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II **Ainelo A, Tamman H, Leppik M, Remme J, Hõrak R.** The toxin GraT inhibits ribosome biogenesis. Molecular microbiology. 2016. 100(4): 719–734.

III **Talavera A, Tamman H, Ainelo A, Hadži S, Garcia-Pino A, Hõrak R, Konijnenberg A, Loris R.** Production, biophysical characterization and crystallization of *Pseudomonas putida* GraA and its complexes with GraT and the *graTA* operator. Acta Crystallographica Section F: Structural Biology Communications. 2017. 73(Pt 8):455–462.

IV **Ainelo A, Porosk R, Kilk K, Rosendahl S, Remme J, Hõrak R.** Proteomic and metabolomic alterations induced by the *Pseudomonas putida* toxin GraT. Manuscript.

My contribution to the publications is following:

Ref I – I performed the *in vitro* experiments and participated in editing the manuscript.

Ref II – I participated in planning the work, performed most of the *in vitro* experiments, and wrote the manuscript.

Ref III – I participated in protein purification for crystallization.

Ref IV – I participated in planning the experiments and construction of plasmids and strains, prepared samples for proteomics and metabolomics, performed data analysis, wrote most of the manuscript.

ABBREVIATIONS

(p)ppGpp	guanosine penta- or tetraphosphate
FIC	filamentation induced by cyclic AMP
GCS	glycine cleavage system
GNAT	GCN5-related *N*-acetyltransferase
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
OAA	oxaloacetate
OD	optical density (absorbance)
ORF	open reading frame
PDB	Protein Data Bank
PEP	phosphoenolpyruvate
RP-HPLC	reverse phase high pressure liquid chromatography
SD	Shine-Dalgarno sequence (ribosome binding site)
TA	toxin-antitoxin
TCA	tricarboxylic acid
UNAG	uridine diphosphate-*N*-acetylglucosamine

Etymology of the toxin-antitoxin systems mentioned in the thesis, in alphabetical order. Toxins are listed first.

AbiEii/AbiEi	abortive infection
AtaT/AtaR	aminoacyl-tRNA acetyltransferase toxin / repressor
BsrG/SR4	*Bacillus* small RNA / small RNA
CbtA/CbeA	cytoskeleton binding toxin / cytoskeleton bundling-enhancing
CcdA/CcdB	coupled cell division
CptA/CptB	cytoskeleton polymerization inhibiting
DarT/DarG	DNA ADP-ribosyl transferase / glycohydrolase
Doc/Phd	death on curing / prevents host death
EzeT	*E. coli* epsilon/zeta, toxin and antitoxin in single polypeptide
FicT/FicA	FIC-domain
GhoT/GhoS	*ghoST* operon, toxin causes ghost cell phenotype
GraT/GraA	growth rate-affecting
Hha/TomB	haemolysin expression modulating protein / toxin overexpression modulator in biofilms
HicA/HicB	hif contiguous (homologous to *H. influenzae hif* locus)
HigB/HigA	host inhibition of growth
HipA/HipB	high persistence
Hok/Sok	host killing / suppression of killing
MazF/MazE	hebrew "ma-ze" – "what is it?"
MosT/MosA	maintenance of SXT
MqsR/MqsA	motility quorum-sensing regulator
ParE/ParD	partitioning of plasmid RK2

PasT(RatA)/PasI	persistence and stress-resistance toxin / immunity (ribosome association toxin)
PezT/PezA	pneumococcal epsilon zeta
RalR/RalA	restriction alleviation, named in phage λ
RelE/RelB	relaxed phenotype B
SocB/SocA	suppressor of *clpXP*
SymE/SymR	SOS-induced *yjiW* gene with similarity to MazE / symbiotic RNA
TacT/TacA	tRNA-acetylating
TisB/IstR	toxicity induced by SOS / inhibitor of SOS-induced toxicity by RNA
ToxN/ToxI	toxin / toxin inhibitor
VapC/VapB	virulence associated protein
VbhT/VbhA	VirB-homolog
YafO/YafN	systematic *E. coli* nomenclature
YafQ/DinJ	systematic *E. coli* nomenclature / damage inducible
YhaV/PrlF	systematic *E. coli* nomenclature / protein localization locus F
YoeB/YefM	systematic *E. coli* nomenclature

INTRODUCTION

Bacterial toxin-antitoxin (TA) modules are curious little systems that in their canonical form comprise a toxin and its cognate antitoxin. These both remain within the same cell that produced them, in the form of an inactive complex. Yet, upon antitoxin degradation, the toxin is liberated from the suppressive TA complex and can inhibit its cellular target. TA systems were discovered more than 30 years ago on a plasmid (Ogura & Hiraga, 1983) and later found to be ubiquitous in prokaryotic chromosomes as well (Pandey & Gerdes, 2005). The main question that has captivated scientists since concerns the role of genomic TA systems in bacterial physiology (Van Melderen, 2010). To be able to assess their importance, both the regulation of TA systems (Garcia-Pino *et al.*, 2010, Brzozowska & Zielenkiewicz, 2013), and also the molecular mechanisms of both toxins and antitoxins have been studied in detail (Yamaguchi *et al.*, 2011, Harms *et al.*, 2018). TA regulation, with an emphasis on toxin liberation conditions, is intriguing due to the potential links between toxin-mediated metabolism inhibition and the stress response (Wang & Wood, 2011). At the same time, researching the toxins' mechanisms of action also provides better understanding about how the bacteria react to these intrinsic metabolism inhibitors.

Pseudomonas putida is a cosmopolitan soil bacterium that can adapt to various growth conditions. The isogenic laboratory strains PaW85 (Bayley *et al.*, 1977) and KT2440 (Nelson *et al.*, 2002) are highly regarded as versatile and safe model organisms. Our studies on the TA biology in *P. putida* PaW85 were initiated by the finding that disruption of a putative TA antitoxin gene could suppress the membrane defect and conditional lethality of mutants deficient in the ColR-ColS two-component signalling system (Putrinš *et al.*, 2011). This was unexpected, as disrupting the antitoxin in a functional TA system would result in toxin liberation and metabolism inhibition instead of a positive effect. However, TA systems had been associated with increased stress tolerance (Wang & Wood, 2011), which hinted at the possible relevance of this locus in the *P. putida* stress response. As first experiments indicated that the system, dubbed GraTA (*growth rate affecting toxin-antitoxin*), is indeed functional and possesses unusual toxin features such as temperature-dependent growth inhibition and a conflicting role in modulating tolerance to different stressors, we decided to further study this TA module and its effect on *P. putida*.

The first part of this thesis gives an overview of different TA system architectures, their potential roles in bacterial biology, and the variety of toxicity mechanisms employed by TA toxins. The experimental chapters consider the *P. putida* toxin GraT, firstly focusing on its effects and cellular interactions on the molecular level, and secondly characterizing its physiological effects using a near-native model system.

REVIEW OF LITERATURE

1. Overview of toxin-antitoxin systems

Microbial toxin-antitoxin (TA) systems are small genetic modules that are canonically composed of two components transcribed from a single operon: a toxin that is able to inhibit cellular processes, and an antitoxin that counters the toxic effects during regular growth. Although the "toxin-antitoxin" designation suggests their involvement in intercellular aggression and defence, both common tactics in the microbial world, these toxins most often function within the same cell that produces them.

1.1. The variety of TA system composition

The toxins of TA systems are always proteins, but the antitoxins can vary in their biochemical nature: some are proteins and some are RNA molecules. The most general classification of whole TA systems is based on the antitoxin's nature and mode of action. Currently, seven different types (designated I–VII) have been described (Page & Peti, 2016, Song & Wood, 2018). The first two have been known and studied for more than thirty years (Ogura & Hiraga, 1983, Gerdes *et al.*, 1986b). The latter five are more recent discoveries and apart from type III, have been defined based on a few or even single findings of peculiar TA systems that do not fit under any other type.

In type I systems, the antitoxins are small RNAs that bind the toxin mRNA and prevent its translation so that the toxic protein is not produced (Figure 1 A). The *hok/sok* system from plasmid R1 was the first such module to be discovered (Gerdes *et al.*, 1986b), and the fact that *sok* antitoxin functions as an antisense RNA was determined soon after (Gerdes *et al.*, 1988).

Type II systems' antitoxins are proteins that sequester the toxins by binding them into a non-toxic complex (Figure 1 B). The first studied TA system, CcdA/CcdB (Ogura & Hiraga, 1983), belongs to this class. Type II TA systems are the most widespread (Coray *et al.*, 2017, Leplae *et al.*, 2011, Makarova *et al.*, 2009) and, due to the relative ease of working with proteins, also the most often studied toxin-antitoxin modules. Type II TA complexes and some single antitoxins additionally serve as transcriptional regulators that repress the TA locus itself, thus limiting the number of TA complexes in the cell (Tam & Kline, 1989, Tian *et al.*, 1996a, Gotfredsen & Gerdes, 1998). This thesis will mostly focus on the features of type II TA systems.

Antitoxins of type III TA systems are again RNA molecules that differ from type I by their mode of action. These antitoxin RNAs do not repress toxin synthesis but instead resemble type II antitoxins as the RNA molecules directly bind the toxins and inhibit their activity (Figure 1 C). This was first demonstrated for the ToxN/ToxI system in *Pectobacterium atrosepticum* (Fineran *et*

al., 2009). The type III antitoxin genes consist of ~35 nt repeats, which are cleaved into functional single antitoxin RNAs by the respective toxins (Goeders *et al.*, 2016).

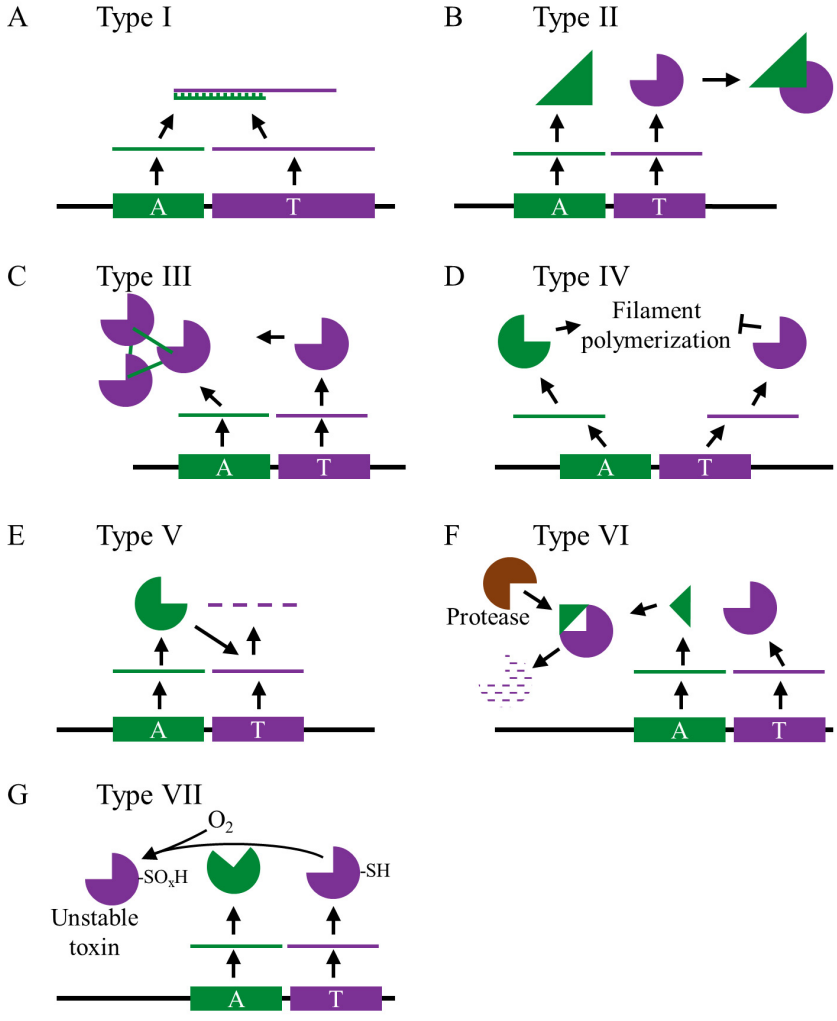

Figure 1. Antitoxins' mechanisms of toxin inhibition. Antitoxin genes, mRNAs and proteins are depicted in green and toxin genes, mRNAs and proteins in purple.

Type IV antitoxins are proteins that uniquely do not interact with the toxins themselves. They function by stabilizing the target of their toxins: the archetypal CbeA (YeeU) antitoxin enhances the bundling of MreB and FtsZ cytoskeletal filaments while the toxin CbtA inhibits their polymerization (Figure 1 D)(Masuda *et al.*, 2012a). The widespread phage abortive infection system AbiE has also been reported to act as a type IV TA module, although the target of the toxin is not known (Dy *et al.*, 2014).

Type V is characterized based on a single known system, the GhoT/GhoS from *E. coli*. The antitoxin GhoS is a protein that specifically cleaves the *ghoT* mRNA, thus preventing toxin translation (Figure 1 E)(Wang *et al.*, 2012). Interestingly, this system is activated *in vivo* by the type II TA toxin MqsR that degrades almost all cellular mRNAs, including the *ghoS* mRNA (Yamaguchi *et al.*, 2009). Notably, the *ghoT* mRNA is among the few that do not contain the preferred MqsR cleavage sites (Wang *et al.*, 2013).

Of type VI systems, again only one example has been found: the *Caulobacter crescentus* SocB/SocA module. The antitoxin SocA is a protein that binds the toxin and functions as a proteolytic adapter, leading to toxin degradation by the protease ClpXP (Figure 1 F)(Aakre *et al.*, 2013).

The latest addition to the diverse family of distinctly working antitoxins is the *E. coli* Hha/TomB system, assigned to type VII. The antitoxin TomB enhances the spontaneous oxidation of a cysteine residue of the toxin Hha in the presence of molecular oxygen. The oxidized Hha is structurally less stable and as such, less toxic (Figure 1 G)(Marimon *et al.*, 2016).

An interesting subset of TA systems are those that are not composed of two counterparts. Three-component systems are quite common, where the extra protein is either a transcriptional regulator of the TA operon (Zielenkiewicz & Ceglowski, 2005, Hallez *et al.*, 2010), an antitoxin enhancer (Smith & Rawlings, 1997) or an antitoxin chaperone (Bordes *et al.*, 2016). A single one-component TA module has been discovered as well, where a type II zeta toxin and its epsilon antitoxin are joined in a single polypeptide (Rocker & Meinhart, 2015).

1.2. Functions of TA systems

TA systems are widespread in prokaryotic genomes (Pandey & Gerdes, 2005). They are present in plasmids, where they were first discovered (Ogura & Hiraga, 1983), and have since been also found on chromosomes of numerous bacteria and archaea (Leplae *et al.*, 2011). While reductive evolution can lead to bacteria like some *Rickettsia* species being devoid of any TA modules (Leplae *et al.*, 2011), the cyanobacterium *Microcystis aeruginosa* has been predicted to contain 113 TA loci (Xie *et al.*, 2018). The number of TA systems is not well correlated with genome sizes (Leplae *et al.*, 2011) and is rather linked with the lifestyle of the bacterium, being larger in free-living bacteria where horizontal gene transfer is common (Pandey & Gerdes, 2005).

On plasmids, the presence of TA systems is easier to explain as already the first work on TA modules demonstrated that the CcdB/CcdA system helps to stabilize mini-F and *oriC* plasmids (Ogura & Hiraga, 1983). Chromosomal TA systems' functions have been a lot more difficult to understand. Opinions about their importance in bacterial physiology have ranged from TA systems being totally useless to them having a major role as stress response regulators (Van Melderen & Saavedra De Bast, 2009, Van Melderen, 2010, Díaz-Orejas *et al.*,

2017, Song & Wood, 2018). Some proposed functions, however, are controversial and have not been successfully replicated. One such example is the *E. coli* MazF-mediated programmed cell death model (Aizenman *et al.*, 1996), which has been challenged by others (Pedersen *et al.*, 2002, Tsilibaris *et al.*, 2007, Ramisetty *et al.*, 2015, Ramisetty *et al.*, 2016). The discrepancies in cell death are likely caused by differences between the specific strains used, as the cell death effects were observed in *E. coli* MC4100 and not in MG1655 (Kolodkin-Gal & Engelberg-Kulka, 2008). The *mazEF* genes are adjacent to the *relA* locus that encodes the synthetase of the alarmone ppGpp, and it was determined that the MC4100 strain is RelA-deficient, which can obscure the cell death experiments (Ramisetty *et al.*, 2016).

1.2.1. Stabilization of mobile genetic elements

The first biological role discovered for TA systems was plasmid stabilization by the CcdB/CcdA system (Ogura & Hiraga, 1983). Plasmid maintenance is conferred by the differential stability of the toxin and the antitoxin. In plasmid-carrying cells, both the toxin and the antitoxin are produced, and the system is dormant. Upon plasmid loss, the antitoxin gets degraded preferentially and the toxin starts to inhibit cell growth (Tsuchimoto *et al.*, 1992, Van Melderen *et al.*, 1994, Lehnherr & Yarmolinsky, 1995). This limits the propagation of plasmid-free bacteria and allows their outcompetition by plasmid-carrying ones. This mechanism is often called post-segregational killing, suggesting that the TA toxins will kill the plasmid-free cells (Gerdes *et al.*, 1986b). However, cell killing has not been demonstrated by natively occurring amounts of toxins and remains an effect seen only when a higher-than-normal amount of toxin is present in the cells (Song & Wood, 2018).

Some chromosomal TA systems are known to have a similar effect as plasmid-borne modules: stabilizing their surrounding genomic regions. In *Vibrio cholerae*, the MosT/MosA system has been found to stabilize the ~100 kb integrative conjugative element SXT (Wozniak & Waldor, 2009). It has also been demonstrated that TA modules can stabilize superintegrons and otherwise expendable genomic regions (Szekeres *et al.*, 2007).

1.2.2. Modulation of bacterial stress tolerance

One of the most controversial fields of TA biology has been the link between TA systems and persister cell formation, which has been considered an important stress tolerance mechanism for prokaryotes. Persisters are a metabolically dormant subpopulation of bacteria that are therefore not susceptible to killing by antibiotics (Bigger, 1944, Balaban *et al.*, 2004). There is strong interest in studying persisters as they cause recurrent infections and can lead to the faster development of antibiotic resistance (Levin-Reisman *et al.*, 2017).

In *E. coli*, it was proposed that successive deletion of 10 TA modules decreases persister frequency (Maisonneuve *et al.*, 2011). The same group then proposed an elegant model where ppGpp synthesis leads to polyphosphate-dependent Lon activation, which degrades TA antitoxins, leaving the toxins to cause dormancy and persistence (Maisonneuve *et al.*, 2013). However, the authors later discovered that the effects attributed to TA system deletions were instead caused by prophage ϕ80 that had infected the multi-TA-deletion and other mutant strains used in establishing the persistence model (Harms *et al.*, 2017). Two independently constructed $\phi 80^{-}$ Δ10TA strains did not display elevated persister levels, thus disproving the TA-centric *E. coli* persistence theory (Harms *et al.*, 2017, Goormaghtigh *et al.*, 2018).

However, there is independent evidence that in some cases, TA systems are still implicated in bacterial persistence. In *Salmonella* Typhimurium, single deletions of 11 TA modules decrease intracellular persister occurrence in mouse macrophages about twofold on average and up to ~10-fold in two cases (Helaine *et al.*, 2014). The type I system toxin HokB is required for Obg-mediated persistence in *E. coli* but at the same time, not in *Pseudomonas aeruginosa* (Verstraeten *et al.*, 2015). Another type I system, TisB/IstR, also enhances *E. coli* persistence (Dörr *et al.*, 2010). The toxin MqsR has been repeatedly associated with persistence, although the observed effects are not always in unison. In *E. coli* MG1655 background, MqsR deletion had no effect on persistence (Shah *et al.*, 2006). On the other hand, the Keio collection deletant of MqsR from *E. coli* BW25113 caused a drop in persister levels (Kim & Wood, 2010). This could be explained by variations in experimental procedures, as there is evidence that the causal link between MqsR and persister formation is conditional. In this assay, the Keio collection MqsR deletion decreased persister levels only when the antibiotic killing was carried out using cells that had spent more than 12 hours in the stationary phase beforehand (Luidalepp *et al.*, 2011). This indicates that the link between TA systems and persister formation is finely tuned and may require particular experimental conditions.

