ratio is slightly lower in the proposed MazF regulon (0.09) compared with the entire genome (0.13; publication III; Table S8). The hypothethical MazF regulon also has a higher share of ORFs that lack in-frame ACA triplets (31%) than the rest of the genome (23%; publication III; Table S7). Neverthe-less, we consider these differences too small to support MazF-driven codon bias. Alternatively, MazF could drive the codon usage of the entire genome, as bacteria try to minimize the damage done by toxins and ensure post-stress regrowth. Although this hypothesis is highly speculative, we decided to test it. We compared ACA to all Thr codon ratios of various *E. coli* and *Shigella* strains, some of which did not have the *mazEF* TA system, and observed similar ACA codon usage in all the bacteria we analysed (publication III; Table S7). We conclude that MazF can cleave all unstructured and unprotected ACA sites independently of the reading frame.

3.9. MazF regulon is not preferentially translated during MazF induction

The best way to confirm or refute the MazF regulon is to directly measure the protein production of MazF expressing cells. A previous study reported low levels of translation in MazF expression culture using [^{35}S]methionine in-corporation [67]. The proposed MazF regulon genes may represent the majority

of newly synthesized proteins. We used SILAC (stable isotope labeling with amino acids in cell culture) to analyse the proteome of *E. coli* MazF expression culture [196]. *E. coli* was pre-grown in "light" medium and switched to "heavy" medium upon induction of MazF synthesis from a plasmid. Glucose was the carbon source during pre-growth, L-arabinose was both the carbon source and inductor for MazF synthesis during the toxin expression phase of the experiment. Proteins were quantified using mass spectrometry. The mass spectrometry work was performed by Merilin Saarma and Sergo Kasvandik. Comparing the heavy/light ratios of proteins provides the relative amount of new proteins that are produced during the expression of the toxin. 409 proteins were identified in all three replicates of MazF expression cultures and 1035 in three replicates of an empty expression vector control (publication III; Data Set S4). This implies that the synthesis of hundreds of proteins was arrested in response to MazF expression. The vast majority of the proteins we managed to detect following MazF induction have very low heavy/light ratios at the 20 min time point, and reveal only a marginal increase at the 60 min time point (publication III; Figure 4). The heavy/light ratios of the control culture increase significantly between the 20 min and 60 min time points and the heavy/light ratios at 20 min are much higher than for MazF. Taken together, this data indicates that translation is halted for almost all genes during MazF overexpression.

We detected 71 proteins that belong to the hypothetical MazF regulon. Their expression profiles in MazF induction and control cultures were the same as for the rest of the proteins (publication III; Figure 4), thus refuting the hypothesis that the translation of this group of proteins is selectively promoted by MazF. Also, SILAC revealed only eight proteins encoded by transcripts for which we saw cleavage only in 5' UTR, and seven of these had low heavy/light ratios. We conclude that under our experimental conditions neither of the MazF regulons exists. Culviner and Laub [69] also report a general arrest of translation during the expression of MazF based on reduced ribosome densities at the 3' ends of the transcripts. Also, their ribosome profiling experiments did not reveal increased translation of transcripts with truncated 5' UTR.

We identified only a handful of proteins whose production remained uninhibited in MazF-expressing bacteria (publication III; Table S9). The heavy/light ratio of eight proteins increased at least two fold between 20 min and 60 min time points. Seven of these had a low heavy/light ratio after 60 min of MazF production, thus indicating a slow translation rate. One protein, AraC, reached a heavy/light ratio of two at the 60 min time point. This result is not surprising because AraC is upregulated in response to our inductor and carbon source L-arabinose. AraC controls the expression of genes involved in L-arabinose metabolism [197]. Three other proteins – MazF, IraP, and UspF – had a heavy/light ratio above two at the 60 min time point, but this ratio was also the same at the 20 min time point (publication III; Figure 4A). A high level of the over-expressed MazF is to be expected. The two other proteins are involved in stress response: UspF promotes adhesion and IraP stabilizes alternative sigma factor σ^S [198,199]. Genes that encode these proteins contain very few ACA sites,

uspF has two ACA sites and *iraP* has only one ACA site, which could partially explain their tolerance to MazF cleavage. As expected, we did not detect any cleavage at their ACA sites. It is possible that these sites are located in double-stranded regions of mRNA.

4. CONCLUSIONS

Our data do not support the existence of specialised MazF-generated translational machinery. We show that the MazF and MqsR toxins of *E. coli* act as degraders of unstructured RNA. The endoribonuclease toxins of *E. coli* are usually thought to arrest growth through mRNA cleavage, however, our results suggest that extensive cleavage of rRNA precursors also contributes to growth inhibition (Figure 4).

The most important results of our work are:

- The MazF and MqsR toxins of *E. coli* primarily act as degraders of unstructured RNA (publication **III**).
- The MazF and MqsR toxins of *E. coli* extensively cleave precursor rRNAs, but cannot attack rRNA in mature ribosomes (publication **II**).
- Expression of MazF strongly impairs the production of the majority of proteins (publication **III**).
- The 3' end of 16S rRNA is not cleaved by MazF in mature ribosomes and does not lead to the generation of specialised ribosomes (publication **II**).
- Most transcripts that belong to the hypothetical MazF regulon are cleaved in ORF and none of the transcripts are specifically translated during MazF expression (publication **III**).
- There is no bias against the ACA codon in the hypothetical MazF regulon (publication **II** and **III**).
- All cells in a culture of *Escherichia coli* recover from 90 min of MazF, MqsR, RelE, or HipA production. The recovery of a subpopulation of bacteria is delayed after the expression of MazF and MqsR (publication **I**).
- Toxins can activate the transcription of non-cognate toxin-antitoxin systems and *relBEF* is required for the transcriptional activation of *mazEF* during amino acid starvation (publication **I, III**).

REFERENCES

1. Vesper O, Amitai S, Belitsky M, Byrgazov K, Kaberdina AC, Engelberg-Kulka H, and Moll I. Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in Escherichia coli. *Cell*. 2011;147(1): 147–157.
2. Sauert M, Wolfinger MT, Vesper O, Müller C, Byrgazov K, and Moll I. The MazF-regulon: a toolbox for the post-transcriptional stress response in Escherichia coli. *Nucleic Acids Res*. 2016;44(14): 6660–6675.
3. Yamaguchi Y, Park J-H, and Inouye M. Toxin-Antitoxin Systems in Bacteria and Archaea. *Annu Rev Genet*. 2011;45(1): 61–79.
4. Hayes F, and Van Melderen L. Toxins-antitoxins: Diversity, evolution and function. *Crit Rev Biochem Mol Biol*. 2011;46(5): 386–408.
5. Masuda H, and Inouye M. Toxins of prokaryotic toxin-antitoxin systems with sequence-specific endoribonuclease activity. *Toxins (Basel)*. 2017;9(4): E140.
6. Goeders N, and Van Melderen L. Toxin-Antitoxin Systems as Multilevel Interaction Systems. *Toxins (Basel)*. 2014;6(1): 304–324.
7. Harms A, Brodersen DE, Mitarai N, and Gerdes K. Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Mol Cell*. 2018;70(5): 768–784.
8. Gerdes K, and Wagner EGH. RNA antitoxins. *Curr Opin Microbiol*. 2007;10(2): 117–124.
9. Goeders N, Chai R, Chen B, Day A, and Salmond GPC. Structure, evolution, and functions of bacterial type III toxin-antitoxin systems. *Toxins (Basel)*. 2016;8(10): E282.
10. Gerdes K, Rasmussen PB, and Molin S. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. *Proc Natl Acad Sci*. 1986;83(10): 3116–3120.
11. Ogura T, and Hiraga S. Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc Natl Acad Sci*. 1983;80(15): 4784–4788.
12. Pandey DP, and Gerdes K. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res*. 2005;33(3): 966–976.
13. Leplae R, Geeraerts D, Hallez R, Guglielmini J, Drze P, and Van Melderen L. Diversity of bacterial type II toxin-antitoxin systems: A comprehensive search and functional analysis of novel families. *Nucleic Acids Res*. 2011;39(13): 5513–5525.
14. Xie Y, Wei Y, Shen Y, Li X, Zhou H, Tai C, Deng Z, and Ou HY. TADB 2.0: An updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Res*. 2018;46(D1): D749–D753.
15. Ramage HR, Connolly LE, and Cox JS. Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: Implications for pathogenesis, stress responses, and evolution. *PLoS Genet*. 2009;5(12): e1000767.
16. Van Melderen L, and Saavedra De Bast M. Bacterial toxin-Antitoxin systems: More than selfish entities? *PLoS Genet*. 2009;5(3): e1000437.
17. Van Melderen L. Toxin-antitoxin systems: Why so many, what for? *Curr Opin Microbiol*. 2010;13(6): 781–785.
18. Lobato-Márquez D, Díaz-Orejas R, and García-del Portillo F. Toxin-antitoxins and bacterial virulencea. *FEMS Microbiol Rev*. 2016;40(5): 592–609.
19. Page R, and Peti W. Toxin-antitoxin systems in bacterial growth arrest and persistence. *Nat Chem Biol*. 2016;12(4): 208–214.