TA systems are not constrained to persistence but are implied in several different stress responses as well. In particular, the *E. coli* MqsR/MqsA system has been studied in regard to stress situations. It was first linked to biofilm formation even before its characterization as a TA module, by the findings that the toxin MqsR is upregulated in biofilms (Ren *et al.*, 2004) and participates in biofilm induction by autoinductor 2 (González Barrios *et al.*, 2006). Later studies showed that the actual effector is the antitoxin MqsA, which atypically binds promoters of other genes besides its own locus (Brown *et al.*, 2009). Most notably, MqsA represses the stationary phase sigma factor RpoS (Wang *et al.*, 2011). Antitoxin degradation induced by oxidative stress activates RpoS synthesis, which in turn enhances cyclic-di-GMP synthesis, leading to decreased motility and increased biofilm formation (Wang *et al.*, 2011).

1.2.3. Defence against bacteriophages

TA modules play a role in the fight between bacteria and bacteriophages by participating in the abortive infection process. It is notable that limiting phage propagation is conferred by TA systems present both in plasmids and in chromosomes of bacteria. The most thoroughly studied abortive infection TA module is the type III ToxN/ToxI, which is carried on a plasmid in *P. atrosepticum* (Fineran *et al.*, 2009). Its presence lowers the plaquing efficiency of various native *Pectobacterium* phages (Fineran *et al.*, 2009) due to cellular RNA degradation, which hinders the phage infection cycle (Blower *et al.*, 2011a). ToxN/ToxI effects are not specific to *Pectobacterium*, as introducing the system on a plasmid can also protect other enteric bacteria against some of their phages (Fineran *et al.*, 2009). It is notable that the effect of ToxN on the cell is essentially reversible (Fineran *et al.*, 2009) and so at least here it is not a case of altruistic suicide, as was proposed for the *hok*/*sok* system (Pecota & Wood, 1996). Later, several other abortive infection loci have been determined to function as TA modules in different bacteria as well (Blower *et al.*, 2012, Otsuka & Yonesaki, 2012, Samson *et al.*, 2013, Dy *et al.*, 2014).

2. TA toxins' targets in the cells

With the exception of systems where the antitoxin transcriptionally regulates genes other than the TA locus itself (Wang *et al.*, 2011, Hu *et al.*, 2012), the toxin is responsible for the physiological effects of TA systems. As these systems are prevalent on plasmids and other mobile genetic elements that have the potential to migrate between bacterial species, it is not surprising that TA toxins target the most conserved and dogmatic cellular processes (Harms *et al.*, 2018). Among these, the most common target is the translational apparatus, while DNA replication and cell wall disruption are inhibited less often. It is speculated that translation is an evolutionarily favoured target, because its inhibition is relatively less toxic and allows for easier reversal of the effects in case of accidental toxin activation (Guglielmini & Van Melderen, 2011, Harms *et al.*, 2018). All the toxin targets discussed below are summarized in Figure 2.

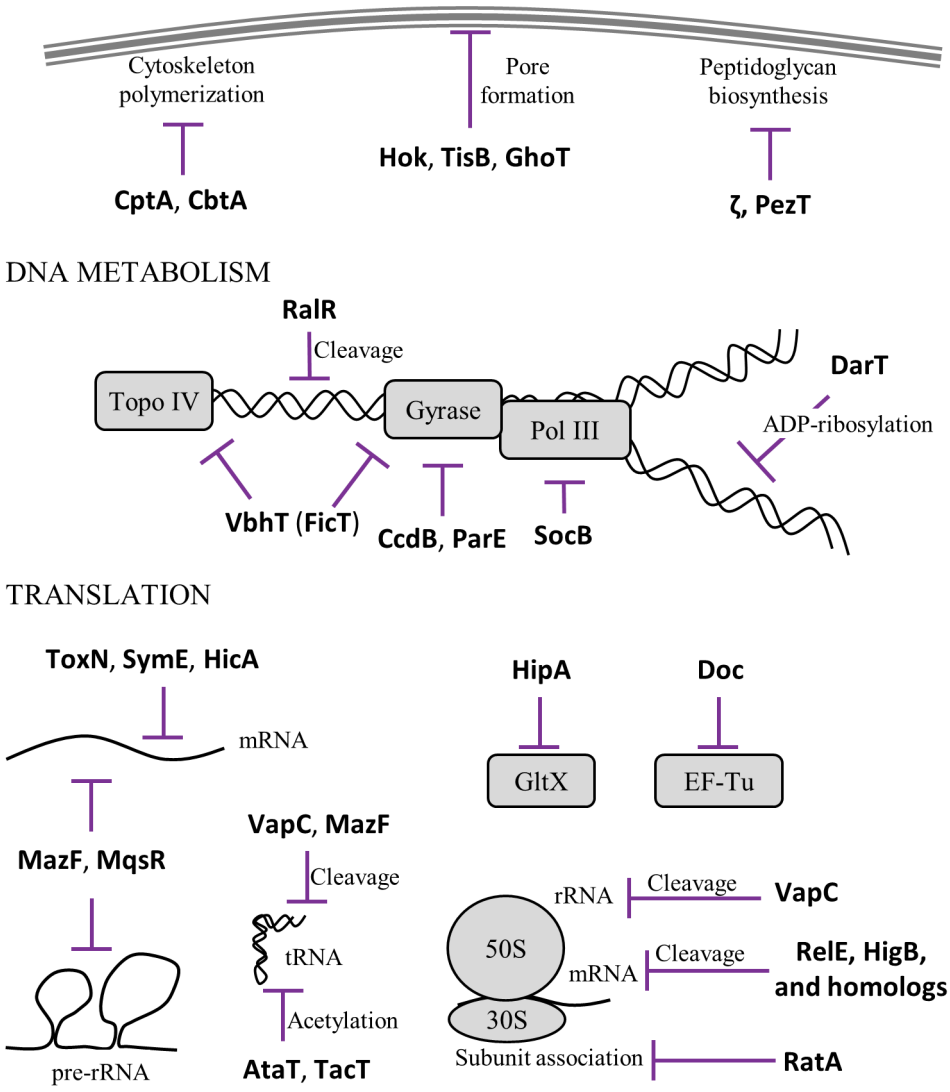

Figure 2. TA toxins' targets in the cell. Similarly acting toxins are grouped together. GltX – Glutamyl-tRNA synthetase.

2.1. Inhibition of DNA metabolism

TA toxins have been found to interfere with DNA metabolism enzymes and even to directly attack DNA. One of the earliest-studied toxins, CcdB (Ogura & Hiraga, 1983), was found to inhibit DNA gyrase (Miki *et al.*, 1992, Bernard & Couturier, 1992). CcdB is able to bind the GyrA subunit dimer and thus inhibit DNA supercoiling activity (Maki *et al.*, 1996). CcdB can also associate with the full $GyrA_2$-$GyrB_2$ heterotetramer in complex with DNA and trap the enzyme in the pre-religation step, causing DNA cleavage (Bernard *et al.*, 1993).

Later, the ParE toxins were found to have similar effects on DNA gyrase despite lack of sequence similarity with CcdB (Jiang *et al.*, 2002) and their clustering to a separate TA toxin superfamily (Anantharaman & Aravind, 2003). Similarly to CcdB, ParE interacts with free GyrA as well as with the $GyrA_2B_2$ complex and is also able to induce DNA cleavage by the gyrase complex (Yuan *et al.*, 2010). Although the CcdB and ParE toxins achieve the same results, their specific binding sites on GyrA are distinct, as ParE can interact with CcdB-saturated GyrA (Yuan *et al.*, 2010). Further underlining their difference, ParE requires ATP hydrolysis to induce DNA cleavage, which is not a necessity for CcdB (Yuan *et al.*, 2010).

Another family of toxins that interfere with DNA metabolism are the FIC domain-containing proteins. FIC enzymes canonically act as AMPylases (Yarbrough *et al.*, 2009), although some of them exhibit other activities. A notable exception to the rule is the TA toxin Doc that is instead a kinase (Castro-Roa *et al.*, 2013, Cruz *et al.*, 2014)(discussed further in 2.3). The first discovered FIC-fold AMPylase TA toxin, VbhT (Engel *et al.*, 2012) was found to adenylylate the DNA gyrase B-subunit GyrB and its paralog, the topoisomerase IV B-subunit ParE (not to be confused with the previously discussed toxins) (Harms *et al.*, 2015). ParE is likely the main target of FicT toxins, as three tested homologs reliably inhibited Topo IV whereas gyrase inhibition levels varied. The combined blocking of the two topoisomerases leads to a reversible arrest in cell growth (Harms *et al.*, 2015).

While disruption of topoisomerase activity is a widespread phenomenon in the TA world, several mechanisms of DNA metabolism inhibition by TA toxins are thus far characterized by single examples. The lone type VI toxin, *C. crescentus* SocB, is also currently unique in its function. SocB interacts with the β sliding clamp of DNA polymerase III and causes replication fork collapse, thus inhibiting DNA replication (Aakre *et al.*, 2013). In addition to disturbing DNA metabolism, there are two toxins that directly attack DNA itself. The *E. coli* type I toxin RalR is to date the only known TA toxin that functions as a nonspecific DNase and it was found to increase fosfomycin resistance (Guo *et al.*, 2014). The other toxin that attacks DNA is DarT from the recently described DarT/DarG system in *Mycobacterium tuberculosis* (Jankevicius *et al.*, 2016). DarT functions as a DNA ADP-ribosyl transferase and specifically modifies thymine residues in single-stranded DNA. This induces the SOS-response and inhibits DNA replication (Jankevicius *et al.*, 2016). Curiously, the

antitoxin DarG can, in addition to binding and inhibiting DarT, also enzymatically remove the ADP-ribosyl moiety from affected DNA. This presents an unusual mix between type II and IV TA systems.

2.2. Disruption of the cell envelope and cytoskeleton

The physical support structures of the bacterial cell also fall under attack by TA toxins. One of the earliest known targets is the inner membrane, which is disturbed by several type I system toxins and the type V toxin GhoT. The first such toxin described was Hok, a small protein with hydrophobic regions. Hok was found to associate with the cell membrane and abolish membrane potential (Gerdes *et al.*, 1986a). Cells killed by Hok display the distinct 'ghost cell' phenotype where the bacteria appear visually translucent in the centre of the cell (Gerdes *et al.*, 1986b). For the similarly-acting TisB toxin, it was determined that upon toxin overexpression, cellular ATP levels decrease and as a consequence, all major biosynthesis processes are quickly inhibited (Unoson & Wagner, 2008). *In vitro* experiments later showed that TisB forms anion-selective pores in the lipid bilayers, which can cause membrane depolarization (Gurnev *et al.*, 2012). Such activity is not limited to toxins of type I systems, as GhoT from the type V GhoT/GhoS system functions in the same way. GhoT is also a small protein with hydrophobic regions that forms membrane pores, causing ghost cell phenotype and lysis when overexpressed (Wang *et al.*, 2012). This is accompanied by a reduction in the proton motive force and ATP levels (Cheng *et al.*, 2014). It was additionally suggested that native amounts of GhoT do not kill the cells (Cheng *et al.*, 2014) and that the pore forming is transient (Kim *et al.*, 2018).

In addition to the membrane, the protective cell wall can be affected by toxins: the zeta family toxins, including ζ and PezT, inhibit the peptidoglycan biosynthesis process. They function as kinases and mostly phosphorylate the peptidoglycan precursor molecule uridine diphosphate-*N*-acetylglucosamine (UNAG), forming UNAG-3P. This reaction causes dual effects: first it lowers the UNAG pool available for peptidoglycan synthesis, and second, UNAG-3P acts as an inhibitor of the downstream enzyme MurA (Mutschler *et al.*, 2011). The ng_ζ1 toxin in *Neisseria gonorrhoeae* is an example of a zeta toxin with broader specificity, as it can phosphorylate several uridine diphosphate sugars that are precursors of peptidoglycan and lipopolysaccharide synthesis (Rocker *et al.*, 2018). In accordance with their molecular mechanism, it was found that the ζ toxin is bactericidal to gram-positive *Bacillus* but bacteriostatic to gram-negative *E. coli* (Zielenkiewicz & Ceglowski, 2005).

The type IV system toxin CbtA (YeeV) binds to and interferes with the cytoskeleton proteins MreB and FtsZ. CbtA inhibits FtsZ polymerization by blocking its GTPase activity. Interestingly, while CbtA inhibits the ATP-dependent polymerization of MreB, it does not abolish MreB ATPase activity (Tan *et al.*, 2011). It was suggested that CbtA instead blocks the interaction

between MreB and some of its accessory proteins (Tan *et al.*, 2011). Another type IV toxin, CptA, also inhibits FtsZ and MreB polymerization, although it is structurally distinct from CbtA. Moreover, CptA is localized in the inner cell membrane whereas CbtA is a soluble protein (Masuda *et al.*, 2012b).

2.3. The various possibilities of translation inhibition

Most of the studied TA toxins inhibit translation (Guglielmini & Van Melderen, 2011, Harms *et al.*, 2018). The variety of specific targets within the whole protein synthesis process and the specific mechanisms that the toxins employ is quite impressive.

2.3.1. Modification of translation factors and initiation inhibition

The first group of toxins to be discussed are enzymes that inactivate translation factors by either phosphorylating or acetylating them. The toxin Doc from the Doc/Phd module was originally thought to affect the 30S ribosomal subunit (Liu *et al.*, 2008) but was later shown to function as a kinase that phosphorylates elongation factor Tu (Castro-Roa *et al.*, 2013, Cruz *et al.*, 2014). Similarly to the discussed FicT toxins (see chapter 2.1), Doc is a FIC-domain protein but binds ATP in an inverted orientation compared to FicTs and therefore rather catalyses the transfer of a phosphate group instead of AMP (Castro-Roa *et al.*, 2013). Phosphorylated EF-Tu-GTP is unable to bind aminoacyl-tRNAs and thus cannot support translation (Castro-Roa *et al.*, 2013).

The same function, phosphorylating EF-Tu, was proposed for the toxin HipA (Schumacher *et al.*, 2009). However, these results were challenged by later works that instead showed phosphorylation of the glutamyl-tRNA synthetase GltX by HipA (Germain *et al.*, 2013, Kaspy *et al.*, 2013) and demonstrated no EF-Tu phosphorylation (Germain *et al.*, 2013). Phosphorylated GltX is unable to aminoacylate $tRNA^{Glu}$ (Germain *et al.*, 2013) and the uncharged tRNA accumulates in the bacterial cells, leading to activation of the stringent response (Kaspy *et al.*, 2013).

Quite recently, two GNAT-fold toxins were identified that acetylate tRNAs and thus inactivate them (Cheverton *et al.*, 2016, Jurėnas *et al.*, 2017). However, the *Salmonella* TacT and *E. coli* O157:H7 AtaT toxins differ in their specificity of tRNA amino acid N-acetylation. TacT modifies charged elongator-tRNAs and thus disrupts the aa-$tRNA^{aa}$:EF-Tu:GTP ternary complex formation (Cheverton *et al.*, 2016), blocking translation at the same stage as the EF-Tu kinase Doc (Castro-Roa *et al.*, 2013). AtaT, on the other hand, specifically targets the initiator Met-$tRNA^{fMet}$, N-acetylating the methionyl group before it can be converted to N-formylmethionine. This most likely prevents the tRNA from binding IF2 and thus forming a productive initiation complex (Jurėnas *et al.*, 2017).

Translation initiation was suggested as the primary target of the RatA toxin from *E. coli* (distinct from the type I antitoxin RatA (Silvaggi *et al.*, 2005)). This protein was found to associate with the ribosomal large subunit and inhibit its association with the small subunit (Zhang & Inouye, 2011). Still, RatA function as a classic TA toxin is somewhat unclear as no antitoxin activity could be detected for the adjacently-encoded RatB gene (Zhang & Inouye, 2011). In another study using the uropathogenic *E. coli* CFT073 where the locus was designated PasT (toxin) – PasI (antitoxin), PasI could reverse the toxic effects but at the same time, a strong expression of PasT was required for toxicity. A low-level expression of PasT was on the other hand shown to support bacterial growth during various stresses (Norton & Mulvey, 2012).

2.3.2. tRNA cleavage

A large number of TA toxins function as endo-RNases and altogether they are able to cleave every major RNA species in the cell. tRNA has been reported as the target of toxins belonging to two different families. It was first shown that two VapC proteins, one from a *Shigella flexneri* virulence plasmid and the other from *Salmonella* Typhimurium LT2, specifically cleave the initiator-tRNA^{fMet} on the 3'-side of the anticodon loop (Winther & Gerdes, 2011). Later, a thorough target search of 48 *M. tuberculosis* VapC homologs was performed by *in vivo* cross-linking. It was found that different VapC toxins mostly cleave a single specific tRNA or two isoacceptor-tRNAs. Constructing a phylogenetic tree of VapC homologs showed that their target preference correlates well with phylogeny, making it possible to predict the target of unstudied VapC proteins by sequence analysis (Winther *et al.*, 2016). The other toxin family that exhibits tRNase activity is MazF. One of the *M. tuberculosis* 9 MazF proteins, MazF-mt9, targets six elongator-tRNAs, including all lysine- and asparagine-accepting tRNAs, and cleaves their single-stranded loop regions (Schifano *et al.*, 2016). This is in contrast with the more specific action of VapC proteins that commonly target only one or two different tRNAs (Winther *et al.*, 2016). Additionally, given suitable experimental conditions, MazF-mt9 is able to cleave selected mRNAs. Still, the primary target of MazF-mt9 are most likely tRNAs, as substrate recognition seems to be based on both RNA sequence and structure (Schifano *et al.*, 2016).

2.3.3. rRNA cleavage

Even the ribosome itself is not safe from attack by TA toxins, which may target both the large and small subunits' rRNA. Some toxins of the MazF family, originally found to cleave mRNAs at ACA sequences without requiring the ribosome (Zhang *et al.*, 2003b), are also able to attack rRNAs. The *E. coli* MazF recognizes an ACA sequence at the 3' end of the 16S rRNA, which is accessible

both in 30S subunits and 70S ribosomes. It was originally demonstrated that cleavage at this site causes loss of the last 43 nucleotides that most importantly contain the anti-Shine-Dalgarno sequence (Vesper *et al.*, 2011). At the same time, it was shown that MazF cleaves the leader regions off a specific subset of mRNAs and it was shown *in vitro* that the MazF-cleaved ribosomes are able to translate those leaderless mRNAs (Vesper *et al.*, 2011). However, a recent global study of *E. coli* MazF cleavage sites did not detect appearance of leaderless mRNAs nor the specialized ribosomes. Instead, they show MazF-mediated cleavage of pre-rRNA and mRNAs, including those of ribosomal proteins, and suggest that the resulting block of ribosome biogenesis is the main cellular effect of MazF (Culviner & Laub, 2018).

Still, the link between MazF and rRNA cleavage in context of the ribosome may be true in other bacterial species, as a similar specificity towards the anti-Shine-Dalgarno region was shown for one of the nine *M. tuberculosis* MazF homologs, MazF-mt3. It is notable that even though the cleavage site is different, UCCUU, it is still found in the anti-SD region. Thus, MazF-mt3 has the potential to abolish regular SD-antiSD association and alter ribosome specificity (Schifano *et al.*, 2014). Additionally, MazF-mt3 was found to cleave 23S rRNA in helix/loop 70, although it is not certain whether this activity is limited to free 23S or applies to mature ribosomes as well (Schifano *et al.*, 2014). Another *Mycobacterium* toxin, MazF-mt6 has a very similar cleavage site, UUCCU, which is also present in the 23S helix/loop 70 but not in 16S rRNA. MazF-mt6 cleaves this site in free 50S subunits and renders them deficient in subunit association (Schifano *et al.*, 2013).

M. tuberculosis harbours 48 VapC/VapB TA modules (Winther *et al.*, 2016) and two of them, VapC20 and VapC26, cleave 23S rRNA in the sarcin-ricin loop, effectively inhibiting translation (Winther *et al.*, 2013, Winther *et al.*, 2016). For VapC20, it was demonstrated that the secondary structure of the sarcin-ricin loop in context of the 70S ribosome is required for cleavage (Winther *et al.*, 2013). Interestingly, these VapCs cleave rRNA in precisely the same position as α-sarcin, even though the molecular mechanisms differ between the enzymes (Winther *et al.*, 2013).

Pre-rRNA is not yet shielded by r-proteins and packed as tightly as rRNA in complete ribosomes and therefore it presents an easier target for degrading enzymes. It is thus perhaps not surprising that the *E. coli* toxins MazF and MqsR, identified as ribosome-independent mRNases (Zhang *et al.*, 2003a, Yamaguchi *et al.*, 2009) can cleave pre-16S and pre-23S rRNAs as well (Mets *et al.*, 2017, Culviner & Laub, 2018). These defective rRNAs are not immediately excluded from the ribosome assembly process and cause the accumulation of aberrant ribosomal subunits. Still, it was hypothesized that these toxins play a role in recycling surplus rRNA when stress conditions require downregulating the ribosome synthesis machinery (Mets *et al.*, 2017).