20. Masachis S, and Darfeuille F. Type I Toxin-Antitoxin Systems: Regulating Toxin Expression via Shine-Dalgarno Sequence Sequestration and Small RNA Binding. 2018; 1–18.
21. Berghoff BA, and Wagner EGH. RNA-based regulation in type I toxin–antitoxin systems and its implication for bacterial persistence. *Curr Genet*. 2017;63(6): 1011–1016.
22. Brielle R, Pinel-Marie ML, and Felden B. Linking bacterial type I toxins with their actions. *Curr Opin Microbiol*. 2016;30: 114–121.
23. Wagner EGH, and Unoson C. The toxin-antitoxin system *tisB-istR1*. *RNA Biol*. 2012;9(12): 1513–1519.
24. Franch T, Gultyaev AP, and Gerdes K. Programmed cell death by hok/sok of plasmid R1: Processing at the hok mRNA 3'-end triggers structural rearrangements that allow translation and antisense RNA binding. *J Mol Biol*. 1997;273(1): 38–51.
25. Daou-Chabo R, Mathy N, Bénard L, and Condon C. Ribosomes initiating translation of the hbs mRNA protect it from 5′-to-3′ exoribonucleolytic degradation by RNase J1. *Mol Microbiol*. 2009;71(6): 1538–1550.
26. Durand S, Gilet L, and Condon C. The Essential Function of B. subtilis RNase III Is to Silence Foreign Toxin Genes. *PLoS Genet*. 2012;8(12): e1003181.
27. Reif C, Löser C, and Brantl S. Bacillus subtilis type I antitoxin SR6 promotes degradation of toxin yont mRNA and is required to prevent toxic yoyJ overexpression. *Toxins (Basel)*. 2018;10(2): E74.
28. Gerdes K, Bech FW, Jørgensen ST, Løbner-Olesen A, Rasmussen PB, Atlung T, Boe L, Karlstrom O, Molin S, and von Meyenburg K. Mechanism of postsegregational killing by the hok gene product of the parB system of plasmid R1 and its homology with the relF gene product of the E. coli relB operon. *EMBO J*. 1986;5(8): 2023–2029.
29. Unoson C, and Wagner EGH. A small SOS-induced toxin is targeted against the inner membrane in Escherichia coli. *Mol Microbiol*. 2008;70(1): 258–270.
30. Gurnev PA, Ortenberg R, Dörr T, Lewis K, and Bezrukov SM. Persister-promoting bacterial toxin TisB produces anion-selective pores in planar lipid bilayers. *FEBS Lett*. 2012;586(16): 2529–2534.
31. Gerdes K. Hypothesis: Type i toxin—antitoxin genes enter the persistence field-a feedback mechanism explaining membrane homoeostasis. *Philos Trans R Soc B Biol Sci*. 2016;371(1707).
32. Patel S, and Weaver KE. Addiction toxin Fst has unique effects on chromosome segregation and cell division in Enterococcus faecalis and Bacillus subtilis. *J Bacteriol*. 2006;188(15): 5374–5384.
33. Weaver KE. The par toxin-antitoxin system from Enterococcus faecalis plasmid pAD1 and its chromosomal homologs. *RNA Biol*. 2012;9(12): 1498–1503.
34. Weaver KE, Reddy SG, Brinkman CL, Patel S, Bayles KW, and Endres JL. Identification and characterization of a family of toxin-antitoxin systems related to the Enterococcus faecalis plasmid pAD1 par addiction module. *Microbiology*. 2009;155(9): 2930–2940.
35. Shokeen S, Patel S, Greenfield TJ, Brinkman C, and Weaver KE. Translational regulation by an intramolecular stem-loop is required for intermolecular RNA regulation of the par addiction module. *J Bacteriol*. 2008;190(18): 6076–6083.
36. Kawano M, Oshima T, Kasai H, and Mori H. Molecular characterization of long direct repeat (LDR) sequences expressing a stable mRNA encoding for a 35-

amino-acid cell-killing peptide and a cis-encoded small antisense RNA in Escherichia coli. *Mol Microbiol*. 2002;45(2): 333–349.
37. Yamaguchi Y, Tokunaga N, Inouye M, and Phadtare S. Characterization of LdrA (Long Direct Repeat A) protein of escherichia coli. *J Mol Microbiol Biotechnol*. 2014;24(2): 91–97.
38. Jahn N, Brantl S, and Strahl H. Against the mainstream: The membrane-associated type I toxin BsrG from Bacillus subtilis interferes with cell envelope biosynthesis without increasing membrane permeability. *Mol Microbiol*. 2015;98(4): 651–666.
39. Kawano M, Aravind L, and Storz G. An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol Microbiol*. 2007;64(3): 738–754.
40. Guo Y, Quiroga C, Chen Q, McAnulty MJ, Benedik MJ, Wood TK, and Wang X. RalR (a DNase) and RalA (a small RNA) form a type I toxin-antitoxin system in Escherichia coli. *Nucleic Acids Res*. 2014;42(10): 6448–6462.
41. Coles M, Djuranovic S, Söding J, Frickey T, Koretke K, Truffault V, Martin J, and Lupas AN. AbrB-like transcription factors assume a swapped hairpin fold that is evolutionarily related to double-Psi β barrels. *Structure*. 2005;13(6): 919–928.
42. Chan WT, Espinosa M, and Yeo CC. Keeping the Wolves at Bay: Antitoxins of Prokaryotic Type II Toxin-Antitoxin Systems. *Front Mol Biosci*. 2016;3: 9.
43. Yang J, Zhou K, Liu P, Dong Y, Gao Z, Zhang J, and Liu Q. Structural insight into the E. coli HigBA complex. *Biochem Biophys Res Commun*. 2016;478(4): 1521–1527.
44. Talavera A, Tamman H, Ainelo A, Konijnenberg A, Hadži S, Sobott F, Garcia-Pino A, Hõrak R, and Loris R. A dual role in regulation and toxicity for the disordered N-terminus of the toxin GraT. *Nat Commun*. 2019;10(1): 972.
45. Kasari V, Kurg K, Margus T, Tenson T, and Kaldalu N. The Escherichia coli mqsR and ygiT genes encode a new toxin-antitoxin pair. *J Bacteriol*. 2010; 192(11): 2908–2919.
46. Yamaguchi Y, Park J-H, and Inouye M. MqsR, a crucial regulator for quorum sensing and biofilm formation, is a GCU-specific mRNA interferase in Escherichia coli. *J Biol Chem*. 2009;284(42): 28746–28753.
47. Christensen-Dalsgaard M, Jørgensen MG, and Gerdes K. Three new RelE-homologous mRNA interferases of Escherichia coli differentially induced by environmental stresses. *Mol Microbiol*. 2010;75(2): 333–348.
48. Koga M, Otsuka Y, Lemire S, and Yonesaki T. Escherichia coli rnlA and rnlB compose a novel toxin-antitoxin system. *Genetics*. 2011;187(1): 123–130.
49. Brzozowska I, and Zielenkiewicz U. Regulation of toxin-antitoxin systems by proteolysis. *Plasmid*. 2013;70(1): 33–41.
50. Ruiz-Echevarría MJ, de la Cueva G, and Díaz-Orejas R. Translational coupling and limited degradation of a polycistronic messenger modulate differential gene expression in the parD stability system of plasmid R1. *MGG Mol Gen Genet*. 1995;248(5): 599–609.
51. Deter HS, Jensen R V., Mather WH, and Butzin NC. Mechanisms for differential protein production in toxin–antitoxin systems. *Toxins (Basel)*. 2017;9(7): E211.
52. Otsuka Y, Miki K, Koga M, Katayama N, Morimoto W, Takahashi Y, and Yonesaki T. IscR regulates RNase LS activity by repressing rnlA transcription. *Genetics*. 2010;185(3): 823–830.