2.3.4. mRNA cleavage

Among the RNase toxin families, mRNA is by far the most common target (Masuda & Inouye, 2017). There are several likely reasons behind this: mRNAs are present in large amounts in growing cells and they are more accessible to toxins when compared to rRNAs that are shielded by proteins and extensive secondary structures. In comparison with tRNAs that are also found in large amounts, mRNAs present a massively wider variety of sequences that different toxins can recognize and are also less structured, again making them more accessible.

2.3.4.1. Ribosome-independent mRNA cleavage

The HicA/HicB system toxin HicA was first suggested to partake in RNA metabolism based on bioinformatic analysis (Makarova *et al.*, 2006). It was later experimentally confirmed that HicA induction leads to digestion of several mRNAs without apparent sequence specificity (Jørgensen *et al.*, 2009). HicA was also shown to cleave tmRNA and its non-translatable mutants, which illustrated that the cleavage is ribosome-independent. However, that work did not distinguish whether HicA itself possesses RNase activity or indirectly induces some other enzyme that cleaves the RNAs (Jørgensen *et al.*, 2009). Later studies with the *Yersinia pestis* HicA3 toxin showed that it cleaves mRNA *in vitro* without added cellular factors, confirming its role as a ribosome-independent RNase (Bibi-Triki *et al.*, 2014).

The toxin MqsR from the MqsR/MqsA system is classified as a member of the RelE superfamily according to its structure (Brown *et al.*, 2009). RelE toxins function by cleaving actively translated mRNAs on the ribosome (Christensen & Gerdes, 2003) and structure analysis suggested the same function for MqsR (Brown *et al.*, 2009). However, MqsR was determined to cleave mRNAs specifically at GCU or GCA sites regardless of reading frame. Interestingly, the exact cutting site is not fixed and cleavages were found both directly before or after the G nucleotide (Yamaguchi *et al.*, 2009, Christensen-Dalsgaard *et al.*, 2010). Translation-independence was confirmed by *in vitro* reactions, where MqsR is active without the presence of ribosomes (Yamaguchi *et al.*, 2009). The stronger recognition site, GCU, is missing from only 14 *E. coli* mRNAs (Yamaguchi *et al.*, 2009). These include 6 genes that are induced during biofilm formation (Yamaguchi *et al.*, 2009) and perhaps more notably, two genes encoding TA toxins: the type I RalR, a DNase (Guo *et al.*, 2014), and the membrane-disturbing type V GhoT (Wang *et al.*, 2012, Wang *et al.*, 2013). MqsR expression was additionally shown to induce the DNA replication-inhibiting protein CspD (Kim *et al.*, 2010), indicating that this system is well integrated into the *E. coli* metabolism inhibition network. Still, the *in vivo* importance of these links is not clear as for example, 4 hours of MqsR expression does not lead to the ghost cell phenotype in *E. coli* (Kasari *et al.*, 2010), as would be expected in case of GhoT activation (Wang *et al.*, 2012).

Perhaps one of the best studied TA toxins is MazF. The *E. coli* MazF (MazF-ec)/MazE protein pair was shown to function as a TA module where MazF overexpression stops bacterial growth and reduces the number of viable cells (Aizenman *et al.*, 1996). It was later determined that MazF-ec blocks protein synthesis by cleaving mRNAs specifically at ACA sequences and that the ribosome is not required for this process (Zhang *et al.*, 2003b). Later works with MazF homologs from other bacteria have shown that their recognition sequence length can vary from 3 to 7 nucleotides. This determines whether the particular MazF has a broad mRNA degrading effect or if it only targets a smaller subset of mRNAs, which might enable a more specific effect on bacterial physiology (Yamaguchi *et al.*, 2011). In *E. coli*, the ACA sequence is missing in only 51 genes, which include two cold-shock proteins (Yamaguchi & Inouye, 2013). Interestingly, identification of 13 proteins that are still translated after MazF induction showed that each of their mRNAs does contain ACA sequences and therefore the *in vivo* cleavage has additional layers of control in addition to primary RNA sequence (Amitai *et al.*, 2009). While the authors group these proteins with regard to their participation in MazF-mediated cell death, these experiments were performed in *E. coli* MC4100 (Amitai *et al.*, 2009), which was later determined to be RelA-deficient and thus not a reliable platform for cell death experiments (Ramisetty *et al.*, 2016). The other extreme is the archaeal 7-nucleotide UUACUCA-specific MazF from *Haloquadra walsbyi* (MazF-hw) (Yamaguchi *et al.*, 2012). MazF-hw was predicted to cut only 183 out of the 2610 protein-encoding mRNAs in *H. walsbyi*. Notably, the only gene that contains 3 MazF-hw recognition sites encodes a putative rhodopsin transcriptional activator, suggesting that MazF-hw induction can downregulate the light-driven protein pumps of the archaeon (Yamaguchi *et al.*, 2012).

The MazF proteins with their variety of RNA specificity can be useful tools in biotechnological applications. One example is protein production in *E. coli* using the ACA-specific MazF-ec and engineered ACA-free mRNA for the protein of interest. Inducing MazF-ec degrades almost all other cellular mRNAs, leading to a very high-yield synthesis of the desired protein (Suzuki *et al.*, 2007).

Another group of toxins that function as translation-independent mRNases are those belonging to type III TA systems (Goeders *et al.*, 2016). The ToxN proteins from several microbes cleave mRNAs at specific 4- or 5-nucleotide A-rich sequences (Short *et al.*, 2013). This is in line with their functional role in processing the corresponding antitoxins' RNAs into active fragments (Blower *et al.*, 2011a, Short *et al.*, 2013). Interestingly, despite low sequence similarity, the type III toxin ToxN is structurally similar to the type II systems' MazF-CcdB superfamily, which includes both RNases and DNA gyrase inhibitors (Blower *et al.*, 2011b).

The type I system toxin SymE is an intriguing example of an RNase toxin as it was found to be homologous to MazE, which is instead an antitoxin. The *E. coli* SymE sequence, however, revealed several additional amino acids

compared to MazE. These included several polar residues located in a region where they could affect nucleic acid binding and hydrolysis. In accordance with these predictions, SymE induction caused degradation of several mRNAs, although the distinction between direct and indirect activity remained untested (Kawano *et al.*, 2007).

2.3.4.2. Ribosome-dependent mRNA cleavage – RelE

Almost all toxins that depend on the ribosome and thus cut only actively translated mRNAs are RelE homologs from the RelE/ParE superfamily (Anantharaman & Aravind, 2003, Pandey & Gerdes, 2005). The first RelE/RelB system was identified as a TA module in *E. coli* where it was seen that producing RelE in an *in vitro* transcription-translation system inhibits the reaction (Gotfredsen & Gerdes, 1998). Soon, translation was specified as the target by measuring the replication, transcription and translation rates upon RelE induction (Christensen *et al.*, 2001). More precise experiments revealed that RelE must function in a catalytic fashion as even a 1:20 toxin:ribosome ratio abolished 90% of translation (Pedersen *et al.*, 2003). Testing with individual components of the *in vitro* translation reaction indicated that RelE targets ribosomes post-initiation. Observed codon-dependency of the inhibition lead the investigators to examine the RelE-affected mRNA, which was found to be cleaved at specific sites (Pedersen *et al.*, 2003). *In vivo* experiments confirmed that RelE cuts mRNA most often between the 2nd and 3rd nucleotides of codons and that G or C in the 3rd but not in the 2nd position results in more efficient cleavage (Christensen & Gerdes, 2003). At the same time, free mRNA is not a target for RelE, highlighting its strictly ribosome-dependent mode of action (Pedersen *et al.*, 2003). Intriguingly, the whole ribosome is not required for RelE activity. The toxin was shown to cleave mRNA on an *in vitro* assembled 30S initiation complex, although with lower efficiency (Pedersen *et al.*, 2003).

E. coli RelE structure was first solved by NMR for a less toxic R81A/R83A mutant in both free and RelB-bound states, which revealed that the potential RelE active site resembles that of RNase SA (Li *et al.*, 2009). Soon after, the crystal structure of RelE bound to the ribosome in pre- and postcleavage state was reported (Neubauer *et al.*, 2009). RelE was found in the ribosomal A-site, in contact with conserved regions of 16S rRNA. There, RelE pulls the mRNA into its active site and attacks the linkage between the 2nd and 3rd nucleotides of the A-site codon, resulting in new RNA 5'-OH and 2'–3' cyclic phosphate ends (Neubauer *et al.*, 2009). The structure also shed light on RelE's codon preference and suggests a pyrimidine-purine-G pattern: a smaller pyrimidine in the first position allows the mRNA easier access to the RelE active site, a larger purine in the second position may give a more stable stacking interaction with RelE Y87, and it is possible that the toxin directly interacts with the 3rd position guanosine (Neubauer *et al.*, 2009). However, a later transcriptomic study used ribosome profiling to show no specific nucleotide preference for the first

nucleotide, a C favoured and G avoided in the 2nd position, and a strong preference for G and especially not C in the 3rd position of the cleaved codons (Hwang & Buskirk, 2017).

2.3.4.3. Ribosome-dependent mRNA cleavage – YoeB

The *E. coli* K-12 genome contains six additional RelE/RelB homologs: YoeB/YefM, YafO/YafN, YafQ/DinJ, HigB/HigA, YhaV/PrlF, and MqsR/MqsA (Harms *et al.*, 2018). Among these, MqsR has been well established as a ribosome-independent RNase (Yamaguchi *et al.*, 2009, Christensen-Dalsgaard *et al.*, 2010). The others all share a ribosome-associated mode of action but in some cases, it is not clear whether they can exhibit an intrinsic RNase activity as well. All the ribosome-associated RelE family proteins share a common tertiary fold (Figure 3) despite low sequence conservation. Nevertheless, closer investigation of their specific activity and catalytic mechanisms reveals several differences between the RelE homologs.

First study regarding the action of the toxin YoeB determined that it cuts only translated regions of mRNA and tmRNA (Christensen *et al.*, 2004). However, an *in vitro* experiment showed that free YoeB is capable of RNA degradation without the need for translation (Kamada & Hanaoka, 2005). This difference was explained by the finding that YoeB possesses a complete RNase fold similar to RNase SA while RelE is missing the necessary conserved catalytic residues (H85 and E54 in RNase SA) and therefore relies on the ribosome to induce RNA hydrolysis (Kamada & Hanaoka, 2005, Li *et al.*, 2009). However, YoeB most likely acts in a ribosome-dependent fashion *in vivo*, as evidenced by its adherence to reading frame in experiments with frameshifted and untranslatable model mRNAs (Christensen-Dalsgaard & Gerdes, 2008). YoeB was found to have similar cleavage specificity to RelE on some tested mRNAs but exhibited a stronger preference for cutting the first and last codons of the ORF, potentially due to the relative slowness of translation initiation and termination in comparison to elongation (Christensen-Dalsgaard & Gerdes, 2008).

Interestingly, co-crystallization of YoeB with the ribosome shows that the toxin binds to the ribosomal A-site as a dimer (Feng *et al.*, 2013). Still, it is not known whether a toxin dimer is also a relevant conformation *in vivo* or if it is just a crystallization artifact (Feng *et al.*, 2013). The toxin monomer facing mRNA makes contacts with both 16S and 23S RNA and the ribosomal protein S12 (Feng *et al.*, 2013), unlike RelE, which only contacts 16S (Neubauer *et al.*, 2009). Additionally, the two toxins interact with the mRNA differently and cause it to obtain different conformations in the A-site (Neubauer *et al.*, 2009, Feng *et al.*, 2013). Unlike RelE, which produces a 2'–3' cyclic phosphate end after cleavage, YoeB produces a 3'-phosphate end similarly to RNase T1 (Feng *et al.*, 2013). The 3D structure also suggests a more relaxed codon selection for YoeB in comparison with RelE (Feng *et al.*, 2013). This agrees with the

observation that in two tested cases, YoeB preferentially cuts mRNAs very closely following the start codon and does not substantially affect the stability of the whole mRNA (Zhang & Inouye, 2009).

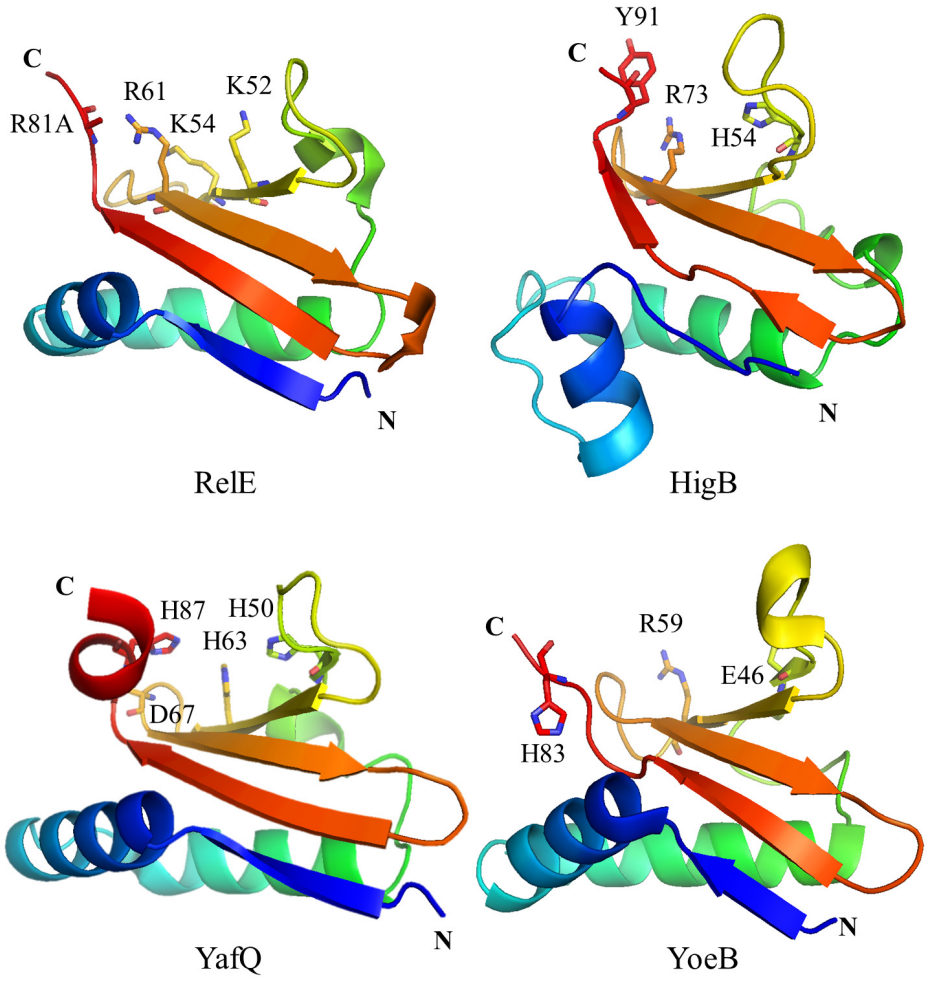

Figure 3. Structures of RelE and homologous toxins. N-termini are blue and C-termini are red. Catalytically relevant amino acids as described by (Neubauer *et al.*, 2009, Griffin *et al.*, 2013, Schureck *et al.*, 2016b, Maehigashi *et al.*, 2015, Kamada & Hanaoka, 2005) are shown as sticks. The structures of *E. coli* RelE (PDB: 4FXE), YafQ (PDB: 4ML0), and YoeB (PDB: 2A6Q) are from the respective toxin-antitoxin complexes. *P. vulgaris* HigB (PDB: 4ZSN) structure is from its complex with the ribosome.

2.3.4.4. Ribosome-dependent mRNA cleavage – YafQ, YhaV, and YafO

The YafQ toxin resembles YoeB in retaining the catalytic histidine and glutamic acid residues that are potentially used for self-sufficient RNase activity (Kamada & Hanaoka, 2005). However, it was later demonstrated that while the histidine residues do play a role in YafQ toxicity, the glutamic acid is functionally replaced by an aspartic acid residue (Figure 3), (Maehigashi *et al.*, 2015). YafQ blocks translation (Motiejūnaitė *et al.*, 2007) and was found to cut mRNAs both *in vitro* in the absence of ribosomes and *in vivo* in a ribosome-dependent manner (Prysak *et al.*, 2009). Ribosome-dependence is evidenced by its binding to the ribosome and a strict mRNA recognition sequence of an in-frame AAA codon followed by a G or an A: out-of-frame AAA-G/A is not cleaved. As in the case of RelE, YafQ cleaves the mRNA between the 2nd and 3rd positions of the codon (Prysak *et al.*, 2009). However, conflicting results have been reported regarding YafQ activity without ribosomes. Another work showed that YafQ is active *in vitro* without added ribosomes and also demonstrated new *in vivo* cleavage sites (Armalytė *et al.*, 2012). A later study, on the other hand, shows no RNA cleavage activity for standalone YafQ and suggests a strictly ribosome-dependent mode of action (Maehigashi *et al.*, 2015). The actual cleavage mechanism on the ribosome is slightly different from both RelE and YoeB, as unlike RelE and YoeB that leave the cleaved mRNA with a 2'–3' cyclic phosphate (Neubauer *et al.*, 2009) or a 3'-phosphate (Feng *et al.*, 2013), respectively, YafQ produces 3'-OH ends (Maehigashi *et al.*, 2015).

The *E. coli* YhaV toxin is similar to the RelE family of ribosome-dependent mRNAses with the exception of a 20-amino acid insertion roughly in the middle of the protein. Like RelE, YhaV lacks the catalytic histidine and glutamic acid residues characteristic to RNase SA but retains conserved arginines that are necessary for RelE activity (Schmidt *et al.*, 2007). This implies a ribosome-dependent mode of action for YhaV as well. Yet, YhaV was found to efficiently and without apparent specificity cleave 16S and 23S rRNAs from purified total RNA *in vitro* (Schmidt *et al.*, 2007). However, that study did not test whether complete ribosomes would also be targeted by YhaV or whether ribosome binding modulates YhaV activity (Schmidt *et al.*, 2007). A later work showed on the contrary that YhaV is active *in vitro* only in the presence of ribosomes and that the *in vivo* detected cleavage sites imply codon-dependence, with most cuts occurring between two codons (Choi *et al.*, 2017). Therefore, it is most likely that YhaV is similar to other RelE homologs and acts as a ribosome-dependent mRNase *in vivo*.

YafO is a relatively less studied RelE homolog. Upon induction, a reduction in overall amounts of specific mRNAs were detected (Zhang *et al.*, 2009), as well as specific cleavage only in the translated regions of mRNAs (Zhang *et al.*, 2009, Christensen-Dalsgaard *et al.*, 2010). As is common for RelE family toxins, the cleavages were mostly localized between the 2nd and 3rd nucleotides of codons (Christensen-Dalsgaard *et al.*, 2010).

2.3.4.5. Ribosome-dependent mRNA cleavage – HigB

The HigB/HigA system was first described as a toxin-antitoxin pair in the Rts1 plasmid from *Proteus vulgaris* (Tian *et al.*, 1996b). Evolutionary analysis later revealed that HigB toxins are closely related to RelE proteins but distinguished by the structure of the operon: *relE* is found after the antitoxin gene *relB* whereas *higB* is the first gene of the operon, preceding the antitoxin gene *higA* (Pandey & Gerdes, 2005). The Rts1 HigB and two *V. cholerae* HigB proteins were shown to mediate codon-dependent mRNA cleavage *in vivo* with the resulting cleavage patterns similar to RelE (Christensen-Dalsgaard & Gerdes, 2006). Another study found more cleavage sites of Rts1 HigB on the widely analysed *lpp* mRNA and suggests a less strict adherence to reading frame with short A-rich stretches being the general target (Hurley & Woychik, 2009). Like RelE, HigB does not degrade mRNA *in vitro* without translating ribosomes. In concert with that, HigB was found to be associated with the ribosome, specifically the 50S subunit (Hurley & Woychik, 2009). The mRNase activity was determined to depend on a highly conserved histidine residue in the C-terminus of HigB, as its mutation to glutamine abolished HigB toxicity (Hurley & Woychik, 2009).