53. Volante A, Carrasco B, Tabone M, and Alonso JC. The interaction of ω2with the RNA polymerase β′ subunit functions as an activation to repression switch. *Nucleic Acids Res*. 2015;43(19): 9249–9261.
54. Hallez R, Geeraerts D, Sterckx Y, Mine N, Loris R, and Van Melderen L. New toxins homologous to ParE belonging to three-component toxin-antitoxin systems in Escherichia coli O157:H7. *Mol Microbiol*. 2010;76(3): 719–732.
55. Smith ASG, and Rawlings DE. The poison-antidote stability system of the broad-host-range Thiobacillus ferrooxidans plasmid pTF-FC2. *Mol Microbiol*. 1997; 26(5): 961–970.
56. Hall AM, Gollan B, and Helaine S. Toxin–antitoxin systems: reversible toxicity. *Curr Opin Microbiol*. 2017;36: 102–110.
57. Yamaguchi Y, and Inouye M. Regulation of growth and death in Escherichia coli by toxin-antitoxin systems. *Nat Rev Microbiol*. 2011;9(11): 779–790.
58. Pedersen K, Zavialov A V, Pavlov MY, Elf J, Gerdes K, and Ehrenberg M. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell*. 2003;112(1): 131–140.
59. Neubauer C, Gao YG, Andersen KR, Dunham CM, Kelley AC, Hentschel J, Gerdes K, Ramakrishnan V, and Brodersen DE. The Structural Basis for mRNA Recognition and Cleavage by the Ribosome-Dependent Endonuclease RelE. *Cell*. 2009;139(6): 1084–1095.
60. Zhang Y, and Inouye M. The inhibitory mechanism of protein synthesis by YoeB, an Escherichia coli toxin. *J Biol Chem*. 2009;284(11): 6627–6638.
61. Prysak MH, Mozdzierz CJ, Cook AM, Zhu L, Zhang Y, Inouye M, and Woychik NA. Bacterial toxin YafQ is an endoribonuclease that associates with the ribosome and blocks translation elongation through sequence-specific and frame-dependent mRNA cleavage. *Mol Microbiol*. 2009;71(5): 1071–1087.
62. Hurley JM, and Woychik NA. Bacterial toxin HigB associates with ribosomes and mediates translation-dependent mRNA cleavage at A-rich sites. *J Biol Chem*. 2009;284(28): 18605–18613.
63. Zhang Y, Yamaguchi Y, and Inouye M. Characterization of YafO, an Escherichia coli toxin. *J Biol Chem*. 2009;284(38): 25522–25531.
64. Tamman H, Ainelo A, Ainsaar K, and Hõrak R. A moderate toxin, grat, modulates growth rate and stress tolerance of pseudomonas putida. *J Bacteriol*. 2014;196(1): 157–169.
65. Ainelo A, Tamman H, Leppik M, Remme J, and Hõrak R. The toxin GraT inhibits ribosome biogenesis. *Mol Microbiol*. 2016;100(4): 719–734.
66. Ainelo A, Porosk R, Kilk K, Rosendahl S, Remme J, and Hõrak R. Pseudomonas putida Responds to the Toxin GraT by Inducing Ribosome Biogenesis Factors and Repressing TCA Cycle Enzymes. *Toxins (Basel)*. 2019;11(2): E103.
67. Zhang Y, Zhang J, Hoeflich KP, Ikura M, Qing G, and Inouye M. MazF Cleaves Cellular mRNAs Specifically at ACA to Block Protein Synthesis in Escherichia coli. *Mol Cell*. 2003;12(4): 913–923.
68. Zhang Y, Zhang J, Hara H, Kato I, and Inouye M. Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase. *J Biol Chem*. 2005;280(5): 3143–3150.
69. Culviner PH, and Laub MT. Global Analysis of the E. coli Toxin MazF Reveals Widespread Cleavage of mRNA and the Inhibition of rRNA Maturation and Ribosome Biogenesis. *Mol Cell*. 2018;70(5): 868–880.e10.

70. Zorzini V, Mernik A, Lah J, Sterckx YGJ, De Jonge N, Garcia-Pino A, De Greve H, Versees W, and Loris R. Substrate recognition and activity regulation of the Escherichia coli mRNA endonuclease MazF. *J Biol Chem*. 2016;291(21): 10950–10960.
71. Temmel H, Müller C, Sauert M, Vesper O, Reiss A, Popow J, Martinez J, and Moll I. The RNA ligase RtcB reverses MazF-induced ribosome heterogeneity in Escherichia coli. *Nucleic Acids Res*. 2017;45(8): 4708–4721.
72. Yamaguchi Y, Nariya H, Park JH, and Inouye M. Inhibition of specific gene expressions by protein-mediated mRNA interference. *Nat Commun*. 2012;3: 607.
73. Schifano JM, Vvedenskaya IO, Knoblauch JG, Ouyang M, Nickels BE, and Woychik NA. An RNA-seq method for defining endoribonuclease cleavage specificity identifies dual rRNA substrates for toxin MazF-mt3. *Nat Commun*. 2014;5: 3538.
74. Schifano JM, Edifor R, Sharp JD, Ouyang M, Konkimalla A, Husson RN, and Woychik NA. Mycobacterial toxin MazF-mt6 inhibits translation through cleavage of 23S rRNA at the ribosomal A site. *Proc Natl Acad Sci*. 2013;110(21): 8501–8506.
75. Schifano JM, Cruz JW, Vvedenskaya IO, Edifor R, Ouyang M, Husson RN, Nickels BE, and Woychik NA. tRNA is a new target for cleavage by a MazF toxin. *Nucleic Acids Res*. 2016;44(3): 1256–1270.
76. Arcus VL, Mckenzie JL, Robson J, and Cook GM. The PIN-domain ribonucleases and the prokaryotic VapBC toxin-antitoxin array. *Protein Eng Des Sel*. 2011;24(1–2): 33–40.
77. Winther KS, and Gerdes K. Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA. *Proc Natl Acad Sci*. 2011;108(18): 7403–7407.
78. Lopes APY, Lopes LM, Fraga TR, Chura-Chambi RM, Sanson AL, Cheng E, Nakajima E, Morganti L, and Martins EAL. VapC from the leptospiral VapBC toxin-antitoxin module displays ribonuclease activity on the initiator tRNA. *PLoS One*. 2014;9(7): e101678.
79. Cruz JW, Sharp JD, Hoffer ED, Maehigashi T, Vvedenskaya IO, Konkimalla A, Husson RN, Nickels BE, Dunham CM, and Woychik NA. Growth-regulating Mycobacterium tuberculosis VapC-mt4 toxin is an isoacceptor-specific tRNase. *Nat Commun*. 2015;6: 7480.
80. Winther K, Tree JJ, Tollervey D, and Gerdes K. VapCs of Mycobacterium tuberculosis cleave RNAs essential for translation. *Nucleic Acids Res*. 2016;44(20): 9860–9871.
81. Winther KS, Brodersen DE, Brown AK, and Gerdes K. VapC20 of mycobacterium tuberculosis cleaves the sarcin-ricin loop of 23S rRNA. *Nat Commun*. 2013;4: 2796.
82. Mets T, Lippus M, Schryer D, Liiv A, Kasari V, Paier A, Maiväli Ü, Remme J, Tenson T, and Kaldalu N. Toxins MazF and MqsR cleave Escherichia coli rRNA precursors at multiple sites. *RNA Biol*. 2017;14(1): 124–135.
83. Schmidt O, Schuenemann VJ, Hand NJ, Silhavy TJ, Martin J, Lupas AN, and Djuranovic S. prlF and yhaV Encode a New Toxin-Antitoxin System in Escherichia coli. *J Mol Biol*. 2007;372(4): 894–905.
84. Jørgensen MG, Pandey DP, Jaskolska M, and Gerdes K. HicA of Escherichia coli defines a novel family of translation-independent mRNA interferases in bacteria and archaea. *J Bacteriol*. 2009;191(4): 1191–1199.