The *P. vulgaris* HigB has by now been the subject of a thorough structural analysis. In agreement with HigB clustering into the RelE superfamily (Pandey & Gerdes, 2005), the first structure showing the HigB-HigA complex revealed that HigB adopts a very similar fold to the RelE/YoeB proteins (Schureck *et al.*, 2014). Crystallization of HigB on the ribosome showed that HigB, as expected, occupies the ribosomal A-site (Schureck *et al.*, 2015). The mRNA is bent towards the toxin, allowing for codon reading and its subsequent cleavage. According to these structures that feature an AAA codon in the A-site, HigB does not contact the 1st nucleotide of the codon, suggesting that codons starting with any nucleotide can be cleaved (Schureck *et al.*, 2015). The 2nd position is read more carefully, by hydrogen bonding between the adenine and HigB. The 3rd adenine is curiously fit in a nucleotide-specific pocket formed by HigB and 16S rRNA (Schureck *et al.*, 2015). In agreement with structural data, cleavage kinetics of codons based on AAA and its substitution variants show that the 1st position is indeed the most flexible and only UAA is cleaved markedly less efficiently (Schureck *et al.*, 2015). The 2nd position is more sensitive to substitutions, with even lower k_{obs} values measured for AGA and AUA than for UAA. In the 3rd position, substituting the adenine for any other three nucleotides lowers the cleavage efficiency about 10-fold, illustrating the strictest control at the last position of the codon. It was shown that a conserved HigB residue, N71, is largely responsible for forming the adenine-specific pocket (Schureck *et al.*, 2015). HigB catalysis is mediated by 4 essential amino acids: H54 and an D-Y-H triad in the extreme C-terminus (amino acids 90–92) (Schureck *et al.*, 2016b). H54 is proposed to act as a general base, attacking the 2'-OH of the 2nd adenine of the codon. The non-essential R73 likely stabilizes the transition state, while

Y91 acts as the general acid to complete the reaction. D90 and H92 likely assist in properly orienting Y91 for catalysis (Schureck *et al.*, 2016b).

As was shown for RelE (Pedersen *et al.*, 2003), HigB is also not strictly dependent on the 70S ribosome and can attack mRNA in the 30S initiation complex *in vitro* (Schureck *et al.*, 2016a). Crystal structures of this complex lacked mRNA due to crystal packing artifacts but nevertheless revealed that HigB employs two sets of basic residues for binding the 30S subunit. This suggests that HigB may target the initiation step in addition to translation elongation *in vivo* (Schureck *et al.*, 2016a).

3. Temperature-sensitive TA systems

Temperature-dependence is not a common trait among TA systems, but some instances have been reported where either high or low temperatures modulate TA activity. Heat shock was shown to decrease the level of toxin mRNA in the type I BsrG/SR4 system of *Bacillus subtilis* (Jahn *et al.*, 2012). BsrG is a small hydrophobic peptide that localizes to the cell membrane and disturbs cell envelope biosynthesis whilst not reducing membrane permeability and cell energy production (Jahn *et al.*, 2015). BsrG temperature dependence was seen at a shift from 37 °C to 48 °C. The higher temperature reduces the toxin mRNA half-life approximately 3.5-fold whilst the antitoxin RNA SR4 is degraded only 30% faster. However, the molecular reasons for this stability change have not been determined (Jahn *et al.*, 2012).

On the other hand, elevated temperature causes the activation of the *E. coli* ribosome-associated mRNase YoeB (Janssen *et al.*, 2015). Raising the temperature from 37 °C to 42 °C induced YoeB-dependent cleavage of an *in vivo* expressed recombinant model mRNA (Janssen *et al.*, 2015). Again, the exact mechanism for the activation is not known. It was determined that while the Lon protease is responsible for degrading YefM, the antitoxin that inhibits YoeB, the heat shock does not increase the amount of Lon in the cells (Janssen *et al.*, 2015). Also, the *in vivo* relevance of this activation is unclear as no temperature- and YoeB-dependent degradation of endogenous *lpp* mRNA was detected (Janssen *et al.*, 2015), even though this mRNA is a known substrate for YoeB (Christensen-Dalsgaard & Gerdes, 2008, Zhang & Inouye, 2009). In agreement with these data, YoeB does not affect the growth rate of the heat-shocked bacteria (Janssen *et al.*, 2015). Instead, the authors suggest that YoeB may perform a quality control function by recycling ribosomes that have stalled due to heat stress (Janssen *et al.*, 2015).

Lower than optimal growth temperatures also affect some TA modules. Unlike for high temperature, all the known cases of cold-affected TA systems result in the toxin being more active. One such example is the unusual *E. coli* EzeT protein. EzeT is a curious example of a zeta toxin and its epsilon antitoxin fused together into a single polypeptide (Rocker & Meinhart, 2015). Despite the continually present antitoxin domain, EzeT displays low UNAG kinase activity

in vitro (Rocker & Meinhart, 2015), as would be expected of zeta family toxins (Mutschler *et al.*, 2011). Expressing only the EzeT C-terminal toxin domain (EzeTΔN83) revealed that it is not toxic at 37 °C and becomes successively more active at temperatures below 30 °C. In this case, the sensitivity is thought to arise from the low thermostability of EzeT, as the full protein's melting temperature was measured to be 38 °C (Rocker & Meinhart, 2015).

The other known TA system more active in colder conditions is the previously discussed *E. coli* YafQ/DinJ (Zhao *et al.*, 2016). It was found that deletion of the antitoxin gene *dinJ* reduced cellular metabolism at 18 °C but not at 37 °C. Quantitative PCR showed that the amount of *yafQ* mRNA does not change at the low temperatures, which indicates that the cold increases YafQ toxicity. Searching for toxicity modulators by transposon mutagenesis revealed 5 genes that restored growth in the cold when disrupted. These encode the RNA polymerase recycling protein RapA, the nucleoid-associated protein HU α subunit, RNase PH, and two proteins of the glycine cleavage system, GcvT and GcvP. The authors provide possible explanations on how the first three could affect YafQ toxicity by modulating RNA metabolism while the role of the Gcv proteins' disruption remains enigmatic (Zhao *et al.*, 2016).

4. Toxin-antitoxin connections to chaperones: the tripartite systems

Recent works suggest that the activation of some TA systems is linked to cellular chaperones. Perhaps the best-known instances are the tripartite toxin-antitoxin-chaperone (TAC) systems, first described in *M. tuberculosis* (Bordes *et al.*, 2011). It was found that in the *higB-higA-Rv1957* operon, the last gene encodes a SecB chaperone homolog. Ectopic expression of the toxin HigB in *E. coli* suppresses bacterial growth, and concomitant expression of neither the antitoxin HigA nor SecB counter HigB toxicity. Only when HigB is expressed along with both HigA and SecB are the cells able to form colonies on solid medium. It was demonstrated that the chaperone SecB interacts with HigA to promote its stability and solubility (Bordes *et al.*, 2011). A bioinformatic search for TAC systems revealed their presence in several gram-negative and -positive bacteria. TAC modules were found in 7 different phyla but with each represented by only a few species (Sala *et al.*, 2013). The chaperone-associated TA systems are not limited to HigB/HigA modules, as also MqsR/MqsA and HicA/HicB systems were found together with SecB homologs (Sala *et al.*, 2013).

Reasons for the chaperone addiction were elucidated when sequence alignments revealed that antitoxins from TAC modules differ from their TA homologs by a variable C-terminal extension (Bordes *et al.*, 2016). This terminus was shown to be necessary for SecB binding to the *M. tuberculosis* HigA. Furthermore, fusing the HigA 42 amino acid C-terminus to an unrelated model protein resulted in its binding with SecB as well (Bordes *et al.*, 2016). Variability of this domain, termed ChAD (Chaperone Addiction Domain), results in the

requirement for a specific chaperone, as SecBs from different TAC modules are either less effective or altogether unable to control non-cognate TAC antitoxins (Bordes *et al.*, 2016).

5. Pseudomonas putida TA systems

Pseudomonas putida is a ubiquitous soil bacterium that is commonly found on plant roots where it forms biofilm and promotes plant growth (Espinosa-Urgel *et al.*, 2000, Espinosa-Urgel *et al.*, 2002). The ~6.2 Mbp genome of the laboratory strain KT2440 encodes a variety of metabolic pathways that enable the bacterium to thrive in different conditions (Nelson *et al.*, 2002). The ability to digest toxic compounds such as phenolics makes *P. putida* a potential bioremediation tool (Zuo *et al.*, 2015, Belda *et al.*, 2016). Given that the conditions in the soil are quite fluctuating over time and that TA modules are more prevalent in free-living bacteria (Pandey & Gerdes, 2005), it is not surprising that *P. putida* KT2440 genome is predicted to contain up to 15 TA loci (Xie *et al.*, 2018). However, only 3 of these have been experimentally studied: MqsR/MqsA (Sun *et al.*, 2017), MazF/MazE (Miyamoto *et al.*, 2016) and the subject of this thesis, the HigB/HigA homologous GraT/GraA (Tamman *et al.*, 2014, Tamman *et al.*, 2016, Ainelo *et al.*, 2016, Talavera *et al.*, 2017).

The MqsR/MqsA locus is thus far characterized in one study. It was confirmed as a functional TA locus with activity in both *P. putida* and *E. coli*. The system was found to affect biofilm formation, although curiously the deletion of either MqsR, MqsA or the whole system all similarly reduced the amount of biofilm. MqsR was also linked to increased persistence, as the deletion of the antitoxin significantly increased survival under ciprofloxacin stress. However, the mode of action of MqsR was not investigated in this work (Sun *et al.*, 2017).

Conversely, the *P. putida* MazF/MazE system was investigated in a mechanistic sense. Miyamoto and colleagues developed a massive parallel sequencing methodology for determining the cleavage specificity of mRNases and used this system as an example of a RNase with unknown specificity (Miyamoto *et al.*, 2016). First it was confirmed that the proteins are indeed a TA pair where MazF degrades RNA and MazE counteracts its effect (Miyamoto *et al.*, 2016). The parallel sequencing method was validated on *E. coli* MazF where it successfully identified the known ACA consensus (Zhang *et al.*, 2003b, Miyamoto *et al.*, 2016). Analysing the *P. putida* MazF revealed that its recognition site is UAC and the cleavage occurs between the U and A nucleotides. It was interestingly pointed out that connection between different MazF recognition sequences and the similarity of the respective toxins is low. The *E. coli* and *P. putida* MazF toxins are only 34.8% identical and have different cleavage motifs, which is not surprising (Miyamoto *et al.*, 2016). At the same time, *P. putida* MazF and the *M. tuberculosis* MazF-mt1 are even less similar proteins with 28.8% identity yet they share the UAC recognition motif and both cleave between U and A (Miyamoto *et al.*, 2016, Zhu *et al.*, 2006).

THE AIMS OF THE THESIS

Toxin-antitoxin systems in bacterial chromosomes are fascinating due to their counterintuitive ability to poison the host cell itself and the potential physiological reasoning behind retaining such modules. The interest in the *Pseudomonas putida* PaW85 GraT/GraA TA system was sparked by previous studies on glucose-dependent autolysis of *P. putida* strains that are deficient in the ColR-ColS two-component signalling system. A transposon mutagenesis screen searching for lysis-suppressing mutants revealed among others an insertion in the PP_1585 locus, encoding a putative antitoxin gene (Putrinš *et al.*, 2011). Given that antitoxin deletions leave the corresponding toxin uninhibited and are thus often difficult or impossible to construct (Shah *et al.*, 2006, Budde *et al.*, 2007), it was intriguing to find an antitoxin disruption that improved the condition of the *colR*-mutant bacteria (Putrinš *et al.*, 2011). Active discussion regarding the action of chromosomal TA systems as stress response regulators (Hayes, 2003, Magnuson, 2007, Van Melderen, 2010, Wang & Wood, 2011, Gerdes & Maisonneuve, 2012) further increased our interest in the subject.

According to the genome annotation, the antitoxin gene PP_1585 is preceded in an operon by PP_1586, encoding a putative toxin. Based on this knowledge and during the course of the work, the following aims were outlined:

- To determine whether these genes encode a functional toxin-antitoxin pair
- To discover the molecular target of the toxin
- To investigate the cellular response to a near-native amount toxin

RESULTS AND DISCUSSION

6. The toxin GraT, encoded by PP_1586, inhibits cell growth at low temperatures (Ref I)

To investigate whether the putative toxin-antitoxin operon is functional, we constructed single deletions of both the antitoxin PP_1585 (Δ*graA*), the toxin PP_1586 (Δ*graT*) and the whole operon (Δ*graTA*). As a by-product, likely generated by a PCR polymerase error, we obtained an antitoxin deletion variant that harboured a point mutation in the toxin that resulted in an E80G amino acid substitution. This strain ($\Delta graA_{T(E80G)}$) was included in initial experiments to find out if the substitution has any effects on toxin activity.

The first bacterial cultivations showed that the Δ*graA* strain grows slightly slower than the wild-type (Ref I, Fig. 1 B). The slower growth is caused by the effects of the toxin GraT, as the Δ*graT* and Δ*graTA* strains behave exactly like the wild-type. Additionally, the $\Delta graA_{T(E80G)}$ strain showed no growth inhibition, indicating that the accidentally-generated E80G substitution is enough to abolish toxicity (Ref I, Fig. 1 B). This knowledge allowed us to use the $\Delta graA_{T(E80G)}$ strain as a control in subsequent experiments to verify whether antitoxin deletion effects are caused by the toxin or other pathways that might involve the antitoxin.

Subsequent cultivation at different temperatures revealed a striking temperature-dependent growth inhibition in the Δ*graA* strain. On solid medium, incubating Δ*graA* at 25 °C instead of the *P. putida* default growth temperature of 30 °C results in minuscule colonies after 24 hours. At 20 °C, the Δ*graA* strain is unable to form colonies in 72 hours (Ref I, Fig. 1 B). Measuring generation times in liquid media shows that all the deletion strains behave exactly like the wild-type at 37 °C (Ref I, Table 2). At 30 °C, the Δ*graA* strain is already 1.3 times slower than the wild-type. As the temperature drops, the difference increases, up to 5-fold at 20 °C while the other strains remain comparable to the wild-type throughout (Ref I, Table 2). These results illustrate that the toxin is the cause for the observed cold-sensitivity. As changes in growth rate were the first verified effect of this TA system, we named the previously uncharacterised system GraTA for Growth Rate-Affecting Toxin-Antitoxin system.

7. GraT is a codon-specific mRNase

The GraT/GraA toxin-antitoxin system belongs to the HigB/HigA family of TA systems. The most similar experimentally described proteins to GraT and GraA are the *V. cholerae* HigB and HigA (Budde *et al.*, 2007) with 37% and 38% sequence identities, respectively (Ref I, Suppl. Fig. 1) and the *P. vulgaris* HigB-HigA with 32% and 29% respective identities (Figure 5 E). The *graTA* locus also follows the atypical organization of *higBA* loci where the toxin gene

precedes the antitoxin (Ref I, Fig. 1A). HigB toxins bind to the ribosome and function as co-translational mRNases [(Christensen-Dalsgaard & Gerdes, 2006, Hurley & Woychik, 2009), discussed in chapter 2.3.4.5].

To test whether GraT also cleaves mRNA, we used a controllable expression system in *E. coli*. As construction of a plasmid carrying *graT* alone turned out to be impossible, we used the whole *graTA* operon but with the Shine-Dalgarno (SD) sequence in front of *graT* replaced with a more efficient one from the pET11c expression plasmid. This modification of the *graTA* operon (plasmid pBBRlacItac-pETSD-graTA) increases the GraT/GraA ratio, as was evidenced by the cold-sensitive growth defect upon induction by IPTG. We then analysed the commonly used *lpp* mRNA integrity by primer extension. To avoid possible cross-activation of *E. coli* TA systems by GraT activity, the experiment was carried out using the *E. coli* Δ10 TA strain that lacks the known TA mRNases (Maisonneuve *et al.*, 2011). GraT induction resulted in a specific cleavage pattern appearing on the *lpp* mRNA (Figure 4). 13 out of 14 clearly defined cleavage positions were between the 2nd and 3rd nucleotides in a codon, which suggests a ribosome-dependent mode of action. The common characteristic of all observed cleavages is that they follow an A nucleotide (Figure 4). This is in line with previous reports on *P. vulgaris* Rts1 HigB selectivity where a single A can be enough to induce cleavage (Hurley & Woychik, 2009). However, while the Rts1 HigB shows a clear preference for A in the 3rd position of the codon (Schureck *et al.*, 2015), GraT seems to be rather indifferent towards the last nucleotide of the codon and instead prefers A in the 2nd position (Figure 4). The Rts1 HigB also avoids codons with U in the first position, while A, C, and, slightly less, G are preferred (Schureck *et al.*, 2015). GraT is similar in the sense that it shows no clear preference among A, C, and G in the first position (Figure 4). Based on these data, a disfavour for U cannot be confirmed, as there were no UAN codons in the analysed *lpp* region. This indicates that whilst GraT and the *P. vulgaris* Rts1 HigB are not identical in their target selection, there are still some common mRNA features that determine the activity of both proteins.

The HigB active site residues are proposed to be H54, acting as a general base, R73, stabilizing the intermediate state, and Y91, a general acid that is oriented and stabilized by D90 and H92 (Schureck *et al.*, 2016b). Of these, R73, D90, Y91, and H92 are conserved in GraT (Figure 5 B, E) and could thus serve identical roles. Instead of histidine, GraT has a glutamate in the corresponding 54th position. A glutamic acid residue (E46) has been proposed to act as the general base in *E. coli* YoeB (Kamada & Hanaoka, 2005) and thus it is possible that the GraT E54 also functions as the general base in mRNA cleavage. Still, given the wide variety of catalytic residues among the mRNase toxins (Schureck *et al.*, 2016b), it is also possible that other GraT residues drive RNA cleavage.

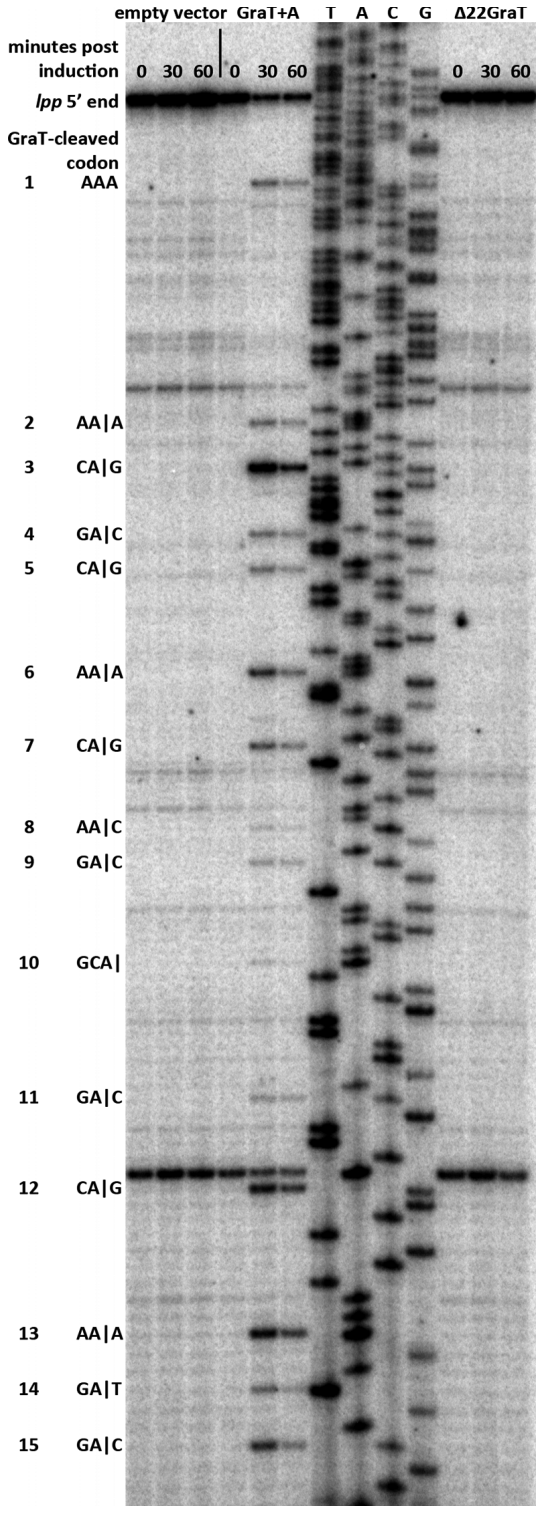

Figure 4. GraT is a codon-specific mRNase. Autoradiography of the *E. coli lpp* mRNA primer extension analysis. MG1655 Δ10TA cultures carrying either the empty pBBRlacItac expression vector or its *graTA* (with SD region from the pET vector) or Δ22*graT*-containing variants were sampled at 0, 30, and 60 minutes after GraT induction at 20 °C. Inducing the full GraT but not the N-terminally truncated Δ22 variant causes mRNA cleavage. The cut sites follow adenines, usually in the 2nd position of codons. Cleaved codons are shown next to the gel image with cleavage sites, if precisely determined, represented by vertical lines.