85. Germain E, Castro-Roa D, Zenkin N, and Gerdes K. Molecular Mechanism of Bacterial Persistence by HipA. *Mol Cell*. 2013;52(2): 248–254.
86. Kaspy I, Rotem E, Weiss N, Ronin I, Balaban NQ, and Glaser G. HipA-mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase. *Nat Commun*. 2013;4: 3001.
87. Castro-Roa D, Garcia-Pino A, De Gieter S, Van Nuland NAJ, Loris R, and Zenkin N. The Fic protein Doc uses an inverted substrate to phosphorylate and inactivate EF-Tu. *Nat Chem Biol*. 2013;9(12): 811–817.
88. Mutschler H, Gebhardt M, Shoeman RL, and Meinhart A. A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis. *PLoS Biol*. 2011;9(3): e1001033.
89. Zhang Y, and Inouye M. RatA (YfjG), an Escherichia coli toxin, inhibits 70S ribosome association to block translation initiation. *Mol Microbiol*. 2011;79(6): 1418–1429.
90. Cheverton AM, Gollan B, Przydacz M, Wong CT, Mylona A, Hare SA, and Helaine S. A Salmonella Toxin Promotes Persister Formation through Acetylation of tRNA. *Mol Cell*. 2016;63(1): 86–96.
91. Jurenas D, Chatterjee S, Konijnenberg A, Sobott F, Droogmans L, Garcia-Pino A, and Van Melderen L. AtaT blocks translation initiation by N-acetylation of the initiator tRNAfMet. *Nat Chem Biol*. 2017;13(6): 640–646.
92. Loris R, Dao-Thi MH, Bahassi EM, Van Melderen L, Poortmans F, Liddington R, Couturier M, and Wyns L. Crystal structure of CcdB, a topoisomerase poison from E. coli. *J Mol Biol*. 1999;285(4): 1667–1677.
93. Jiang Y, Pogliano J, Helinski DR, and Konieczny I. ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of Escherichia coli gyrase. *Mol Microbiol*. 2002;44(4): 971–979.
94. Dao-Thi MH, Van Melderen L, De Genst E, Afif H, Buts L, Wyns L, and Loris R. Molecular basis of gyrase poisoning by the addiction toxin CcdB. *J Mol Biol*. 2005;348(5): 1091–1102.
95. Roberts RC, Ström AR, and Helinski DR. The parDE operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. *J Mol Biol*. 1994;237(1): 35–51.
96. Jaffe A, Ogura T, and Hiraga S. Effects of the ccd function of the F plasmid on bacterial growth. *J Bacteriol*. 1985;163(3): 841–849.
97. Yuan J, Sterckx Y, Mitchenall LA, Maxwell A, Loris R, and Waldor MK. Vibrio cholerae ParE2 poisons DNA gyrase via a mechanism distinct from other gyrase inhibitors. *J Biol Chem*. 2010;285(51): 40397–40408.
98. Harms A, Stanger FV, Scheu PD, de Jong IG, Goepfert A, Glatter T, Gerdes K, Schirmer T, and Dehio C. Adenylylation of Gyrase and Topo IV by FicT Toxins Disrupts Bacterial DNA Topology. *Cell Rep*. 2015;12(9): 1497–1507.
99. Jankevicius G, Ariza A, Ahel M, and Ahel I. The Toxin-Antitoxin System DarTG Catalyzes Reversible ADP-Ribosylation of DNA. *Mol Cell*. 2016;64(6): 1109–1116.
100. Blower TR, Short FL, Rao F, Mizuguchi K, Pei XY, Fineran PC, Luisi BF, and Salmond GPC. Identification and classification of bacterial Type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. *Nucleic Acids Res*. 2012;40(13): 6158–6173.

101. Fineran PC, Blower TR, Foulds IJ, Humphreys DP, Lilley KS, and Salmond GPC. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proc Natl Acad Sci*. 2009;106(3): 894–899.
102. Blower TR, Pei XY, Short FL, Fineran PC, Humphreys DP, Luisi BF, and Salmond GPC. A processed noncoding RNA regulates an altruistic bacterial antiviral system. *Nat Struct Mol Biol*. 2011;18(2): 185–191.
103. Short FL, Pei XY, Blower TR, Ong S-L, Fineran PC, Luisi BF, and Salmond GPC. Selectivity and self-assembly in the control of a bacterial toxin by an antitoxic noncoding RNA pseudoknot. *Proc Natl Acad Sci*. 2013;110(3): E241–E249.
104. Rao F, Short FL, Voss JE, Blower TR, Orme AL, Whittaker TE, Luisi BF, and Salmond GPC. Co-evolution of quaternary organization and novel RNA tertiary interactions revealed in the crystal structure of a bacterial protein-RNA toxin-antitoxin system. *Nucleic Acids Res*. 2015;43(19): 9529–9540.
105. Short FL, Akusobi C, Broadhurst WR, and Salmond GPC. The bacterial Type III toxin-antitoxin system, ToxIN, is a dynamic protein-RNA complex with stability-dependent antiviral abortive infection activity. *Sci Rep*. 2018;8(1): 1013.
106. Samson JE, Spinelli S, Cambillau C, and Moineau S. Structure and activity of AbiQ, a lactococcal endoribonuclease belonging to the type III toxin-antitoxin system. *Mol Microbiol*. 2013;87(4): 756–768.
107. Brown JM, and Shaw KJ. A Novel Family of Escherichia coli Toxin-Antitoxin Gene Pairs. *J Bacteriol*. 2003;185(22): 6600–6608.
108. Masuda H, Tan Q, Awano N, Wu KP, and Inouye M. YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in Escherichia coli. *Mol Microbiol*. 2012;84(5): 979–989.
109. Tan Q, Awano N, and Inouye M. YeeV is an Escherichia coli toxin that inhibits cell division by targeting the cytoskeleton proteins, FtsZ and MreB. *Mol Microbiol*. 2011;79(1): 109–118.
110. Heller DM, Tavag M, and Hochschild A. CbtA toxin of Escherichia coli inhibits cell division and cell elongation via direct and independent interactions with FtsZ and MreB. *PLoS Genet*. 2017;13(9): e1007007.
111. Wen Z, Wang P, Sun C, Guo Y, and Wang X. Interaction of type IV toxin/antitoxin systems in cryptic prophages of Escherichia coli K-12. *Toxins (Basel)*. 2017;9(3): E77.
112. Dy RL, Przybilski R, Semeijn K, Salmond GPC, and Fineran PC. A widespread bacteriophage abortive infection system functions through a Type IV toxin-antitoxin mechanism. *Nucleic Acids Res*. 2014;42(7): 4590–4605.
113. Wang X, Lord DM, Cheng HY, Osbourne DO, Hong SH, Sanchez-Torres V, Quiroga C, Zheng K, Herrmann T, Peti W, Benedik MJ, Page R, and Wood TK. A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nat Chem Biol*. 2012;8(10): 855–861.
114. Kim JS, Schantz AB, Song S, Kumar M, and Wood TK. GhoT of the GhoT/GhoS toxin/antitoxin system damages lipid membranes by forming transient pores. *Biochem Biophys Res Commun*. 2018;497(2): 467–472.
115. Aakre CD, Phung TN, Huang D, and Laub MT. A bacterial toxin inhibits DNA replication elongation through a direct interaction with the β sliding clamp. *Mol Cell*. 2013;52(5): 617–628.
116. Coray DS, Wheeler NE, Heinemann JA, and Gardner PP. Why so narrow: Distribution of anti-sense regulated, type I toxin-antitoxin systems compared with type II and type III systems. *RNA Biol*. 2017;14(3): 275–280.

117. Sberro H, Leavitt A, Kiro R, Koh E, Peleg Y, Qimron U, and Sorek R. Discovery of Functional Toxin/Antitoxin Systems in Bacteria by Shotgun Cloning. *Mol Cell*. 2013;50(1): 136–148.
118. Cambray G, Guerout A-M, and Mazel D. Integrons. *Annu Rev Genet*. 2010;(August): 141–165.
119. Wang X, and Wood TK. Cryptic prophages as targets for drug development. *Drug Resist Updat*. 2016;27: 30–38.
120. Fozo EM, Makarova KS, Shabalina SA, Yutin N, Koonin E V, and Storz G. Abundance of type I toxin-antitoxin systems in bacteria: Searches for new candidates and discovery of novel families. *Nucleic Acids Res*. 2010;38(11): 3743–3759.
121. Pedersen K, and Gerdes K. Multiple hok genes on the chromosome of Escherichia coli. *Mol Microbiol*. 1999;32(5): 1090–1102.
122. Pimentel B, Nair R, Bermejo-Rodriguez C, Preston MA, Agu CA, Wang X, Bernal JA, Sherratt DJ, and de la Cueva-Mendez G. Toxin Kid uncouples DNA replication and cell division to enforce retention of plasmid R1 in Escherichia coli cells. *Proc Natl Acad Sci*. 2014;111(7): 2734–2739.
123. Cooper TF, and Heinemann JA. Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. *Proc Natl Acad Sci*. 2000;97(23): 12643–12648.
124. Saavedra De Bast M, Mine N, and Van Melderen L. Chromosomal toxin-antitoxin systems may act as antiaddiction modules. *J Bacteriol*. 2008;190(13): 4603–4609.
125. Wilbaux M, Mine N, Guérout AM, Mazel D, and Van Melderen L. Functional interactions between coexisting toxin-antitoxin systems of the ccd family in Escherichia coli O157:H7. *J Bacteriol*. 2007;189(7): 2712–2719.
126. Alawneh AM, Qi D, Yonesaki T, and Otsuka Y. An ADP-ribosyltransferase Alt of bacteriophage T4 negatively regulates the Escherichia coliMazF toxin of a toxin-antitoxin module. *Mol Microbiol*. 2016;99(1): 188–198.
127. Pecota DC, and Wood TK. Exclusion of T4 phage by the hok/sok killer locus from plasmid R1. *J Bacteriol*. 1996;178(7): 2044–2050.
128. Garvey P, Fitzgerald GF, and Hill C. Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. *Appl Environ Microbiol*. 1995;61(12): 4321–4328.
129. Otsuka Y. Prokaryotic toxin–antitoxin systems: novel regulations of the toxins. *Curr Genet*. 2016;62(2): 379–382.
130. Otsuka Y, and Yonesaki T. Dmd of bacteriophage T4 functions as an antitoxin against Escherichia coli LsoA and RnlA toxins. *Mol Microbiol*. 2012;83(4): 669–681.
131. Blower TR, Evans TJ, Przybilski R, Fineran PC, and Salmond GPC. Viral Evasion of a Bacterial Suicide System by RNA-Based Molecular Mimicry Enables Infectious Altruism. *PLoS Genet*. 2012;8(10): e1003023.
132. Keren I, Shah D, Spoering A, Kaldalu N, and Lewis K. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. *J Bacteriol*. 2004;186(24): 8172–8180.
133. Christensen SK, Mikkelsen M, Pedersen K, and Gerdes K. RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc Natl Acad Sci*. 2001;98(25): 14328–14333.
134. Christensen SK, Pedersen K, Hansen FG, and Gerdes K. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *J Mol Biol*. 2003;332(4): 809–819.

135. Tsilibaris V, Maenhaut-Michel G, Mine N, and Van Melderen L. What is the benefit to Escherichia coli of having multiple toxin-antitoxin systems in its genome? *J Bacteriol*. 2007;189(17): 6101–6108.
136. Kumar S, and Engelberg-Kulka H. Quorum sensing peptides mediating interspecies bacterial cell death as a novel class of antimicrobial agents. *Curr Opin Microbiol*. 2014;21: 22–27.
137. Engelberg-Kulka H, Amitai S, Kolodkin-Gal I, and Hazan R. Bacterial programmed cell death and multicellular behavior in bacteria. *PLoS Genet*. 2006; 2(10): 1518–1526.
138. Morganroth PA, and Hanawalt PC. Role of DNA replication and repair in thymineless death in Escherichia coli. *J Bacteriol*. 2006;188(14): 5286–5288.
139. Pedersen K, Christensen SK, and Gerdes K. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Mol Microbiol*. 2002;45(2): 501–510.
140. Nariya H, and Inouye M. MazF, an mRNA Interferase, Mediates Programmed Cell Death during Multicellular Myxococcus Development. *Cell*. 2008;132(1): 55–66.
141. Lee B, Holkenbrink C, Treuner-Lange A, and Higgs PI. Myxococcus xanthus developmental cell fate production: Heterogeneous accumulation of developmental Regulatory proteins and reexamination of the role of MazF in developmental lysis. *J Bacteriol*. 2012;194(12): 3058–3068.
142. Boynton TO, Mcmurry JL, and Shimkets LJ. Characterization of Myxococcus xanthus MazF and implications for a new point of regulation. *Mol Microbiol*. 2013;87(6): 1267–1276.
143. Lewis K. Persister Cells. *Annu Rev Microbiol*. 2010;64(1): 357–372.
144. Tuomanen E, Cozens R, Tosch W, Zak O, and Tomasz A. The rate of killing of Escherichia coli by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. *J Gen Microbiol*. 1986;132(5): 1297–1304.
145. Tuomanen E, Durack DT, and Tomasz A. Antibiotic tolerance among clinical isolates of bacteria. *Antimicrob Agents Chemother*. 1986;30(4): 521–527.
146. Korch SB, Henderson TA, and Hill TM. Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. *Mol Microbiol*. 2003;50(4): 1199–1213.
147. Radzikowski JL, Schramke H, and Heinemann M. Bacterial persistence from a system-level perspective. *Curr Opin Biotechnol*. 2017;46: 98–105.
148. Hansen S, Lewis K, and Vulić M. Role of global regulators and nucleotide metabolism in antibiotic tolerance in Escherichia coli. *Antimicrob Agents Chemother*. 2008;52(8): 2718–2726.
149. Keren I, Minami S, Rubin E, and Lewis K. Characterization and transcriptome analysis of mycobacterium tuberculosis persisters. *MBio*. 2011;2(3).
150. Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, and Lewis K. Persisters: A distinct physiological state of E. coli. *BMC Microbiol*. 2006;6: 53.
151. Vázquez-Laslop N, Lee H, and Neyfakh AA. Increased persistence in Escherichia coli caused by controlled expression of toxins or other unrelated proteins. *J Bacteriol*. 2006;188(10): 3494–3497.
152. Dörr T, Vulić M, and Lewis K. Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. *PLoS Biol*. 2010;8(2): e1000317.
153. Maisonneuve E, Shakespeare LJ, Jørgensen MG, and Gerdes K. Bacterial persistence by RNA endonucleases. *Proc Natl Acad Sci U S A*. 2011;108(32): 13206–13211.

154. Harms A, Fino C, Sørensen MA, Semsey S, and Gerdes K. Prophages and growth dynamics confound experimental results with antibiotic-tolerant persister cells. *MBio*. 2017;8(6): e01964-17.
155. Maisonneuve E, Castro-Camargo M, and Gerdes K. (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. *Cell*. 2013;154(5): 1140–1150.
156. Goormaghtigh F, Fraikin N, Putrinš M, Hallaert T, Hauryliuk V, Garcia-Pino A, Sjödin A, Kasvandik S, Udekwu K, Tenson T, Kaldalu N, and Van Melderen L. Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of Escherichia coli Type II Persister Cells. *MBio*. 2018;9(3): e00640-18.
157. Norton JP, and Mulvey MA. Toxin-Antitoxin Systems Are Important for Niche-Specific Colonization and Stress Resistance of Uropathogenic Escherichia coli. *PLoS Pathog*. 2012;8(10): e1002954.
158. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, and Holden DW. Internalization of salmonella by macrophages induces formation of non-replicating persisters. *Science (80-)*. 2014;343(6167): 204–208.
159. Wang X, Kim Y, Hong SH, Ma Q, Brown BL, Pu M, Tarone AM, Benedik MJ, Peti W, Page R, and Wood TK. Antitoxin MqsA helps mediate the bacterial general stress response. *Nat Chem Biol*. 2011;7(6): 359–366.
160. Soo VWC, and Wood TK. Antitoxin MqsA represses curli formation through the master biofilm regulator CsgD. *Sci Rep*. 2013;3: 3186.
161. Hu Y, Benedik MJ, and Wood TK. Antitoxin DinJ influences the general stress response through transcript stabilizer CspE. *Environ Microbiol*. 2012;14(3): 669–679.
162. Lin CY, Awano N, Masuda H, Park JH, and Inouye M. Transcriptional Repressor HipB Regulates the Multiple Promoters in Escherichia coli. *J Mol Microbiol Biotechnol*. 2013;23(6): 440–447.
163. Wen W, Liu B, Xue L, Zhu Z, Niu L, and Sun B. Autoregulation and virulence control by the toxin-antitoxin system SavRS in Staphylococcus aureus. *Infect Immun*. 2018;86(5): e00032-18.
164. Ren D, Kordis AA, Sonenshine DE, and Daines DA. The ToxAvapA toxin-antitoxin locus contributes to the survival of nontypeable Haemophilus influenzae during infection. Chakravortty D, editor. *PLoS One*. 2014;9(3): e91523.
165. Ren D, Walker AN, and Daines DA. Toxin-antitoxin loci vapBC-1 and vapXD contribute to survival and virulence in nontypeable Haemophilus influenzae. *BMC Microbiol*. 2012;12(1): 263.
166. Lobato-Márquez D, Moreno-Córdoba I, Figueroa V, Díaz-Orejas R, and García-del Portillo F. Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside eukaryotic cells. *Sci Rep*. 2015;5(1): 9374.
167. Wang Y, Wang H, Hay AJ, Zhong Z, Zhu J, and Kan B. Functional RelBE-family toxin-antitoxin pairs affect biofilm maturation and intestine colonization in Vibrio cholerae. *PLoS One*. 2015;10(8): e0135696.
168. Tiwari P, Arora G, Singh M, Kidwai S, Narayan OP, and Singh R. MazF ribonucleases promote Mycobacterium tuberculosis drug tolerance and virulence in guinea pigs. *Nat Commun*. 2015;6(1): 6059.
169. Weaver KE, Clewell DB, and An F. Identification, characterization, and nucleotide sequence of a region of Enterococcus faecalis pheromone-responsive plasmid pAD1 capable of autonomous replication. *J Bacteriol*. 1993;175(7): 1900–1909.