7.1. GraT is structurally very similar to HigB, except for its N-terminus (Ref III)

To gain more insight into the mechanisms of GraT, we aimed to purify the protein for *in vitro* experiments. However, the His_6-tagged GraT is very prone to precipitation and has thus far resisted solubilization and purification attempts. At the same time, it is readily soluble and manageable in complex with GraA. Thus, the 3D structure of the GraT-GraA complex was determined (Figure 5 A) by X-ray crystallography at a resolution of 2.2 Å (Ref III).

The X-ray diffraction data reveals that generally, the structures of GraT and GraA are very similar to HigB and HigA of the Rts1 plasmid (Figure 5 C, D). However, to our surprise, the first 22 amino acids of the 92-aa GraT protein are untraceable in the crystal form (Figure 5 A, B, D). This is in contrast with the Rts1 HigB structures that have been resolved in their entire length both on and off the ribosome (Schureck *et al.*, 2015). Notably, both the toxins have a length of 92 amino acids and the sequence identity among the first 22 aa is 36%, even slightly higher than the overall structure-based alignment identity of 32% (Figure 5 E). The lack of resolution in the GraT N-terminus hints at the possibility that it does not have a defined structure and is instead disordered, at least when in complex with GraA.

This finding prompted questions regarding the implications and importance of this potentially disordered region. To investigate whether it is necessary for toxicity, we deleted the region encoding the 22 first amino acids of GraT from the chromosomes of the wild-type and the Δ*graA* strain. Growth analyses show that the Δ*graA*Δ22*graT* strain has lost the Δ*graA* characteristic cold-sensitivity (Figure 6), indicating that the N-terminus is required for GraT-mediated toxicity. Additionally, the truncated GraT variant showed no mRNA cleavage activity when tested in parallel to the wild-type protein (Figure 4). We hypothesize that abolished ribosome binding is the reason behind the loss of activity. On HigB, the catalytic amino acids are located C-terminally (Schureck *et al.*, 2016b) in a region that is structured in GraT as well (Figure 5 B, D). However, the N-terminal region is important for HigB interaction with the ribosome, as the HigB lysine residues at positions 6, 8, and 11 contact 16S rRNA and their mutation diminishes HigB toxicity (Schureck *et al.*, 2016a). In this light it is possible that the GraT N-terminus also partakes in ribosome binding, even though the HigB-specific lysine residues are not conserved in GraT (Figure 5 E).

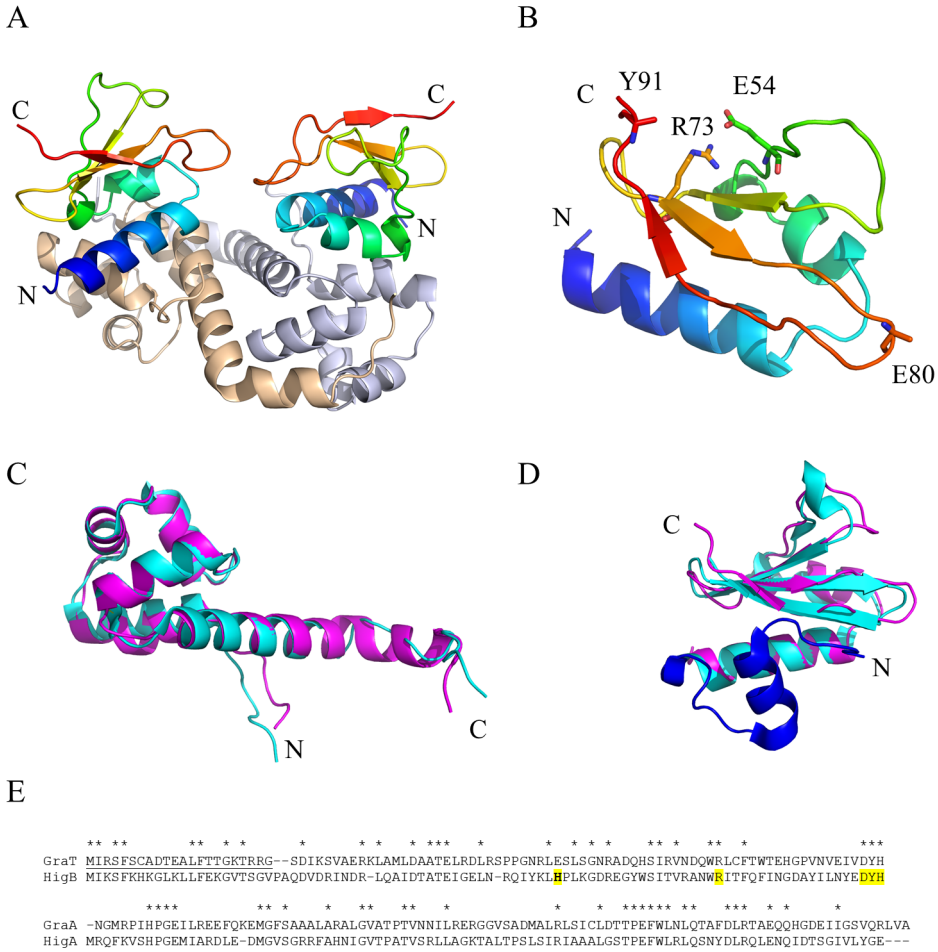

Figure 5. The structure of the GraTA complex and comparison of GraA and GraT with *P. vulgaris* HigA and HigB. A. Cartoon representation of the GraT-GraA$_2$-GraT complex. GraA monomers are coloured light brown and grey. GraT monomers are rainbow-coloured with the N-terminus (starting from the 23rd amino acid) blue and C-terminus red. GraT termini are indicated by N and C. B. Cartoon representation of GraT from the GraT-GraA$_2$-GraT complex. The potential catalytic amino acids and the glutamate exchanged for glycine in the GraT$_{E80G}$ mutant are shown as sticks. C. Superposition of GraA (purple) and *P. vulgaris* HigA (light blue, PDB: 4MCX). D. Superposition of GraT (purple) and *P. vulgaris* HigB (light blue, PDB: 4MCX). The HigB N-terminal domain corresponding to the unresolved GraT region is coloured dark blue. E. Structure-based alignments of GraT and GraA to *P. vulgaris* HigB and HigA. The unresolved GraT N-terminus is underlined, identical amino acids are indicated by an asterisk. HigB catalytically relevant amino acids are highlighted in yellow.

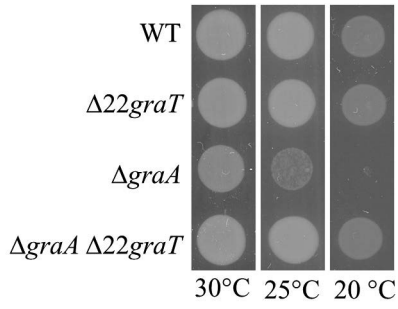

Figure 6. The potentially disordered N-terminus of GraT is required for toxicity *in vivo.* Antitoxin deletion from *P. putida* chromosome (strain Δ*graA*) results in GraT-mediated cold-sensitive growth defect, which is abolished by truncation of GraT N-terminus (strain Δ*graA* Δ22*graT*). The *P. putida* wild-type PaW85 (WT), Δ*graA* and their Δ22*graT* derivative strains Δ22*graT* and Δ*graA* Δ22*graT* were grown on solid LB medium at indicated temperatures for 24 hours. Approximately 10^5 cells were inoculated per spot.

7.2. GraT toxicity is enhanced by the chaperone DnaK (Ref II)

To shed light on the reasons behind the GraT-inflicted cold-sensitivity, we performed a transposon mutagenesis of the Δ*graA* strain to find factors that would alleviate GraT toxicity. Transposon mutants were selected by their ability to form colonies at 20 °C. Out of approximately 250,000 cells plated onto gluconate minimal media, 170 cold-tolerant mutant colonies emerged. Of these, 109 had either the transposon insertion or a random point mutation in the *graT* gene and had fully lost cold-sensitivity compared to the wild-type. Sequencing the remaining 61 mutants revealed only one recurrent target: three independent transposon insertions were detected in the end of the chaperone gene *dnaK*. More specifically, the resulting mutant DnaK versions were lacking 32, 41, and 66 C-terminal amino acids (Ref II, Fig. 5A). The other transposon mutants were single hits that did not cluster into meaningful groups, showed less cold-sensitivity suppression than the *dnaK* insertions, and were thus not analysed further.

The extreme C-terminus of DnaK is a disordered yet highly conserved region of the chaperone that is not strictly required neither for substrate binding nor ATPase activity (Buchberger *et al.*, 1995, Moro *et al.*, 2002, Aponte *et al.*, 2010, Smock *et al.*, 2011). In fact, there are conflicting reports regarding the effect of this domain on DnaK activity. One study found that deleting the 35 C-terminal amino acid residues increases DnaK refolding activity (Aponte *et al.*, 2010) while another found instead that the presence of the C-terminus supports refolding (Smock *et al.*, 2011).

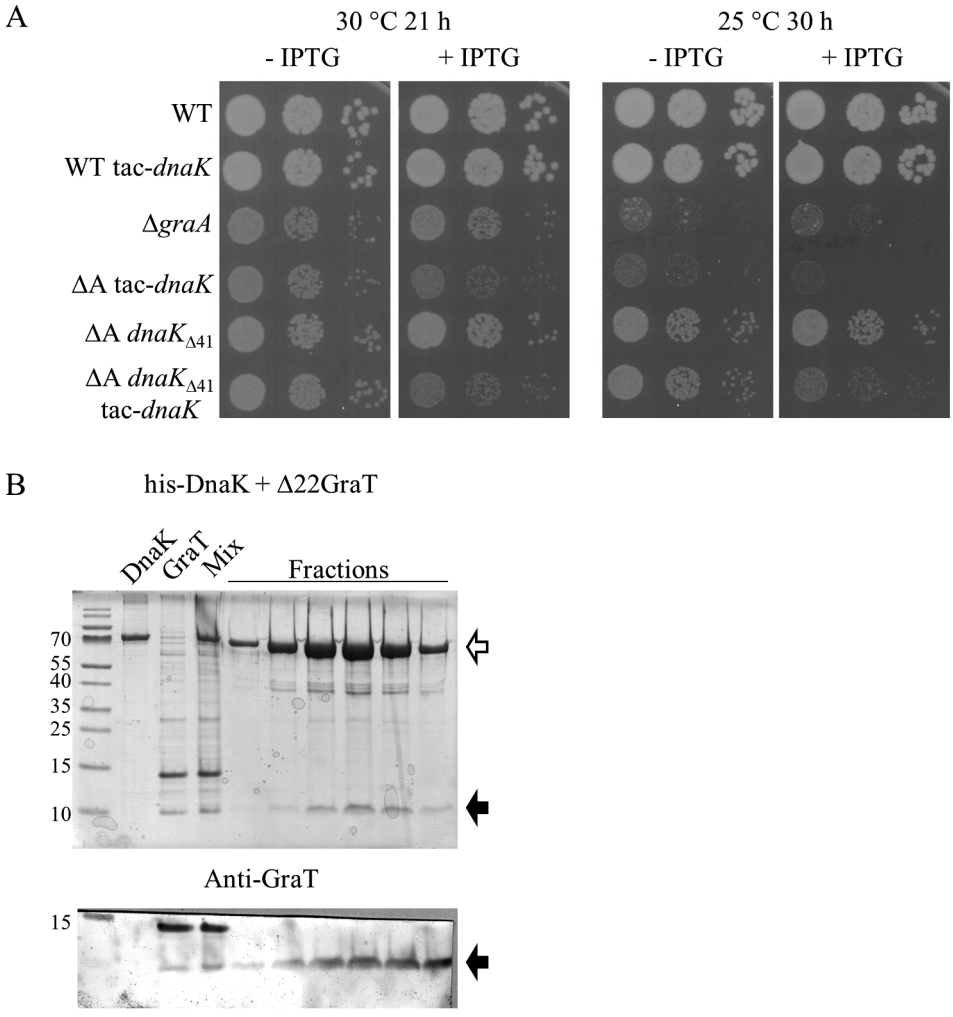

Figure 7. DnaK interactions with GraT. A. Growth assays of *P. putida* wild-type, Δ*graA*, and Δ*graA* $dnaK_{\Delta 41}$ strains and their *dnaK* overexpression derivatives (-tac-*dnaK*) on LB agar plates. DnaK overexpression induced by 0.5 mM IPTG, growth times, and temperatures are indicated above the panels. B. Pull-down assay. His_6-DnaK pulls down the N-terminally truncated toxin Δ22GraT from the cell lysate (Mix) prepared by mixing *E. coli* culture lysates that overexpressed the respective proteins (lanes DnaK and GraT). Upper panel shows Coomassie-stained lysates (DnaK, GraT, Mix) and elution fractions from the Ni^{2+} affinity column. Lower panel represents a Western blotting of an identical gel with anti-GraT antibodies. His_6-DnaK and Δ22GraT are respectively indicated by empty and solid arrows.

Curiously, the level of GraT suppression correlated with the length of DnaK disruption: the more C-terminal amino acids were missing, the better was Δ*graA* growth at 20 °C (Ref II, Fig. 5 B). To confirm that the DnaK disruption is the cause of the reduced cold-sensitivity, we constructed independent DnaK truncation strains lacking the 41 C-terminal amino acids in both wild-type and Δ*graA* backgrounds (*dnaK*$_{\Delta 41}$ and ΔA *dnaK*$_{\Delta 41}$, respectively). These show that the DnaK C-terminal truncation indeed reduces the effect of GraT comparably to the corresponding Δ*graA dnaK*$_{Tn41}$ mutant and does not affect growth in wild-type background (Ref II, Fig. 5 B). Unfortunately, deleting the whole *dnaK* gene from *P. putida* was impossible, suggesting its essentiality. On the other hand, overexpression of full-length *dnaK* in both Δ*graA* and Δ*graA dnaK*$_{\Delta 41}$ strains causes stronger cold-sensitivity, while it does not affect the wild-type (Figure 7 A). This suggests that DnaK facilitates GraT toxicity.

The finding that DnaK and especially its C-terminal domain supports GraT toxicity raised the question whether the two proteins interact directly with each other. To investigate this, we used His$_6$-tagged DnaK variants to pull down tagless GraT from cell lysates. As native GraT is toxic, we expressed an inactive GraT variant that lacks the last amino acid, a conserved histidine residue (GraT$_{\Delta 1C}$). Nickel affinity purification experiments revealed that GraT alone does not bind the column and is thus suitable for the pull-down assay (Ref II, Fig. 6 B). Co-purification indicated that both the full-length DnaK as well as its derivatives lacking 41 or 66 C-terminal amino acids pull down GraT from the cell lysates, as confirmed by Western blotting (Ref II, Fig. 6 A, D, E). The fact that the C-terminally truncated DnaK still binds GraT is not too unexpected, as the DnaK substrate-binding domain is largely intact in these C-terminal mutants (Ref II, Fig. 5 A).

We were also interested in GraT features that affect its binding to DnaK. To test whether the obvious candidate, the putatively disordered N-terminus, is necessary for DnaK interaction, we carried out the pull-down experiment using full-length DnaK and a GraT variant that lacked the first 22 amino acids (Δ22GraT). As DnaK could pull down this truncated GraT variant as well (Figure 7 B), the N-terminus is not the main DnaK substrate region in GraT. This is further supported by the fact that the GraT-GraA complex does not bind DnaK (Ref II, Fig. 6 C) even though the GraT N-terminus would be accessible from the complex (Figure 5 A). Therefore, it is likely that the GraT region necessary for DnaK binding falls to the area that is shielded by GraA.

The *P. vulgaris* HigB N-terminus is directly in contact with 16S rRNA and these interactions are necessary for both ribosome binding and toxicity (Schureck *et al.*, 2016a). Given that GraT acts as a codon-specific mRNase (Figure 4) as do other HigB toxins (Christensen-Dalsgaard & Gerdes, 2006, Hurley & Woychik, 2009) that are fully folded in their N-terminus (Schureck *et al.*, 2015, Yang *et al.*, 2016, Hadži *et al.*, 2017), it is likely that GraT is also fully folded when bound to the ribosome. The finding that DnaK supports GraT toxicity (Ref II, Fig. 5 B; Figure 7 A) suggests that GraT itself does not fold upon binding the ribosome and must rather be folded with the aid of DnaK. However,

disruption of the DnaK C-terminus does not fully abolish GraT toxicity (Ref II, Fig. 5 B), most likely because the substrate-binding domain is still largely intact in the DnaK mutants. The truncated DnaK most likely retains most of its chaperone activity, as the DnaK C-terminus deletion does not affect the wild-type remarkably (Ref II, Fig. 5 B) while our inability to delete the entire *dnaK* gene suggests that DnaK is an essential protein in *P. putida.* Thus, we hypothesize that DnaK is required for GraT to obtain a toxic structure and the C-terminal region of the chaperone is important to enhance the folding process. Such a supporting role of the DnaK C-terminus is consistent with the results obtained by Smock and colleagues (Smock *et al.*, 2011) but disagree with those of Aponte *et al*. (Aponte *et al.*, 2010). The involvement of the chaperone DnaK in GraT folding agrees with the potential disorder suggested by lack of GraT N-terminal structure in GraTA crystals (Figure 5 A) and the low toxic effect displayed by GraT upon DnaK C-terminal deletion (Ref II, Fig. 5 B).

8. Cellular effects of GraT

The common technique for studying toxins – overexpressing the toxin and then investigating the effects (Zhang *et al.*, 2003a, Christensen-Dalsgaard & Gerdes, 2006, Hurley & Woychik, 2009) is in no doubt useful for determining their molecular mechanisms of action. At the same time, artificially high amounts of toxin provide little insights into the biological consequences that native chromosomal TA systems may have. A system like the Δ*graA* strain where the toxin is in its native genomic locus therefore provides us with a very valuable tool to study toxin effects. As the antitoxin GraA represses the *graTA* promoter (Ref I, Fig. 4 A) and is thus far the only known regulator of *graTA* transcription, we can assume that in cells that completely lack GraA, the promoter is as active as can be. Therefore, the Δ*graA* strain produces the maximal amount of toxin that is possible from its native promoter. We can consider that antitoxin deletion mimics the situation of efficient antitoxin degradation in the wild-type, which would result in both liberation of the toxin and derepression of the TA locus. It is especially convenient that the severity of GraT effect increases at lower temperatures. This allows us to use experimental conditions that represent a suitable balance between the level of toxicity and cell growth that is sufficient to obtain experimental material. Growth analyses indicate that 25 °C is a good choice, as it slows Δ*graA* growth significantly but not enough to overly complicate further work. Altogether we believe that observing the Δ*graA* strain at low growth temperatures is a good technique to approximate the effects physiological toxin activation.

8.1. GraT increases membrane permeability and affects stress tolerance (Ref I)

Our work on the GraTA system started from the finding that *graA* disruption relieved the conditional lysis phenotype of the membrane-stressed ColRS mutant grown on glucose (Putrinš *et al.*, 2011). This raised the question whether *graA* deletion in wild-type background also affects the membrane. Staining the bacteria with a LIVE/DEAD kit containing SYTO9 and propidium iodide (PI) followed by flow cytometry revealed that the Δ*graA* population contained significantly more PI-permeable cells than the wild-type, which indicates increased membrane permeability due to GraT (Ref I, Fig. 5 A). It is notable that both the *graA* and *colR* deletions on their own increase membrane permeability, yet partially cancel out each other's effect when combined, which is particularly evidenced by the abolishment of cell lysis. However, PI permeability is a rather general phenotype and two mutations that disturb the membrane via distinct mechanisms do not necessarily have cumulative effects. The *colR*-deficient bacteria do not tolerate expression of the glucose porin OprB1 that increases in cells under glucose limitation (Putrinš *et al.*, 2011). The specific reason why GraT as a quite general mRNase disturbs the membrane is not clear. However, in the *colR* Δ*graA* double mutant GraT likely decreases the expression of membrane proteins, including OprB1, and thus relieves the protein load of the membrane to a level more tolerable to the *colR* mutant. Interestingly, a similar effect was demonstrated for the mRNases HigB, HicA and YafQ that upon overexpression suppress the lethality caused by loss of the *E. coli* envelope stress regulator σ^E (Daimon *et al.*, 2015).