170. Wozniak RAF, and Waldor MK. A toxin-antitoxin system promotes the maintenance of an integrative conjugative element. *PLoS Genet*. 2009;5(3): e1000439.
171. Hopper S, Wilbur JS, Vasquez BL, Larson J, Clary S, Mehr IJ, Seifert HS, and So M. Isolation of Neisseria gonorrhoeae mutants that show enhanced trafficking across polarized T84 epithelial monolayers. *Infect Immun*. 2000;68(2): 896–905.
172. Michaux C, Hartke A, Martini C, Reiss S, Albrecht D, Budin-Verneuil A, Sanguinetti M, Engelmann S, Hain T, Verneuil N, and Giard J-C. Involvement of Enterococcus faecalis small RNAs in stress response and virulence. *Infect Immun*. 2014;82(9): 3599–3611.
173. Pinel-Marie M-L, Brielle R, and Felden B. Dual toxic-peptide-coding Staphylococcus aureus RNA under antisense regulation targets host cells and bacterial rivals unequally. *Cell Rep*. 2014;7(2): 424–435.
174. Komi KK, Ge YM, Xin XY, Ojcius DM, Sun D, Hu WL, Zhao X, Lin X, and Yan J. ChpK and MazF of the toxin-antitoxin modules are involved in the virulence of Leptospira interrogans during infection. *Microbes Infect*. 2015;17(1): 34–47.
175. De Jonge N, Garcia-Pino A, Buts L, Haesaerts S, Charlier D, Zangger K, Wyns L, De Greve H, and Loris R. Rejuvenation of CcdB-Poisoned Gyrase by an Intrinsically Disordered Protein Domain. *Mol Cell*. 2009;35(2): 154–163.
176. Nikolic N, Bergmiller T, Vandervelde A, Albanese TG, Gelens L, and Moll I. Autoregulation of mazEFexpression underlies growth heterogeneity in bacterial populations. *Nucleic Acids Res*. 2018;46(6): 2918–2931.
177. Schumacher MA, Min JK, Link TM, Guan Z, Xu W, Ahn YH, Soderblom EJ, Kurie JM, Evdokimov A, Moseley MA, Lewis K, and Brennan RG. Role of Unusual P Loop Ejection and Autophosphorylation in HipA-Mediated Persistence and Multidrug Tolerance. *Cell Rep*. 2012;2(3): 518–525.
178. Semanjski M, Germain E, Bratl K, Kiessling A, Gerdes K, and Macek B. The kinases HipA and HipA7 phosphorylate different substrate pools in Escherichia coli to promote multidrug tolerance. *Sci Signal*. 2018;11(547): eaat5750.
179. Wang X, Lord DM, Hong SH, Peti W, Benedik MJ, Page R, and Wood TK. Type II toxin/antitoxin MqsR/MqsA controls type V toxin/antitoxin GhoT/GhoS. *Environ Microbiol*. 2013;15(6): 1734–1744.
180. Yang M, Gao C, Wang Y, Zhang H, and He ZG. Characterization of the interaction and cross-regulation of three Mycobacterium tuberculosis RelBE modules. *PLoS One*. 2010;5(5): e10672.
181. Zhu L, Sharp JD, Kobayashi H, Woychik NA, and Inouye M. Noncognate Mycobacterium tuberculosis toxin-antitoxins can physically and functionally interact. *J Biol Chem*. 2010;285(51): 39732–39738.
182. Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiß S, Sittka A, Chabas S, Reiche K, Hackermüller J, Reinhardt R, Stadler PF, and Vogel J. The primary transcriptome of the major human pathogen Helicobacter pylori. *Nature*. 2010; 464(7286): 250–255.
183. Cannistraro VJ, and Kennell D. The 5′ ends of RNA oligonucleotides in Escherichia coli and mRNA degradation. *Eur J Biochem*. 1993;213(1): 285–293.
184. Siibak T, Peil L, Dönhöfer A, Tats A, Remm M, Wilson DN, Tenson T, and Remme J. Antibiotic-induced ribosomal assembly defects result from changes in the synthesis of ribosomal proteins. *Mol Microbiol*. 2011;80(1): 54–67.
185. Siibak T, Peil L, Xiong L, Mankin A, Remme J, and Tenson T. Erythromycin- and chloramphenicol-induced ribosomal assembly defects are secondary effects of protein synthesis inhibition. *Antimicrob Agents Chemother*. 2009;53(2): 563–571.

186. Sirdeshmukh R, and Schlessinger D. Why is processing of 23 S ribosomal RNA in Escherichia coli not obligate for its function? *J Mol Biol*. 1985;186(3): 669–672.
187. Dahlberg AE, Dahlberg JE, Lund E, Tokimatsu H, Rabson AB, Calvert PC, Reynolds F, and Zahalak M. Processing of the 5' end of Escherichia coli 16S ribosomal RNA. *Proc Natl Acad Sci U S A*. 1978;75(8): 3598–3602.
188. Srivastava AK, and Schlessinger D. Coregulation of processing and translation: mature 5' termini of Escherichia coli 23S ribosomal RNA form in polysomes. *Proc Natl Acad Sci U S A*. 1988;85(19): 7144–7148.
189. Oron-Gottesman A, Sauert M, Moll I, and Engelberg-Kulka H. A stress-induced bias in the reading of the genetic code in Escherichia coli. *MBio*. 2016;7(6): e01855-16.
190. Venturelli OS, Tei M, Bauer S, Chan LJG, Petzold CJ, and Arkin AP. Programming mRNA decay to modulate synthetic circuit resource allocation. *Nat Commun*. 2017;8: 15128.
191. Kim Y, Wang X, Zhang XS, Grigoriu S, Page R, Peti W, and Wood TK. Escherichia coli toxin/antitoxin pair MqsR/MqsA regulate toxin CspD. *Environ Microbiol*. 2010;12(5): 1105–1121.
192. Mok WWK, Park JO, Rabinowitz JD, and Brynildsen MP. RNA futile cycling in model persisters derived from mazF accumulation. *MBio*. 2015;6(6): e01588-15.
193. Deana A, Celesnik H, and Belasco JG. The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal. *Nature*. 2008; 451(7176): 355–358.
194. Thomason MK, Bischler T, Eisenbart SK, Förstner KU, Zhang A, Herbig A, Nieselt K, Sharma CM, and Storz G. Global transcriptional start site mapping using dRNA-seq reveals novel antisense RNAs in Escherichia coli. *J Bacteriol*. 2014;197(1): 18–28.
195. Wagner GP, Kin K, and Lynch VJ. Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. *Theory Biosci*. 2012;131(4): 281–285.
196. Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, and Mann M. Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Mol Cell Proteomics*. 2002;1(5): 376–386.
197. Gallegos MT, Schleif R, Bairoch A, Hofmann K, and Ramos JL. Arac/XylS family of transcriptional regulators. *Microbiol Mol Biol Rev*. 1997;61(4): 393–410.
198. Saveanu C, Miron S, Borza T, Craescu CT, Labesse G, Gagyi C, Popescu A, Schaeffer F, Namane A, Laurent-winter C, Barzu O, and Gilles A-M. Structural and nucleotide-binding properties of YajQ and YnaF, two Escherichia coli proteins of unknown function. *Protein Sci*. 2002;11: 2551–2560.
199. Bougdour A, Wickner S, and Gottesman S. Modulating RssB activity: IraP, a novel regulator of σSstability in Escherichia coli. *Genes Dev*. 2006;20(7): 884–897.

SUMMARY IN ESTONIAN

Escherichia coli RNA fragmenteerimine MazF ning MqsR toksiinide poolt

Bakterite toksiin-antitoksiin süsteemid on väikesed geneetilised moodulid, mis kodeerivad toksilist valku ja seda neutraliseerivat antitoksiini. Antitoksiini taseme langedes inhibeerib toksiin teda tootva raku kasvu. Esialgselt avastati toksiin-antitoksiin süsteemid plasmiididelt kui neid stabiliseerivad üksused. Toksiinid on võrreldes antitoksiiniga stabiilsemad ning säilivad plasmiidi kaotuse korral rakus palju kauem. Seetõttu on plasmiidi kaotanud bakterite kasv pärsitud ja kasvueelise saavad plasmiidi pärinud rakud. Peatselt avastati, et toksiin-antitoksiin süsteemid on laialt levinud ka kromosomaalses DNA-s. Vaatamata laialdastele uuringutele ei ole kromosomaalsete toksiin-antitoksiin süsteemide roll veel üheselt selge. Osad hüpoteesid näevad kromosomaalseid toksiin-antitoksiin süsteeme ainult liikuvate geneetiliste üksuste integreerumise ülejääkidena. Samas seostavad mitmed uuringud neid bakteriofaagide vastase kaitse, stressivastuse, antibiootikumidele tolerantsuse ja bakterite virulentsusega. Toksiin-antitoksiin süsteemide uurimine võib seetõttu aidata meil paremini mõista bakteriaalseid infektsioone ja pakkuda uusi lahendusi nendega toime tulekuks.

Toksiinid ründavad erinevaid rakulisi protsesse nagu translatsioon, replikatsioon ja energia tootmine. Seejuures on RNA lõikamine ülekaalukalt kõige levinum toksiinide toimemehhanism. Toksiinid lagundavad RNA-d kas ribosoomidega seondunult või iseseisvalt. Ribosoomist sõltumatud ribonukleaassed toksiinid lõikavad tavaliselt kindlat RNA järjestust, näiteks *Escherichia coli* MazF toksiin tunneb ära ACA ja *E. coli* MqsR toksiin GCU järjestuse. Klassikaliselt on ribonukleaasseid toksiine peetud mittespetsiifilisteks mRNA lagundajateks, mis halvavad raku kasvu rünnates enamikke valku kodeerivatest transkriptidest. Hiljutised uuringud näitasid, et osad MazF ja VapC perekonna toksiinidest inhibeerivad translatisooni ka rRNA-d või tRNA-d lõigates. *Escherichia coli* MazF toksiini puhul on näidatud 16S rRNA lõikamist, kuid väidetavalt ei ole selle eesmärgiks translatsiooni inhibeerimine, vaid selle ümberprogrammeerimine. Nende uuringute kohaselt lõikab *E. coli* MazF stressi tingimustes küpsete ribosoomide 16S rRNA 3’ otsast ära 43 nukleotiidi pikkuse fragmendi. Arvatakse, et selliselt kärbitud ribosoomid toodavad valke 5’ otsast kärbitud spetsiifilistelt mRNA-delt. Spekuleeritakse, et MazF lagundab enamiku mRNA-dest, kuid tekitab ka kärbitud 5’ otstega stressivastuse geenide mRNA-de alamhulga – MazF reguloni. Sedasi aitaks MazF stressi korral raku kasvu aeglustada ja samas tagaks oluliste stressigeenide avaldumise.

Meie tuvastasime *E. coli* MazF ja MqsR toksiinide üleekspressiooni katsetes pikkade rRNA fragmentide teket ning otsustasime lähemalt uurida nende toksiinide rolli rRNA lõikamisel. Me kaardistasime MazFi ja MqsRi lõikekohad rRNAs. Selleks kasutasime spetsiaalset RNA sekveneerimist, mis suudab vahet teha toksiinide ja rakuliste ribonukleaaside poolt tekitatud RNA otstel. Kaardis-

tatud lõikekohad 16S ja 23S RNAs ei ole küpsetes ribosoomides toksiinide poolt lõigatavad: nad asuvad kas sügaval alaühiku sees või rRNA kaheahelalistes piirkondade. Kõik meie andmed viitavad sellele, et toksiinid lõikavad protsessimata rRNA-d, mitte küpseid ribosoomi alaühikuid. Eellas-rRNA lagundamine välistab uute ribosoomide tootmise ja suunab ressursse ümber raku muudeks vajadusteks.

Lisaks uurisime mRNA lõikamist MazF ja MqsR poolt, et jõuda selgusele, kas spetsiifiline 5' kärbitud stressi-mRNA-de grupp eksisteerib või mitte. Kasutasime toksiini üleekspresseerimist ja sellele järgnevat RNA sekveneerimist, et kaardistada toksiinide lõikekohad mRNAs. Mõlemad toksiinid lõikasid ulatuslikult mRNAsid: MazF lõikas pooli ja MqsRi ühte kolmandikku avatud lugemisraamidest. Leidsime, et enamikke niinimetatud MazF reguloni transkriptidest lõigati MazF-i poolt efektiivselt ka avatud lugemisraamis. Meie tulemuste kohaselt lõikavad need kaks toksiini mRNA tükikesteks, millest järeldame, et mingit MazF reguloni pole olemas. Ka proteoomika tulemused kinnitasid, et nn. stressivalke, mille tootmist MazF peaks soodustama, tegelikult MazFi ekspressiooni tingimustes ei toodeta.

Kokkuvõtteks, meie andmed ei toeta hüpoteesi, mille kohaselt põhjustab MazF toksiin translatsiooni ümberprogrammeerimise. Meie tulemused näitavad, et MazF ja MqsR on mittespetsiifilised RNaasid, mis lõikavad kõike kättesaadavat struktureerimata RNA-d. Järeldame, et nende toksiinide funktsioon on raku kasvu peatamine läbi uute valkude ja ribosoomide sünteesi inhibeerimise.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Niilo, for all of his support, guidance, and patience throughout my journey towards becoming a PhD. I would like to thank Tanel for all of his advice and for allowing me to fulfill my goals in his lab. A big thanks also goes to Axi for all the insightful and entertaining discussions. In addition, my thesis could not have been completed without David teaching me the art of programming with Python and Ülo introducing me to the R programming language. Science is a joint effort, so I would like to thank all those with whom I collaborated.

I have been really lucky, as I have had the pleasure of working with the nicest and coolest people. Current and past co-workers (and friends) from TÜTI, you rock! Special thanks go to Triin, Airiin, Kalle, Margit, and Kristjan to whom I constantly complained about my struggles and bragged about my successes. I would also like to thank all my friends, who have helped me to stay sane during the more intense times and reminded me that there is more to life than hard scientific data.

Last, but not least, I would like to thank my family for their never-ending support to my unconventional ambitions.

PUBLICATIONS

CURRICULUM VITAE

Name: Toomas Mets
Date of birth: March 25, 1988, Põlva, Eesti
Citizenship: Estonia
Languages: Estonian, English, Russian, German
Contact: Uus 36–34, 50603, Tartu, Estonia
metsatom@gmail.com
+372 5389 0689

Education:

1995–2007 Räpina Ühisgümnaasium
2007–2010 Bachelor studies in Gene Technology, University of Tartu
2010–2012 Masters studies in Gene Technology, University of Tartu, *cum laude*
2012–… Doctoral studies in Biomedical Engineering, University of Tartu

Employment:

2009 specialist, Institute of Technology, University of Tartu
2010 specialist, Institute of Technology, University of Tartu
2016–2017 specialist, Institute of Technology, University of Tartu

Scientific work:

Since 2009, I have been researching bacterial toxin-antitoxin systems in the laboratory of Professor Tanel Tenson. I have mostly focused on studying the RNA cleavage by MazF and MqsR toxins of *E. coli* and the recovery of bacteria from the overexpression of toxins.

List of publications:

Kasari V, **Mets T**, Tenson T, and Kaldalu N. Transcriptional cross-activation between toxin-antitoxin systems of Escherichia coli. BMC Microbiol. 2013; 13(1): 45.

Mets T, Lippus M, Schryer D, Liiv A, Kasari V, Paier A, Maiväli Ü, Remme J, Tenson T, and Kaldalu N. Toxins MazF and MqsR cleave Escherichia coli rRNA precursors at multiple sites. RNA Biol. 2017;14(1): 124–135.

Mets T, Kasvandik S, Saarma M, Maiväli Ü, Tenson T, and Kaldalu N. Fragmentation of Escherichia coli mRNA by MazF and MqsR. Biochimie. 2018; 156: 79–91.

ELULOOKIRJELDUS

Nimi: Toomas Mets
Sünniaeg: 25. märts 1988, Põlva, Eesti
Kodakondsus: Eesti
Keelteoskus: eesti keel, inglise keel, vene keel, saksa keel
Kontaktinfo: Uus 36–34, 50603, Tartu, Eesti
metsatom@gmail.com
+372 5389 0689

Haridus:

1995–2007 Räpina Ühisgümnaasium
2007–2010 bakalaureuseõpe (geenitehnoloogia), Tartu Ülikool
2010–2012 magistriõpe (geenitehnoloogia), Tartu Ülikool, *cum laude*
2012–… doktoriõpe (tehnika ja tehnoloogia, biomeditsiini suund), tehnoloogiinstituut, Tartu Ülikool

Töökogemus:

2009 spetsialist, tehnoloogiainstituut, Tartu ülikool
2010 spetsialist, tehnoloogiainstituut, Tartu ülikool
2016–2017 spetsialist, tehnoloogiainstituut, Tartu ülikool

Teadustegevus:

Olen alates 2009. aastast tegelenud professor Tanel Tensoni laboris bakteriaalsete toksiin-antitoksiin süsteemide uurimisega. Peamistelt olen uurinud RNA lõikamist *E. coli* toksiinide MazF ja MqsR poolt ning bakterite taastumise toksiinide üleekspressioonist.