The GraT E80G substitution variant does not cause a cold-sensitive growth defect (Ref I, Table 2) nor increase membrane permeability (Ref I, Fig. 5 A, B) of the Δ*graA* strain but at the same time reduces the *colR* mutant strain's membrane defect even more than wild-type GraT. This indicates that growth rate reduction is not the reason why GraT suppresses the *colR*-deficient phenotype. However, it is not clear what effect the $GraT_{E80G}$ protein can have in the cell. It is difficult to imagine another molecular mechanism of action in addition to mRNA degradation, and it is more likely that the *colR* mutant suppression by $GraT_{E80G}$ and the growth rate reduction by GraT result from different levels of mRNase activity. Glu80 is located in the loop between β-sheets 2 and 3 (Figure 5 B) and in the *P. vulgaris* HigB, mutating the corresponding Asn80 to alanine does not affect toxin activity noticeably (Schureck *et al.*, 2016b). It is possible that in our case, the substitution to glycine allows for greater conformational freedom, rendering GraT largely but not totally inactive.

As TA systems are often associated with stress tolerance (Ren *et al.*, 2004, Wang *et al.*, 2011, Norton & Mulvey, 2012, Kwan *et al.*, 2015), we also tested the Δ*graA* strain for resistance to different stressors. Bacteria were grown on media with compounds that inhibit either transcription, translation, replication or cell wall synthesis, or cause oxidative or osmotic stress. Interestingly, the

Δ*graA* bacteria were more sensitive to some stresses whilst being more resistant to others (Ref I, Fig. 7). GraT increased sensitivity to paraquat and hydrogen peroxide that cause oxidative stress, the translation inhibitor tetracycline and osmotic stress caused by 500 mM NaCl. This could be due to the increased membrane permeability of the Δ*graA* bacteria (Ref I, Fig. 5B), which would increase uptake of the compounds and also make the cells less resilient to osmotic stress. At the same time, GraT increased tolerance to streptomycin and kanamycin that inhibit translation, mitomycin C and ciprofloxacin that inhibit replication (Ref I, Fig. 7), and ceftazidime (data not shown) that inhibits cell wall synthesis. The increase in tolerance could be attributed to the slower growth, as antibiotics are more effective on fast-growing bacteria (Tuomanen *et al.*, 1986). However, the growth rate reduction is accompanied by membrane integrity disruption, indicating that toxin activation during stress results in a trade-off and can be either beneficial or harmful for the bacterium depending on the particular stressor.

8.2. GraT inhibits ribosome biogenesis (Ref II)

8.2.1. 50S and 30S subunits accumulate in GraT-affected cells

GraT was first classified as a ribosome-dependent mRNase by sequence similarity (Ref I) and later confirmed to cut mRNAs codon-specifically (Figure 4). Additionally, GraT was the first toxin reported to cause cold-sensitivity (Ref I). The fact that GraT was linked to both ribosomes and cold-sensitivity was interesting, as cold-sensitivity is a common result of defects in ribosome biogenesis (Shajani *et al.*, 2011). We thus decided to investigate whether the toxin affects the profile of ribosomal particles in the cells, which is an easy way to assess the status of ribosome assembly.

The bacteria were cultured at 30, 25, and 20 °C and harvested during logarithmic growth when $OD_{580} \approx 1.0$. Ribosomal particle profiles were analysed by sucrose gradient centrifugation. At 30 °C, the wild-type and the Δ*graA* showed similar ribosomal profiles with distinguishable polysomes, a major peak of 70S ribosomes, and small amounts of 50S and 30S subunits (Ref II, Fig. 1 D, G). This is in accordance with the fact that GraT does not inhibit cell growth significantly at 30 °C (Ref II, Fig. 1 A).

At 25 °C, GraT somewhat restricts growth in rich liquid medium (Ref II, Fig. 1 B). This is reflected also in the ribosomal profile: the Δ*graA* bacteria show increased amounts of free 50S and 30S subunits and relatively less 70S ribosomes when compared to the wild-type (Ref II, Fig. 1 E, H). At 20 °C, GraT effects intensify, with growth being significantly slower and the free subunits' peaks even more prominent than at 25 °C (Ref II, Fig. 1 F, I). To verify that the observed subunit accumulation is indeed caused by GraT activity, we performed the same experiments with the $\Delta graA_{T(E80G)}$ strain where the chromosomal *graT* locus harbours a point mutation. This strain behaved indistinguishably from the

wild-type (Ref II, Fig. 1 J, K, L), indicating that the subunit accumulation is a result of GraT action.

Interestingly, the observed ribosomal profiles are not typical for ribosome-dependent mRNase toxins. YoeB expression does not change the profile notably (Zhang & Inouye, 2009). Rts1 HigB, *E. coli* RelE and YafO rather diminish the amount of free 50S and 30S subunits (Hurley & Woychik, 2009, Zhang *et al.*, 2009). The only other mRNase that shows a small increase in 50S and 30S subunit amounts and even the appearance of a sub-30S particle is the *E. coli* YafQ (Prysak *et al.*, 2009). This is especially intriguing, as YafQ is the only other canonical TA toxin that has been reported to cause a cold-sensitive growth phenotype (Zhao *et al.*, 2016). YafQ and GraT are both ribosome-dependent mRNases but their cleavage specificity differs, as YafQ prefers codons ending in A (Armalytė *et al.*, 2012) while GraT favours A in the 2nd position (Figure 4). The difference is especially apparent in the *E. coli lpp* mRNA cleavage patterns, where GraT produces numerous specific fragments (Figure 4) and YafQ expression only results in one major cleavage product (Armalytė *et al.*, 2012). The GraT *lpp* cleavage pattern is more similar to that of Rts1 HigB (Hurley & Woychik, 2009). Yet, HigB causes an opposite effect on the ribosomal profile, with lower amounts of free subunits (Hurley & Woychik, 2009). However, the HigB data is based on toxin overexpression in an arabinose-inducible system (Hurley & Woychik, 2009), whereas GraT is produced from its native genomic locus, most likely in significantly lower amounts. This means that the physiological situations are inherently different: a sudden induction of mRNA-degrading toxin and total growth arrest versus a constant level of mRNA cleavage during cultivation. Thus, it is possible that the results are not in fact conflicting but rather the consequences of the chosen method.

8.2.2. The accumulated subunits' rRNA maturation is incomplete

Ribosomal subunit accumulation in bacteria can have different reasons. One possibility is that they are otherwise functional subunits that are unable to bind to each other during translation initiation. An example of a toxin that causes such an effect by a so far unknown mechanism is the RatA (PasT) (Zhang & Inouye, 2011). Alternatively, the accumulated particles could either be dysfunctional degradation intermediates of ribosomes, or immature subunits whose maturation is inhibited or delayed.

To investigate the essence of the accumulated subunits, both 70S ribosomes and the free 50S and 30S subunits were purified from both the wild-type and Δ*graA* bacteria grown at 25 °C. The first test was to analyse the rRNA 5' ends as a marker for subunit maturity. The final 7 or 3 nucleotides are trimmed from the 5' end of 23S rRNA when the almost complete subunit is assembled into a 70S ribosome for the first time (Srivastava & Schlessinger, 1988, Allas *et al.*, 2003). The rRNAs were extracted from both the free subunits found in the cells as well as subunits dissociated from mature 70S ribosomes. These were subjected to primer extension analysis to assess 5' end processing.

Both 23S and 16S rRNAs purified from 70S ribosomes were uniformly trimmed at the 5' ends in both wild-type and Δ*graA* (Ref II, Fig. 2). However, compared to the wild-type, the free subunits' 23S rRNA in Δ*graA* show relatively more molecules with either 3 or 7 additional nucleotides and relatively smaller amounts of rRNA has been cleaved to maturity (Ref II, Fig. 2 A). The free subunits' 16S rRNA in Δ*graA* is also notably less processed, as the relative amount of mature-length fragment is much smaller than in either the RNA from 70S subunits or the wild-type free 30S and several longer pre-16S species are visible on the gel image (Ref II, Fig. 2 B). These results indicate that the accumulated subunits are incomplete and thus, GraT inhibits ribosome biogenesis.

The Δ*graA* strain's accumulated subunits are nearly complete, as evidenced by their sedimentation at practically 50S and 30S. This suggests that GraT inhibits one of the final stages of subunit assembly. During the process of ribosome biogenesis, the ribosomal RNAs are subject to not only stepwise trimming but also extensive chemical modification of nucleosides. These are also synthesized at certain stages in subunit assembly (Siibak & Remme, 2010) and thus, quantification of nucleoside modifications can help to more precisely determine the stage of ribosome biogenesis inhibited by GraT.

To quantify nucleoside modifications, the large and small subunits' rRNA nucleosides were prepared from both wild-type and Δ*graA* free subunits and mature ribosomes. The nucleosides were separated and quantified by RP-HPLC, using the wild-type mature subunits' data as the standard for full rRNA modification levels. There were no differences between modification levels in wild-type and Δ*graA* rRNAs purified from 70S ribosomes (Ref II). This indicates that GraT does not cause a complete defect of any particular nucleoside modification. Additionally, both the wild-type and Δ*graA* free subunits' modification levels were compared to wild-type 70S. The wild-type free subunits serve as the baseline corresponding to normal amounts of subunit precursors in the cells. This allowed distinguishing between naturally less-occurring modifications and GraT-specific delayed modifications in the Δ*graA* free subunits.

In 23S rRNA, the only considerable difference was that the Δ*graA* free subunits had almost twofold less 5-methyluridine (m^5U) than the wild-type free subunits (Ref II, Fig. 3 A). In *E. coli*, m^5U is generated at early to intermediate stages of ribosome biogenesis (Siibak & Remme, 2010) and is present in two positions in 23S rRNA (Kaczanowska & Rydén-Aulin, 2007). The approximately twofold m^5U reduction in Δ*graA* could mean that GraT specifically inhibits the synthesis of m^5U at one of these sites but unfortunately, HPLC analysis does not provide site-specific data.

In the free subunits' 16S rRNA, Δ*graA* bacteria had significantly less N2-methylguanosine (m^2G) and N6,N6-dimethyladenosine ($m^6{}_2A$) (Ref II, Fig. 3 B). Both m^2G and $m^6{}_2A$ are synthesized in the late stages of small subunit assembly in *E. coli* (Siibak & Remme, 2010). Moreover, synthesis of the two adjacent $m^6{}_2A$ nucleotides has been proposed to act as a control step in late small subunit biogenesis that allows only properly assembled particles to progress to translation (Connolly *et al.*, 2008).

The observed deficiencies in Δ*graA* rRNA nucleoside modifications do not point to a specific step in ribosome maturation that GraT inhibits. Still, the lowered amounts of mostly late-stage modifications serve to confirm that the free 50S and 30S subunits are not fully matured. Thus, GraT action causes inhibition of the later stages of ribosome biogenesis.

8.3. Whole cell proteomic changes in Δ*graA* (Ref IV)

The previously described different ribosomal particle profile changes in response to mRNase toxins illustrate well that the effects of these toxins can vary significantly depending on the experimental setup. It is not surprising that strong and sudden induction of a toxin elicits a different cellular response than constitutive stress imposed by a smaller amount of toxin that only partially slows growth. To better characterize the bacterial response to growing under GraT effect, where mRNAs are cleaved and ribosome biogenesis is disturbed, the whole cellular proteome of Δ*graA* bacteria was analysed in comparison with wild-type *Pseudomonas putida*. Both strains were cultivated at 30 °C, where GraT only slows growth very slightly, and 25 °C, where the growth is markedly slower and defects manifest in ribosome biogenesis (Ref II). Bacteria were grown to exponential phase ($OD_{580} \approx 1.0$) as in the ribosome assays. The proteomes were measured by label-free mass spectrometry at the Proteomics Core Facility of the University of Tartu.

Of the total 5527 proteins annotated in *Pseudomonas putida* KT2440 (The UniProt Consortium, 2017), between 2557 and 2601 were detected and passed data filtering for pairwise comparisons between groups (Ref IV, Fig. 1). First, we focused on proteins that showed a twofold or larger difference that was statistically significant after applying the Benjamini-Hochberg multiple testing correction at a false discovery rate of 0.05. Additionally, the strains comparison datasets were searched for operons where their encoded proteins show concerted regulation.

Comparing the proteomes of single strains grown at 30 °C and 25 °C, it was somewhat surprising that neither wild-type nor Δ*graA* showed any temperature-dependent changes in protein levels (Ref IV, Fig. 1 C, D). This illustrates that the response to a temperature shift to 25 °C is not reliant on major changes of protein levels, as all the differences remain below the statistical significance threshold.

Analysing the differences between the wild-type and Δ*graA* strains at either growth temperature revealed that at 30 °C, GraT caused significant upregulation of 4 and downregulation of 2 proteins (Ref IV, Table 3). At 25 °C, 6 proteins were down- and 7 upregulated in the Δ*graA* strain (Ref IV, Table 3). The results show that the extent of proteomic response to GraT is slightly increased at lower growth temperatures but not by a large margin (Ref IV, Fig. 1 A, B). This was somewhat surprising, as that the main phenotype, growth inhibition, is markedly stronger at 25 °C (Ref I, Table 2). Given that at 30 °C, we see very

little growth inhibition (Ref I, Table 2) yet the proteomic response is similar to that at 25 °C, we can hypothesize that GraT is already active at 30 °C but the cell is able to overcome its effects, as discussed in the following chapters.

8.3.1. Δ*graA* response to the ribosome biogenesis defect

As part of the proteome investigation, we hoped that it could provide insight into the reasons behind the ribosome biogenesis defect, ideally in the form of some significantly downregulated protein that could be linked to ribosome biogenesis. On the contrary, data indicated that GraT clearly causes an up-regulation of several proteins related to ribosome biogenesis. Already at 30 °C, the RNA helicase DeaD and RNase III are upregulated approximately 2.5-fold (Ref IV, Table 3). DeaD participates in the final steps of 50S subunit maturation (Charollais *et al.*, 2004, Peil *et al.*, 2008) and RNase III processes pre-rRNA (Ginsburg & Steitz, 1975). Additionally, the *rnc* gene encoding RNase III is co-transcribed with the *era* gene encoding the Era GTPase that also participates in ribosome biogenesis (Inoue *et al.*, 2003). Era was found to be about 1.8-fold upregulated at both growth temperatures, although the effect was not significant (Ref IV, Table S2). The operon analysis at 25 °C also revealed upregulation of YbeY, an RNase also involved in ribosome biogenesis (Jacob *et al.*, 2013) but with an unknown specific function (Smith *et al.*, 2018). Based on these findings, we compiled a list of ribosome biogenesis factors and specifically looked for changes in their amounts. The corresponding volcano plots indicate that the general trend among ribosome biogenesis factors is towards upregulation under GraT stress at both temperatures (Ref IV, Fig. 3 A, B). Notably, the chaperones DnaK, GroEL, and GroES involved in ribosome assembly stand out as mostly downregulated at both temperatures (Ref IV, Table S3). Altogether, the proteomic response does not uncover the cause of the GraT-mediated ribosome biogenesis defect. Rather, it indicates that cellular mechanisms work to alleviate the toxic effect of GraT, resulting in upregulation of several ribosome biogenesis factors. It is tempting to speculate that the downregulation of chaperones similarly serves to counter GraT toxicity, given that DnaK enhances the GraT-mediated growth suppression. The fact that the ribosome biogenesis factors are similarly regulated at both 30 °C and 25 °C suggests that GraT is already affecting ribosome assembly at 30 °C but the cells are able to counter the toxicity, resulting in no accumulation of free ribosomal subunits (Ref II, Fig. 1 G). However, at 25 °C, the cells are less able to rescue the inherently cold-sensitive process of ribosome biogenesis and the characteristic subunit precursors start accumulating in Δ*graA* bacteria (Ref II, Fig. 1 H).

Quite recently, inhibition of rRNA maturation and ribosome biogenesis was suggested as the primary reason for growth inhibition by the *E. coli* RNase toxin MazF. A global analysis of MazF cleavage specificity determined that MazF cleaves pre-rRNA as well as the ribosomal proteins' mRNAs and thus efficiently blocks ribosome biogenesis (Culviner & Laub, 2018). However,

MazF is a ribosome-independent RNase and as such, it is not surprising that it cleaves both pre-rRNA and mRNAs. In contrast, GraT is most likely a ribosome-dependent mRNase, which would exclude pre-rRNA as a target. This is supported by the finding that the Δ*graA* accumulated free subunits' rRNAs show no particular degradation (Ref II, Fig. 4). The possibility that GraT significantly impacts the production of ribosomal proteins by cleaving their mRNAs is not supported by the proteome analysis, as the levels of ribosomal proteins in Δ*graA* remain on par with the wild-type (Ref IV, Fig. S1, Table S1). Thus, while it is intriguing that ribosome biogenesis emerges as the target of another toxin, the molecular reasons behind the comparable phenotypes must differ between MazF and GraT.

8.3.2. Carbon metabolism alterations in Δ*graA* bacteria

In addition to elevated levels of ribosome biogenesis factors, the proteome dataset showed several changes in the amounts of carbon metabolism enzymes. At 25 °C, three out of six significantly downregulated proteins are enzymes that take part in the tricarboxylic acid (TCA) cycle: isocitrate dehydrogenase Idh, α-ketoglutarate dehydrogenase subunit SucA, and succinyl-CoA synthetase subunit SucD (Ref IV, Table 3). Notably, these enzymes all cluster together in the TCA cycle and catalyse three subsequent reactions, which summarily convert isocitrate to succinate. These three downregulated enzymes are responsible for the decarboxylation steps of TCA cycle, producing NADH and CO_2, and substrate-level phosphorylation, producing GTP. This clear effect on the TCA cycle prompted us to specifically analyse other TCA cycle enzyme levels in the proteomes as well. The plot of central carbon metabolism enzymes participating in the TCA cycle and selected side reactions (Ref IV, Fig. 3 C, D, proteins are listed in Ref IV, Table S3) indicate that the general trend, especially at 25 °C, is towards downregulation. Thus, the central carbon metabolism seems to be downregulated in GraT-affected bacteria.

One of the significantly downregulated proteins in Δ*graA* at 25 °C is the glycine dehydrogenase GcvP (Ref IV, Table 3), a part of the glycine cleavage system (GCS). Other GCS proteins GcvH and GcvT are also approximately 2-fold downregulated, although not statistically significantly (Ref IV, Table S2, Table S1). This downregulation is potentially in line with the significantly increased amounts of the PP_1236 protein that is a putative transcriptional repressor of GCS (Ref IV, Table 3) but this link has not been proven experimentally. GCS catalyses the breakdown of glycine, which results in formation of 5,10-methylenetetrahydrofolate, an important cofactor in one-carbon metabolism (Kikuchi *et al.*, 2008). It is notable that like the significantly downregulated TCA cycle enzymes, the GCS reactions also produce CO_2 and NADH, suggesting that their production is specifically lowered in Δ*graA* bacteria. Interestingly, disruptions of *gcvP* or *gcvT* genes were found to suppress the toxicity of YafQ, the other ribosome mRNase toxin that causes

cold-sensitivity (Zhao *et al.*, 2016). Unfortunately, the reasons behind this effect were not elucidated in any way. Still, their results raise the question whether GCS downregulation could alleviate GraT effects as well. In our analogous transposon mutagenesis assay designed to find GraT-suppressor mutants, we detected no insertions in the *gcv* genes (Ref II, data not shown). However, this does not rule out the possibility that GCS downregulation or disruption partially alleviates GraT toxicity but not enough to reach the detection threshold in the transposon assay.

To gain further insights into the state of carbon metabolism under GraT effects, we compared the amounts of TCA cycle intermediates in wild-type and Δ*graA* bacteria by mass spectrometry. Surprisingly, only two significant differences were detected at 25 °C: oxaloacetate (OAA) levels were 4-fold higher and malate levels 1.8-fold lower in Δ*graA* (Ref IV, Table 4). This was unexpected, as the highest differences in protein levels were observed in enzymes leading from isocitrate to succinate, yet the amounts of isocitrate, α-ketoglutarate, and succinate remained unchanged (Ref IV, Table 4). Still, the fact that OAA and malate levels are altered by GraT stress is interesting, as these two compounds have been described as allosteric regulators of metabolic enzymes. Malate inhibits PEP carboxylase in *E. coli* (Morikawa *et al.*, 1980), which raises the possibility that less malate in Δ*graA P. putida* results in less repression of PEP carboxylase, an enzyme that produces oxaloacetate. OAA itself is a repressor of several enzymes in and around the TCA cycle: the succinate dehydrogenase complex (Molenaar *et al.*, 2000), the malic enzyme MaeB (Sanwal & Smando, 1969), and PEP synthetase (Chulavatnatol & Atkinson, 1973). It is interesting to note that among these four allosterically regulated enzymes, only PEP synthetase shows possible differential regulation with 2.5-fold lower levels in Δ*graA* ($p=0.0011$). The remaining three allosterically regulated enzymes are among the few TCA-related ones that show no discernible protein level changes in response to GraT.