Teaduspublikatsioonid:

Kasari V, **Mets T**, Tenson T, and Kaldalu N. Transcriptional cross-activation between toxin-antitoxin systems of Escherichia coli. BMC Microbiol. 2013; 13(1): 45.

Mets T, Lippus M, Schryer D, Liiv A, Kasari V, Paier A, Maiväli Ü, Remme J, Tenson T, and Kaldalu N. Toxins MazF and MqsR cleave Escherichia coli rRNA precursors at multiple sites. RNA Biol. 2017;14(1): 124–135.

Mets T, Kasvandik S, Saarma M, Maiväli Ü, Tenson T, and Kaldalu N. Fragmentation of Escherichia coli mRNA by MazF and MqsR. Biochimie. 2018; 156: 79–91.

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger**. Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
2. **Taavi Lehto**. Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression. Tartu 2011, 155 p.
3. **Hannes Luidalepp**. Studies on the antibiotic susceptibility of *Escherichia coli*. Tartu 2012, 111 p.
4. **Vahur Zadin**. Modelling the 3D-microbattery. Tartu 2012, 149 p.
5. **Janno Torop**. Carbide-derived carbon-based electromechanical actuators. Tartu 2012, 113 p.
6. **Julia Suhorutšenko**. Cell-penetrating peptides: cytotoxicity, immunogenicity and application for tumor targeting. Tartu 2012, 139 p.
7. **Viktoryia Shyp**. G nucleotide regulation of translational GTPases and the stringent response factor RelA. Tartu 2012, 105 p.
8. **Mardo Kõivomägi**. Studies on the substrate specificity and multisite phosphorylation mechanisms of cyclin-dependent kinase Cdk1 in *Saccharomyces cerevisiae*. Tartu, 2013, 157 p.
9. **Liis Karo-Astover**. Studies on the Semliki Forest virus replicase protein nsP1. Tartu, 2013, 113 p.
10. **Piret Arukuusk**. NickFects–novel cell-penetrating peptides. Design and uptake mechanism. Tartu, 2013, 124 p.
11. **Piret Villo**. Synthesis of acetogenin analogues. Asymmetric transfer hydrogenation coupled with dynamic kinetic resolution of α-amido-β-keto esters. Tartu, 2013, 151 p.
12. **Villu Kasari**. Bacterial toxin-antitoxin systems: transcriptional cross-activation and characterization of a novel *mqsRA* system. Tartu, 2013, 108 p.
13. **Margus Varjak**. Functional analysis of viral and host components of alphavirus replicase complexes. Tartu, 2013, 151 p.
14. **Liane Viru**. Development and analysis of novel alphavirus-based multifunctional gene therapy and expression systems. Tartu, 2013, 113 p.
15. **Kent Langel**. Cell-penetrating peptide mechanism studies: from peptides to cargo delivery. Tartu, 2014, 115 p.
16. **Rauno Temmer**. Electrochemistry and novel applications of chemically synthesized conductive polymer electrodes. Tartu, 2014, 206 p.
17. **Indrek Must**. Ionic and capacitive electroactive laminates with carbonaceous electrodes as sensors and energy harvesters. Tartu, 2014, 133 p.
18. **Veiko Voolaid**. Aquatic environment: primary reservoir, link, or sink of antibiotic resistance? Tartu, 2014, 79 p.
19. **Kristiina Laanemets**. The role of SLAC1 anion channel and its upstream regulators in stomatal opening and closure of *Arabidopsis thaliana.* Tartu, 2015, 115 p.

20. **Kalle Pärn**. Studies on inducible alphavirus-based antitumour strategy mediated by site-specific delivery with activatable cell-penetrating peptides. Tartu, 2015, 139 p.
21. **Anastasia Selyutina**. When biologist meets chemist: a search for HIV-1 inhibitors. Tartu, 2015, 172 p.
22. **Sirle Saul**. Towards understanding the neurovirulence of Semliki Forest virus. Tartu, 2015, 136 p.
23. **Marit Orav**. Study of the initial amplification of the human papillomavirus genome. Tartu, 2015, 132 p.
24. **Tormi Reinson**. Studies on the Genome Replication of Human Papillomaviruses. Tartu, 2016, 110 p.
25. **Mart Ustav Jr**. Molecular Studies of HPV-18 Genome Segregation and Stable Replication. Tartu, 2016, 152 p.
26. **Margit Mutso**. Different Approaches to Counteracting Hepatitis C Virus and Chikungunya Virus Infections. Tartu, 2016, 184 p.
27. **Jelizaveta Geimanen**. Study of the Papillomavirus Genome Replication and Segregation. Tartu, 2016, 168 p.
28. **Mart Toots**. Novel Means to Target Human Papillomavirus Infection. Tartu, 2016, 173 p.
29. **Kadi-Liis Veiman**. Development of cell-penetrating peptides for gene delivery: from transfection in cell cultures to induction of gene expression *in vivo*. Tartu, 2016, 136 p.
30. **Ly Pärnaste**. How, why, what and where: Mechanisms behind CPP/cargo nanocomplexes. Tartu, 2016, 147 p.
31. **Age Utt**. Role of alphavirus replicase in viral RNA synthesis, virus-induced cytotoxicity and recognition of viral infections in host cells. Tartu, 2016, 183 p.
32. **Veiko Vunder**. Modeling and characterization of back-relaxation of ionic electroactive polymer actuators. Tartu, 2016, 154 p.
33. **Piia Kivipõld**. Studies on the Role of Papillomavirus E2 Proteins in Virus DNA Replication. Tartu, 2016, 118 p.
34. **Liina Jakobson**. The roles of abscisic acid, CO_2, and the cuticle in the regulation of plant transpiration. Tartu, 2017, 162 p.
35. **Helen Isok-Paas**. Viral-host interactions in the life cycle of human papillomaviruses. Tartu, 2017, 158 p.
36. **Hanna Hõrak**. Identification of key regulators of stomatal CO_2 signalling via O_3-sensitivity. Tartu, 2017, 160 p.
37. **Jekaterina Jevtuševskaja**. Application of isothermal amplification methods for detection of *Chlamydia trachomatis* directly from biological samples. Tartu, 2017, 96 p.
38. **Ülar Allas.** Ribosome-targeting antibiotics and mechanisms of antibiotic resistance. Tartu, 2017, 152 p.
39. **Anton Paier.** Ribosome Degradation in Living Bacteria. Tartu, 2017, 108 p.
40. **Vallo Varik.** Stringent Response in Bacterial Growth and Survival. Tartu, 2017, 101 p.

41. **Pavel Kudrin.** In search for the inhibitors of *Escherichia coli* stringent response factor RelA. Tartu, 2017, 138 p.
42. **Liisi Henno.** Study of the human papillomavirus genome replication and oligomer generation. Tartu, 2017, 144 p.
43. **Katrin Krõlov.** Nucleic acid amplification from crude clinical samples exemplified by *Chlamydia trachomatis* detection in urine. Tartu, 2018, 118 p.
44. **Eve Sankovski.** Studies on papillomavirus transcription and regulatory protein E2. Tartu, 2018, 113 p.
45. **Morteza Daneshmand.** Realistic 3D Virtual Fitting Room. Tartu, 2018, 233 p.
46. **Fatemeh Noroozi.** Multimodal Emotion Recognition Based Human-Robot Interaction Enhancement. Tartu, 2018, 113 p.
47. **Krista Freimann.** Design of peptide-based vector for nucleic acid delivery in vivo. Tartu, 2018, 103 p.
48. **Rainis Venta.** Studies on signal processing by multisite phosphorylation pathways of the *S. cerevisiae* cyclin-dependent kinase inhibitor Sic1. Tartu, 2018, 155 p.
49. **Inga Põldsalu.** Soft actuators with ink-jet printed electrodes. Tartu, 2018, 85 p.
50. **Kadri Künnapuu**. Modification of the cell-penetrating peptide PepFect14 for targeted tumor gene delivery and reduced toxicity. Tartu, 2018, 114 p.