Taken together, proteomics and metabolite measurements indicate a concerted downregulation of the TCA cycle in Δ*graA* bacteria, achieved either by lowered amounts of the respective enzymes or their allosteric inhibition by increased amounts of oxaloacetate (Ref IV, Fig. 4). The accumulation of oxaloacetate is likely due to decreased flux through the TCA cycle and also its reduced utilization in biosynthetic processes. To find a relatively specific effect like this was somewhat surprising, given the quite low sequence specificity of GraT-mediated mRNA cleavage. However, it should be stressed that the Δ*graA* bacteria are only somewhat limited in growth at 25 °C in rich medium, and the cells are thus coping with the stress caused by GraT. Especially given that the proteomics data shows not only significant downregulations but an approximately similar amount of upregulated proteins, it is most likely that the TCA downregulation is just that – adaptive regulation and not a direct result of GraT cleaving the mRNAs of specific proteins. Decreasing the metabolic rate at the central TCA node would serve to balance it with the lower overall translation efficiency that results from the constitutive low-level mRNA cleavage by GraT.

CONCLUSIONS

Toxin-antitoxin systems and their conditional ability to reduce bacterial growth or even induce dormancy are intriguing due to their potential implications in the stress response. Knowledge about the toxins' mechanisms of action and their physiological effects provide important insight into the biology of TA systems. This thesis summarizes our findings on the first studied TA toxin in the soil bacterium *Pseudomonas putida* – the toxin GraT. We were fortunate in being able to study the effects of GraT in an antitoxin deletion model strain, which provides a more physiological setting compared to the often-utilized toxin overexpression systems.

The main results concerning the molecular function of GraT can be summarized as follows:

- GraT cleaves mRNAs in a codon-specific fashion, in agreement with its homology to similarly-acting HigB-family toxins. The preferred cleavage sites are codons with an adenine in the 2nd position.
- Structural data suggests that the GraT N-terminus is intrinsically disordered, which is unique among toxins described so far. This 22-amino acid region of the 92-aa toxin is required for GraT toxicity and most likely affects GraT binding to the ribosome.
- GraT toxicity is enhanced by the chaperone DnaK, likely by assisting toxin folding. This is the first time that a central cellular chaperone has been found to support TA toxicity.

The physiological effects observed during GraT-inflicted stress are as follows:

- GraT inhibits cell growth, the effect increases as incubation temperature lowers.
- GraT increases membrane permeability.
- GraT increases tolerance to some stressors while reducing tolerance for others.
- GraT inhibits ribosome biogenesis, illustrated by accumulation of incomplete ribosomal subunits.
- The whole cell proteome of GraT-affected bacteria revealed that:
  - Ribosome biogenesis factors are upregulated, likely in a compensatory fashion.
  - Central carbon metabolism is downregulated around the TCA cycle.

The results obtained from the proteomic comparisons are especially interesting as they allow for speculation regarding the biological relevance of the GraTA module to *P. putida.* Instead of leading to the discovery of the target of the toxin, the proteomics hint at processes working to alleviate the effects of GraT. One potential inference from this would be that GraT is not a part of the *P. putida* stress tolerance network and its activation is in any case an

unfavourable process that needs to be suppressed or alleviated. On the other hand, the observations that GraT increases tolerance to some stress-inducing chemicals hints at its potential to be beneficial for bacteria during some natural stress conditions. The native trigger of GraTA activation is still unknown, but it is reasonable to assume that the cell would respond to this trigger in more ways than just GraTA activation. In this case, artificially activating GraTA by antitoxin deletion would be perceived as erroneous by the cell and activation of toxicity-countering mechanisms, as detected in our proteome analysis, is expected. Therefore, it is possible that the GraTA system with its potential to modulate stress tolerance is indeed necessary for *P. putida* to hold its ground against specific stressful conditions.

SUMMARY IN ESTONIAN

Pseudomonas putida toksiini GraT mõju bakteri füsioloogiale

Bakterite toksiin-antitoksiin (TA) süsteemide operonid koosnevad klassikaliselt kahest väikesest geenist, mis kodeerivad vastavalt toksiini ja seda neutraliseerivat antitoksiini. Need püsivad bakterirakus kahjutu kompleksina, kuid kui antitoksiin lagundatakse, inhibeerib vabanev toksiin oma sihtmärki. TA operonid avastati enam kui 30 aastat tagasi plasmiididelt, kus need toimivad plasmiidi alalhoiusüsteemidena (Ogura & Hiraga, 1983). Plasmiidi kaotanud rakku jäävad alles toksiin-antitoksiin kompleksid, millest antitoksiin kui reeglina ebastabiilsem valk esimesena laguneb. Vabanenud toksiin pärsib raku elutegevust ning nii konkureeritakse plasmiidita rakud populatsioonist välja (Jensen & Gerdes, 1995). Hiljem leiti, et ka bakterite kromosoomid kannavad hulgaliselt TA süsteeme (Pandey & Gerdes, 2005, Leplae *et al.*, 2011). Vaatamata intensiivsetele uuringutele ei ole genoomsete TA süsteemide roll sugugi selge, kuigi püstitatud ja testitud on mitmeid hüpoteese (Van Melderen, 2010). Kuna TA süsteeme on bakterites arvukalt, ei ole neil ilmselt ühist bioloogilist rolli. Erinevate genoomsete TA süsteemide puhul on kirjeldatud nende osalust kindlate genoomiregioonide stabiliseerimisel (Szekeres *et al.*, 2007), bakteriofaagide vastases kaitses (Fineran *et al.*, 2009) ning ka stressivastuse vahendamises (Ren *et al.*, 2004, Wang & Wood, 2011, Helaine *et al.*, 2014).

TA süsteemi aktiveerumise all mõistetakse toksiini vabanemist antitoksiini kontrolli alt ning väheste eranditega mõjutavad TA süsteemid bakteri elutegevust just toksiini kaudu. Kuna TA lookused on tihti seotud mobiilsete geneetiliste elementidega ja võivad nende koosseisus levida eri liiki bakterite vahel, on toksiinide sihtmärkideks ennekõike fundamentaalsed ja konserveerunud füsioloogilised protsessid ning struktuurid. Toksiinid mõjutavad rakuümbrise terviklikkust (Gerdes *et al.*, 1986a, Mutschler *et al.*, 2011, Tan *et al.*, 2011) ning DNA metabolismi (Miki *et al.*, 1992, Yuan *et al.*, 2010, Harms *et al.*, 2015). Enam on siiski kirjeldatud toksiine, mis takistavad erinevaid valgusünteesi etappe (Guglielmini & Van Melderen, 2011, Harms *et al.*, 2018). Transferaasidena toimivad toksiinid fosforüülivad EF-Tu-d ning glutamüül-tRNA süntetaasi (Germain *et al.*, 2013, Kaspy *et al.*, 2013, Castro-Roa *et al.*, 2013, Cruz *et al.*, 2014) või atsetüleerivad tRNA-sid (Cheverton *et al.*, 2016, Jurėnas *et al.*, 2017). Suur hulk toksiine toimivad RNaasidena, mis lõikavad kas tRNA-sid (Winther & Gerdes, 2011, Schifano *et al.*, 2016), rRNA-d nii pre-rRNA kujul (Mets *et al.*, 2017, Culviner & Laub, 2018) kui ka ribosoomi koosseisus (Schifano *et al.*, 2013, Winther *et al.*, 2013) ning mRNA-sid nii iseseisvalt (Zhang *et al.*, 2003b, Kawano *et al.*, 2007, Yamaguchi *et al.*, 2009, Short *et al.*, 2013, Bibi-Triki *et al.*, 2014) kui ka translatsiooni käigus ribosoomsõltuvalt (Pedersen *et al.*, 2003, Christensen-Dalsgaard & Gerdes, 2006, Christensen-Dalsgaard & Gerdes, 2008, Prysak *et al.*, 2009, Christensen-Dalsgaard *et al.*, 2010). See kirju loetelu erinevalt toimivatest toksiinidest näitab

ilmekalt, et konkreetse TA süsteemi rolli selgitamisel on olulisel kohal nii toksiini kui ka antitoksiini molekulaarsete toimemehhanismide kirjeldamine.

Töö mullabakteri *Pseudomonas putida* GraTA toksiin-antitoksiin süsteemiga sai alguse avastusest, et TA antitoksiinina annoteeritud seni uurimata geeni katkestus kõrvaldab ColR-ColS kahekomponentse signaaliraja mutandi membraanidefekti ning tingimusliku lüüsi (Putrinš *et al.*, 2011). Kuna TA süsteeme oli seostatud stressitaluvusega ning antitoksiini katkestuse korral võiks eeldada toksiini aktiivsust, huvitusime leitud TA lookuse rollist *P. putida* stressivastuses. Kokkuvõttes said töö jooksul püstitatud eesmärkideks:

- teha kindlaks, kas tegemist on funktsionaalse TA süsteemiga,
- tuvastada toksiini sihtmärk rakus,
- kirjeldada bakteri vastust toksiinihulgale, mis vastab hinnanguliselt TA süsteemi looduslikule aktivatsioonile.

Olles tuvastanud, et tegemist on funktsionaalse TA süsteemiga, mille toksiin pidurdab kasvutemperatuuri langedes raku kasvu, nimetasime selle GraT-GraA süsteemiks (*ingl. k. growth rate-affecting toxin-antitoxin*, kasvukiirust mõjutav toksiin-antitoksiin). Edasised toksiini sihtmärgi ja molekulaarsete funktsioonide uuringud saab kokku võtta järgnevalt:

- GraT lõikab mRNA-sid koodonsõltuvalt, mis on kooskõlas homoloogiliste HigB-perekonna toksiinide sama toimega. GraT lõikab eelistatult koodoneid, mille teises positsioonis on adeniin.
- GraT N-terminaalne osa on ilmselt struktureerimata, mis on seni kirjeldatud toksiinide seas ainulaadne, struktuuritud regioonid esinevad pigem antitoksiinides. 92-aminohappelise toksiini esimest 22 aminohapet hõlmav struktureerimata ala on vajalik GraT toksilisuseks ning on tõenäoliselt oluline ribosoomiga seondumisel.
- Šaperonvalk DnaK võimendab GraT toksilisust, abistades tõenäoliselt toksiini voltumist. Varem ei ole kesksete bakteriaalsete šaperonide positiivset mõju TA toksiinidele kirjeldatud.

GraT põhjustatud füsioloogiliste muutuste kirjeldamiseks on selles töös kasutatud mudelsüsteemina ilma antitoksiini geeni *graA*-ta bakteritüve Δ*graA*. Sageli ei ole antitoksiini eemaldamine võimalik vabaneva toksiini aktiivsuse tõttu (Shah *et al.*, 2006, Budde *et al.*, 2007), kuid Δ*graA* tüve konstrueerimine on võimalik tänu toksiini mõju temperatuurisõltuvusele: *P. putida*-le optimaalsel temperatuuril 30 °C ei ole selle tüve kasv märkimisväärselt erinev metsiktüvest, kuid juba 20 °C juures ei ole Δ*graA* võimeline tardsöötmel kolooniaid moodustama. Selline tüvi, kus toksiini toodetakse tema looduslikus lookuses, mis on vaba antitoksiini represseerivast mõjust, vastab teoreetilisele olukorrale, kus TA süsteemi aktivatsiooni tõttu on rakus kogu antitoksiin lagundatud. Võrreldes sageli toksiinide mõju kirjeldamiseks kasutatavate toksiini üleekspressiooni süsteemidega on meie mudel lähedasem looduslikule

olukorrale ning selle abil on seetõttu parem kirjeldada toksiini loomulikku mõju bakterile. Toksiini GraT mõjud bakteri füsioloogiale on järgmised:

- GraT suurendab rakumembraani läbilaskvust.
- GraT mõjutab *P. putida* stressitaluvust kahetiselt, sest toksiini toimel mõningate stressiallikate taluvus suureneb, kuid teiste oma väheneb.
- GraT inhibeerib ribosoomide biogeneesi, mis väljendub ebaküpsete ribosoomi subühikute kuhjumises bakterirakus.
- GraT mõju all kasvanud rakkude täisproteoomi analüüs tuvastas kaks peamist vastust:
    - Ribosoomi biogeneesi abifaktorite hulk on tõusnud, mis tõenäoliselt aitab bakteritel GraT toksilisust vähendada.
    - Tsentraalne süsinikumetabolism on tsitraaditsükli ümber alla reguleeritud.

Kuigi proteoomiuuringud ei toonud selgust küsimusse, kuidas nähtud füsioloogilised efektid võiksid olla põhjustatud GraT mRNaassest aktiivsusest, on siiski huvitav, et jälgitav oli vastureaktsioon GraT-toimelisele ribosoomi biogeneesidefektile. Ühelt poolt võiks selle põhjal oletada, et GraTA süsteem ei ole *P. putida* füsioloogiale omane ning tegemist on pigem iseka süsteemiga, mille aktiivsus tuleb maha suruda. Teisalt ei tea me hetkel, missugustes tingimustes toimub GraTA süsteemi looduslik aktivatsioon. Kui eeldada, et GraT aktiveerub vastusena mingile konkreetsele stressile, mis ilmselt indutseerib ka muud raku kaitsevastused, siis on arusaadav, et meie loodud antitoksiini deletsioontüvi kujutab endast ebaloomulikku olukorda. Nimelt testime me GraT toimet olukorras, kus loomulikku GraTA süsteemi aktiveerivat stressisignaali tegelikult ei ole. Selles valguses ei ole üllatav, et bakteri füsioloogia üritab GraT toksilisust vähendada, näiteks ribosoomi biogeneesifaktorite hulga suurendamisega. Seega ei välista saadud tulemused võimalust, et GraTA süsteem võib teatud tingimustel siiski *P. putida* stressitaluvust ja seega kohasust suurendada.

REFERENCES

Aakre, C. D., Phung, T. N., Huang, D. & Laub, M. T., (2013) A bacterial toxin inhibits DNA replication elongation through a direct interaction with the β sliding clamp. *Molecular cell* **52**: 617–628.

Ainelo, A., Tamman, H., Leppik, M., Remme, J. & Hõrak, R., (2016) The toxin GraT inhibits ribosome biogenesis. *Molecular microbiology* **100**: 719–734.

Aizenman, E., Engelberg-Kulka, H. & Glaser, G., (1996) An *Escherichia coli* chromosomal „addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: a model for programmed bacterial cell death. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 6059–6063.

Allas, Ü., Liiv, A. & Remme, J., (2003) Functional interaction between RNase III and the *Escherichia coli* ribosome. *BMC molecular biology* **4**: 8.

Amitai, S., Kolodkin-Gal, I., Hananya-Meltabashi, M., Sacher, A. & Engelberg-Kulka, H., (2009) *Escherichia coli* MazF leads to the simultaneous selective synthesis of both „death proteins" and „survival proteins". *PLoS genetics* **5**: e1000390.

Anantharaman, V. & Aravind, L., (2003) New connections in the prokaryotic toxin-antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system. *Genome biology* **4**: R81.

Aponte, R. A., Zimmermann, S. & Reinstein, J., (2010) Directed evolution of the DnaK chaperone: mutations in the lid domain result in enhanced chaperone activity. *Journal of molecular biology* **399**: 154–167.

Armalytė, J., Jurėnaitė, M., Beinoravičiūtė, G., Teišerskas, J. & Sužiedėlienė, E., (2012) Characterization of *Escherichia coli dinJ-yafQ* toxin-antitoxin system using insights from mutagenesis data. *Journal of bacteriology* **194**: 1523–1532.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S., (2004) Bacterial Persistence as a Phenotypic Switch. *Science* **305**: 1622.

Bayley, S. A., Duggleby, C. J., Worsey, M. J., Williams, P. A., Hardy, K. G. & Broda, P., (1977) Two modes of loss of the Tol function from *Pseudomonas putida* mt-2. *Molecular & general genetics : MGG* **154**: 203–204.

Belda, E., van Heck, R. G. A., José Lopez□Sanchez, M., Cruveiller, S., Barbe, V., Fraser, C., Klenk, H. P., Petersen, J., Morgat, A., Nikel, P. I., Vallenet, D., Rouy, Z., Sekowska, A., Martins dos Santos, V. A. P., de Lorenzo, V., Danchin, A. & Médigue, C., (2016) The revisited genome of *Pseudomonas putida* KT2440 enlightens its value as a robust metabolic chassis. *Environmental microbiology* **18**: 3403–3424.

Bernard, P. & Couturier, M., (1992) Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *Journal of molecular biology* **226**: 735–745.

Bernard, P., Kézdy, K. E., Van Melderen, L., Steyaert, J., Wyns, L., Pato, M. L., Higgins, P. N. & Couturier, M., (1993) The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. *Journal of molecular biology* **234**: 534–541.

Bibi-Triki, S., Li de la Sierra-Gallay, I., Lazar, N., Leroy, A., Van Tilbeurgh, H., Sebbane, F. & Pradel, E., (2014) Functional and Structural Analysis of HicA3-HicB3, a Novel Toxin-Antitoxin System of *Yersinia pestis*. *Journal of bacteriology* **196**: 3712–3723.

Bigger, J. W., (1944) Treatment of Staphylococcal Infections with Penicillin by Intermittent Sterilisation. *Lancet*: 497–500.

Blower, T. R., Pei, X. Y., Short, F. L., Fineran, P. C., Humphreys, D. P., Luisi, B. F. & Salmond, G. P., (2011a) A processed noncoding RNA regulates an altruistic bacterial antiviral system. *Nature Structural and Molecular Biology* **18**: 185.

Blower, T. R., Salmond, G. P. & Luisi, B. F., (2011b) Balancing at survival's edge: the structure and adaptive benefits of prokaryotic toxin-antitoxin partners. *Current opinion in structural biology* **21**: 109–118.

Blower, T. R., Short, F. L., Rao, F., Mizuguchi, K., Pei, X. Y., Fineran, P. C., Luisi, B. F. & Salmond, G. P., (2012) Identification and classification of bacterial Type III toxin–antitoxin systems encoded in chromosomal and plasmid genomes. *Nucleic acids research* **40**: 6158–6173.

Bordes, P., Cirinesi, A.-M., Ummels, R., Sala, A., Sakr, S., Bitter, W. & Genevaux, P., (2011) SecB-like chaperone controls a toxin–antitoxin stress-responsive system in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences* **108**: 8438–8443.

Bordes, P., Sala, A. J., Ayala, S., Texier, P., Slama, N., Cirinesi, A.-M., Guillet, V., Mourey, L. & Genevaux, P., (2016) Chaperone addiction of toxin–antitoxin systems. *Nature communications* **7**: 13339.

Brown, B. L., Grigoriu, S., Kim, Y., Arruda, J. M., Davenport, A., Wood, T. K., Peti, W. & Page, R., (2009) Three Dimensional Structure of the MqsR:MqsA Complex: A Novel TA Pair Comprised of a Toxin Homologous to RelE and an Antitoxin with Unique Properties. *PLoS pathogens* **5**: e1000706.

Brzozowska, I. & Zielenkiewicz, U., (2013) Regulation of toxin-antitoxin systems by proteolysis. *Plasmid* **70**: 33–41.

Buchberger, A., Theyssen, H., Schröder, H., McCarty, J. S., Virgallita, G., Milkereit, P., Reinstein, J. & Bukau, B., (1995) Nucleotide-induced Conformational Changes in the ATPase and Substrate Binding Domains of the DnaK Chaperone Provide Evidence for Interdomain Communication. *Journal of Biological Chemistry* **270**: 16903–16910.

Budde, P. P., Davis, B. M., Yuan, J. & Waldor, M. K., (2007) Characterization of a *higBA* toxin-antitoxin locus in *Vibrio cholerae*. *Journal of bacteriology* **189**: 491–500.

Castro-Roa, D., Garcia-Pino, A., De Gieter, S., van Nuland, N. A., Loris, R. & Zenkin, N., (2013) The Fic protein Doc uses an inverted substrate to phosphorylate and inactivate EF-Tu. *Nature chemical biology* **9**: 811–817.

Charollais, J., Dreyfus, M. & Iost, I., (2004) CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic acids research* **32**: 2751–2759.

Cheng, H. Y., Soo, V. W., Islam, S., McAnulty, M. J., Benedik, M. J. & Wood, T. K., (2014) Toxin GhoT of the GhoT/GhoS toxin/antitoxin system damages the cell membrane to reduce adenosine triphosphate and to reduce growth under stress. *Environmental microbiology* **16**: 1741–1754.

Cheverton, Angela M., Gollan, B., Przydacz, M., Wong, Chi T., Mylona, A., Hare, Stephen A. & Helaine, S., (2016) A *Salmonella* Toxin Promotes Persister Formation through Acetylation of tRNA. *Molecular cell* **63**: 86–96.

Choi, W., Yamaguchi, Y., Lee, J. W., Jang, K. M., Inouye, M., Kim, S. G., Yoon, M. H. & Park, J. H., (2017) Translation-dependent mRNA cleavage by YhaV in *Escherichia coli*. *FEBS letters* **591**: 1853–1861.

Christensen-Dalsgaard, M. & Gerdes, K., (2006) Two *higBA* loci in the *Vibrio cholerae* superintegron encode mRNA cleaving enzymes and can stabilize plasmids. *Molecular microbiology* **62**: 397–411.

Christensen-Dalsgaard, M. & Gerdes, K., (2008) Translation affects YoeB and MazF messenger RNA interferase activities by different mechanisms. *Nucleic acids research* **36**: 6472–6481.

Christensen-Dalsgaard, M., Jorgensen, M. G. & Gerdes, K., (2010) Three new RelE-homologous mRNA interferases of *Escherichia coli* differentially induced by environmental stresses. *Molecular microbiology* **75**: 333–348.

Christensen, S. K. & Gerdes, K., (2003) RelE toxins from Bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. *Molecular microbiology* **48**: 1389–1400.

Christensen, S. K., Maenhaut-Michel, G., Mine, N., Gottesman, S., Gerdes, K. & Van Melderen, L., (2004) Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: involvement of the *yefM-yoeB* toxin-antitoxin system. *Molecular microbiology* **51**: 1705–1717.

Christensen, S. K., Mikkelsen, M., Pedersen, K. & Gerdes, K., (2001) RelE, a global inhibitor of translation, is activated during nutritional stress. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 14328–14333.

Chulavatnatol, M. & Atkinson, D. E., (1973) Phosphoenolpyruvate synthetase from *Escherichia coli* Effects of adenylate energy charge and modifier concentrations. *Journal of Biological Chemistry* **248**: 2712–2715.

Connolly, K., Rife, J. P. & Culver, G., (2008) Mechanistic insight into the ribosome biogenesis functions of the ancient protein KsgA. *Molecular microbiology* **70**: 1062–1075.

Coray, D. S., Wheeler, N. E., Heinemann, J. A. & Gardner, P. P., (2017) Why so narrow: Distribution of anti-sense regulated, type I toxin-antitoxin systems compared with type II and type III systems. *RNA biology* **14**: 275–280.

Cruz, J. W., Rothenbacher, F. P., Maehigashi, T., Lane, W. S., Dunham, C. M. & Woychik, N. A., (2014) Doc toxin is a kinase that inactivates elongation factor Tu. *Journal of Biological Chemistry* **289**: 7788–7798.

Culviner, P. H. & Laub, M. T., (2018) Global Analysis of the *E. coli* Toxin MazF Reveals Widespread Cleavage of mRNA and the Inhibition of rRNA Maturation and Ribosome Biogenesis. *Molecular cell*.

Daimon, Y., Narita, S.-i. & Akiyama, Y., (2015) Activation of TA system toxins suppresses lethality caused by the loss of σE in *Escherichia coli*. *Journal of bacteriology*.

Díaz-Orejas, R., Espinosa, M. & Yeo, C. C., (2017) The Importance of the Expendable: Toxin–Antitoxin Genes in Plasmids and Chromosomes. *Frontiers in microbiology* **8**: 1479.

Dörr, T., Vulić, M. & Lewis, K., (2010) Ciprofloxacin Causes Persister Formation by Inducing the TisB toxin in *Escherichia coli*. *PLOS Biology* **8**: e1000317.

Dy, R. L., Przybilski, R., Semeijn, K., Salmond, G. P. & Fineran, P. C., (2014) A widespread bacteriophage abortive infection system functions through a Type IV toxin–antitoxin mechanism. *Nucleic acids research* **42**: 4590–4605.

Engel, P., Goepfert, A., Stanger, F. V., Harms, A., Schmidt, A., Schirmer, T. & Dehio, C., (2012) Adenylylation control by intra-or intermolecular active-site obstruction in Fic proteins. *Nature* **482**: 107.

Espinosa-Urgel, M., Kolter, R. & Ramos, J.-L., (2002) Root colonization by *Pseudomonas putida*: love at first sight. *Microbiology* **148**: 341–343.

Espinosa-Urgel, M., Salido, A. & Ramos, J.-L., (2000) Genetic Analysis of Functions Involved in Adhesion of *Pseudomonas putida* to Seeds. *Journal of bacteriology* **182**: 2363–2369.

Feng, S., Chen, Y., Kamada, K., Wang, H., Tang, K., Wang, M. & Gao, Y.-G., (2013) YoeB–ribosome structure: a canonical RNase that requires the ribosome for its specific activity. *Nucleic acids research* **41**: 9549–9556.

Fineran, P. C., Blower, T. R., Foulds, I. J., Humphreys, D. P., Lilley, K. S. & Salmond, G. P., (2009) The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 894–899.

Garcia-Pino, A., Balasubramanian, S., Wyns, L., Gazit, E., De Greve, H., Magnuson, R. D., Charlier, D., van Nuland, N. A. & Loris, R., (2010) Allostery and intrinsic disorder mediate transcription regulation by conditional cooperativity. *Cell* **142**: 101–111.

Gerdes, K., Bech, F., Jørgensen, S., Løbner-Olesen, A., Rasmussen, P., Atlung, T., Boe, L., Karlstrom, O., Molin, S. & Meyenburg, K., (1986a) Mechanism of post-segregational 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. *The EMBO journal* **5**: 2023–2029.

Gerdes, K., Helin, K., Christensen, O. W. & Løbner-Olesen, A., (1988) Translational control and differential RNA decay are key elements regulating postsegregational expression of the killer protein encoded by the parB locus of plasmid R1. *Journal of molecular biology* **203**: 119–129.

Gerdes, K. & Maisonneuve, E., (2012) Bacterial persistence and toxin-antitoxin loci. *Annual review of microbiology* **66**: 103–123.

Gerdes, K., Rasmussen, P. B. & Molin, S., (1986b) Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 3116–3120.

Germain, E., Castro-Roa, D., Zenkin, N. & Gerdes, K., (2013) Molecular mechanism of bacterial persistence by HipA. *Molecular cell* **52**: 248–254.

Ginsburg, D. & Steitz, J. A., (1975) The 30 S ribosomal precursor RNA from *Escherichia coli*. A primary transcript containing 23 S, 16 S, and 5 S sequences. *Journal of Biological Chemistry* **250**: 5647–5654.

Goeders, N., Chai, R., Chen, B., Day, A. & Salmond, G. P., (2016) Structure, evolution, and functions of bacterial type III toxin-antitoxin systems. *Toxins* **8**: 282.

González Barrios, A. F., Zuo, R., Hashimoto, Y., Yang, L., Bentley, W. E. & Wood, T. K., (2006) Autoinducer 2 Controls Biofilm Formation in *Escherichia coli* through a Novel Motility Quorum-Sensing Regulator (MqsR, B3022). *Journal of bacteriology* **188**: 305–316.

Goormaghtigh, F., Fraikin, N., Putrinš, M., Hallaert, T., Hauryliuk, V., Garcia-Pino, A., Sjödin, A., Kasvandik, S., Udekwu, K., Tenson, T., Kaldalu, N. & Van Melderen, L., (2018) Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of *Escherichia coli* Type II Persister Cells. *mBio* **9**.

Gotfredsen, M. & Gerdes, K., (1998) The *Escherichia coli relBE* genes belong to a new toxin–antitoxin gene family. *Molecular microbiology* **29**: 1065–1076.

Griffin, M. A., Davis, J. H. & Strobel, S. A., (2013) Bacterial Toxin RelE: A Highly Efficient Ribonuclease with Exquisite Substrate Specificity Using Atypical Catalytic Residues. *Biochemistry* **52**: 8633–8642.

Guglielmini, J. & Van Melderen, L., (2011) Bacterial toxin-antitoxin systems: Translation inhibitors everywhere. *Mobile genetic elements* **1**: 283–290.

Guo, Y., Quiroga, C., Chen, Q., McAnulty, M. J., Benedik, M. J., Wood, T. K. & Wang, X., (2014) RalR (a DNase) and RalA (a small RNA) form a type I toxin–antitoxin system in *Escherichia coli*. *Nucleic acids research* **42**: 6448–6462.

Gurnev, P. A., Ortenberg, R., Dörr, T., Lewis, K. & Bezrukov, S. M., (2012) Persister-promoting bacterial toxin TisB produces anion-selective pores in planar lipid bilayers. *FEBS letters* **586**: 2529–2534.

Hadži, S., Garcia-Pino, A., Haesaerts, S., Jurėnas, D., Gerdes, K., Lah, J. & Loris, R., (2017) Ribosome-dependent *Vibrio cholerae* mRNAse HigB2 is regulated by a β-strand sliding mechanism. *Nucleic acids research* **45**: 4972–4983.

Hallez, R., Geeraerts, D., Sterckx, Y., Mine, N., Loris, R. & Van Melderen, L., (2010) New toxins homologous to ParE belonging to three-component toxin–antitoxin systems in *Escherichia coli* O157: H7. *Molecular microbiology* **76**: 719–732.

Harms, A., Brodersen, D. E., Mitarai, N. & Gerdes, K., (2018) Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Molecular cell*.

Harms, A., Fino, C., Sørensen, M. A., Semsey, S. & Gerdes, K., (2017) Prophages and Growth Dynamics Confound Experimental Results with Antibiotic-Tolerant Persister Cells. *mBio* **8**: e01964–01917.

Harms, A., Stanger, Frédéric V., Scheu, Patrick D., de Jong, Imke G., Goepfert, A., Glatter, T., Gerdes, K., Schirmer, T. & Dehio, C., (2015) Adenylylation of Gyrase and Topo IV by FicT Toxins Disrupts Bacterial DNA Topology. *Cell Reports* **12**: 1497–1507.

Hayes, F., (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* **301**: 1496–1499.

Helaine, S., Cheverton, A. M., Watson, K. G., Faure, L. M., Matthews, S. A. & Holden, D. W., (2014) Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* **343**: 204–208.

Hu, Y., Benedik, M. J. & Wood, T. K., (2012) Antitoxin DinJ influences the general stress response through transcript stabilizer CspE. *Environmental microbiology* **14**: 669–679.

Hurley, J. M. & Woychik, N. A., (2009) Bacterial toxin HigB associates with ribosomes and mediates translation-dependent mRNA cleavage at A-rich sites. *The Journal of biological chemistry* **284**: 18605–18613.

Hwang, J.-Y. & Buskirk, A. R., (2017) A ribosome profiling study of mRNA cleavage by the endonuclease RelE. *Nucleic acids research* **45**: 327–336.

Inoue, K., Alsina, J., Chen, J. & Inouye, M., (2003) Suppression of defective ribosome assembly in a *rbfA* deletion mutant by overexpression of Era, an essential GTPase in *Escherichia coli*. *Molecular microbiology* **48**: 1005–1016.

Jacob, Asha I., Köhrer, C., Davies, Bryan W., RajBhandary, Uttam L. & Walker, Graham C., (2013) Conserved Bacterial RNase YbeY Plays Key Roles in 70S Ribosome Quality Control and 16S rRNA Maturation. *Molecular cell* **49**: 427–438.

Jahn, N., Brantl, S. & Strahl, H., (2015) Against the mainstream: the membrane-associated type I toxin BsrG from *Bacillus subtilis* interferes with cell envelope biosynthesis without increasing membrane permeability. *Molecular microbiology* **98**: 651–666.

Jahn, N., Preis, H., Wiedemann, C. & Brantl, S., (2012) BsrG/SR4 from *Bacillus subtilis*– the first temperature-dependent type I toxin–antitoxin system. *Molecular microbiology* **83**: 579–598.

Jankevicius, G., Ariza, A., Ahel, M. & Ahel, I., (2016) The Toxin-Antitoxin System DarTG Catalyzes Reversible ADP-Ribosylation of DNA. *Molecular cell* **64**: 1109–1116.

Janssen, B. D., Garza-Sánchez, F. & Hayes, C. S., (2015) YoeB toxin is activated during thermal stress. *MicrobiologyOpen* **4**: 682–697.

Jensen, R. B. & Gerdes, K., (1995) Programmed cell death in bacteria: proteic plasmid stabilization systems. *Molecular microbiology* **17**: 205–210.

Jiang, Y., Pogliano, J., Helinski, D. R. & Konieczny, I., (2002) ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. *Molecular microbiology* **44**: 971–979.

Jørgensen, M. G., Pandey, D. P., Jaskolska, M. & Gerdes, K., (2009) HicA of *Escherichia coli* defines a novel family of translation-independent mRNA interferases in bacteria and archaea. *Journal of bacteriology* **191**: 1191–1199.

Jurėnas, D., Chatterjee, S., Konijnenberg, A., Sobott, F., Droogmans, L., Garcia-Pino, A. & Van Melderen, L., (2017) AtaT blocks translation initiation by N-acetylation of the initiator tRNA fMet. *Nature chemical biology* **13**: 640.

Kaczanowska, M. & Rydén-Aulin, M., (2007) Ribosome biogenesis and the translation process in *Escherichia coli*. *Microbiology and molecular biology reviews : MMBR* **71**: 477–494.

Kamada, K. & Hanaoka, F., (2005) Conformational change in the catalytic site of the ribonuclease YoeB toxin by YefM antitoxin. *Molecular cell* **19**: 497–509.

Kasari, V., Kurg, K., Margus, T., Tenson, T. & Kaldalu, N., (2010) The *Escherichia coli mqsR* and *ygiT* Genes Encode a New Toxin-Antitoxin Pair. *Journal of bacteriology* **192**: 2908–2919.

Kaspy, I., Rotem, E., Weiss, N., Ronin, I., Balaban, N. Q. & Glaser, G., (2013) HipA-mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase. *Nature communications* **4**: 3001.

Kawano, M., Aravind, L. & Storz, G., (2007) An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Molecular microbiology* **64**: 738–754.

Kikuchi, G., Motokawa, Y., Yoshida, T. & Hiraga, K., (2008) Glycine cleavage system: reaction mechanism, physiological significance, and hyperglycinemia. *Proceedings of the Japan Academy, Series B* **84**: 246–263.

Kim, J.-S., Schantz, A. B., Song, S., Kumar, M. & Wood, T. K., (2018) GhoT of the GhoT/GhoS toxin/antitoxin system damages lipid membranes by forming transient pores. *Biochemical and biophysical research communications* **497**: 467–472.

Kim, Y., Wang, X., Zhang, X. S., Grigoriu, S., Page, R., Peti, W. & Wood, T. K., (2010) *Escherichia coli* toxin/antitoxin pair MqsR/MqsA regulate toxin CspD. *Environmental microbiology* **12**: 1105–1121.

Kim, Y. & Wood, T. K., (2010) Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. *Biochemical and biophysical research communications* **391**: 209–213.

Kolodkin-Gal, I. & Engelberg-Kulka, H., (2008) The Extracellular Death Factor: Physiological and Genetic Factors Influencing Its Production and Response in *Escherichia coli*. *Journal of bacteriology* **190**: 3169–3175.

Kwan, B. W., Lord, D. M., Peti, W., Page, R., Benedik, M. J. & Wood, T. K., (2015) The MqsR/MqsA toxin/antitoxin system protects *Escherichia coli* during bile acid stress. *Environmental microbiology* **17**: 3168–3181.

Lehnherr, H. & Yarmolinsky, M. B., (1995) Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 3274–3277.

Leplae, R., Geeraerts, D., Hallez, R., Guglielmini, J., Dreze, P. & Van Melderen, L., (2011) Diversity of bacterial type II toxin-antitoxin systems: a comprehensive search and functional analysis of novel families. *Nucleic acids research* **39**: 5513–5525.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N. & Balaban, N. Q., (2017) Antibiotic tolerance facilitates the evolution of resistance. *Science* **355**: 826.

Li, G.-Y., Zhang, Y., Inouye, M. & Ikura, M., (2009) Inhibitory Mechanism of *Escherichia coli* RelE-RelB Toxin-Antitoxin Module Involves a Helix Displacement Near an mRNA Interferase Active Site. *Journal of Biological Chemistry* **284**: 14628–14636.

Liu, M., Zhang, Y., Inouye, M. & Woychik, N. A., (2008) Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 5885–5890.

Luidalepp, H., Jõers, A., Kaldalu, N. & Tenson, T., (2011) Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. *Journal of bacteriology* **193**: 3598–3605.

Maehigashi, T., Ruangprasert, A., Miles, S. J. & Dunham, C. M., (2015) Molecular basis of ribosome recognition and mRNA hydrolysis by the *E. coli* YafQ toxin. *Nucleic acids research* **43**: 8002–8012.

Magnuson, R. D., (2007) Hypothetical Functions of Toxin-Antitoxin Systems. *Journal of bacteriology* **189**: 6089–6092.

Maisonneuve, E., Castro-Camargo, M. & Gerdes, K., (2013) (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. *Cell* **154**: 1140–1150.

Maisonneuve, E., Shakespeare, L. J., Jorgensen, M. G. & Gerdes, K., (2011) Bacterial persistence by RNA endonucleases. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 13206–13211.

Makarova, K. S., Grishin, N. V. & Koonin, E. V., (2006) The HicAB cassette, a putative novel, RNA-targeting toxin-antitoxin system in archaea and bacteria. *Bioinformatics* **22**: 2581–2584.

Makarova, K. S., Wolf, Y. I. & Koonin, E. V., (2009) Comprehensive comparative-genomic analysis of Type 2 toxin-antitoxin systems and related mobile stress response systems in prokaryotes. *Biology Direct* **4**: 19.

Maki, S., Takiguchi, S., Horiuchi, T., Sekimizu, K. & Miki, T., (1996) Partner Switching Mechanisms in Inactivation and Rejuvenation of *Escherichia coli* DNA Gyrase by F Plasmid Proteins LetD (CcdB) and LetA (CcdA). *Journal of molecular biology* **256**: 473–482.

Marimon, O., Teixeira, J. M. C., Cordeiro, T. N., Soo, V. W. C., Wood, T. L., Mayzel, M., Amata, I., García, J., Morera, A., Gay, M., Vilaseca, M., Orekhov, V. Y., Wood, T. K. & Pons, M., (2016) An oxygen-sensitive toxin–antitoxin system. *Nature communications* **7**: 13634.

Masuda, H. & Inouye, M., (2017) Toxins of Prokaryotic Toxin-Antitoxin Systems with Sequence-Specific Endoribonuclease Activity. *Toxins* **9**: 140.

Masuda, H., Tan, Q., Awano, N., Wu, K. P. & Inouye, M., (2012a) YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in *Escherichia coli*. *Molecular microbiology* **84**: 979–989.

Masuda, H., Tan, Q., Awano, N., Yamaguchi, Y. & Inouye, M., (2012b) A novel membrane-bound toxin for cell division, CptA (YgfX), inhibits polymerization of cytoskeleton proteins, FtsZ and MreB, in *Escherichia coli*. *FEMS microbiology letters* **328**: 174–181.

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

Miki, T., Park, J. A., Nagao, K., Murayama, N. & Horiuchi, T., (1992) Control of segregation of chromosomal DNA by sex factor F in *Escherichia coli*: mutants of DNA gyrase subunit A suppress *letD* (*ccdB*) product growth inhibition. *Journal of molecular biology* **225**: 39–52.

Miyamoto, T., Kato, Y., Sekiguchi, Y., Tsuneda, S. & Noda, N., (2016) Characterization of MazF-Mediated Sequence-Specific RNA Cleavage in *Pseudomonas putida* Using Massive Parallel Sequencing. *PloS one* **11**: e0149494.

Molenaar, D., van der Rest, M. E., Drysch, A. & Yücel, R., (2000) Functions of the membrane-associated and cytoplasmic malate dehydrogenases in the citric acid cycle of *Corynebacterium glutamicum*. *Journal of bacteriology* **182**: 6884–6891.

Morikawa, M., Izui, K., Taguchi, M. & Katsuki, H., (1980) Regulation of *Escherichia coli* phosphoenolpyruvate carboxylase by multiple effectors in vivo. *The Journal of Biochemistry* **87**: 441–449.

Moro, F., Fernández, V. & Muga, A., (2002) Interdomain interaction through helices A and B of DnaK peptide binding domain. *FEBS letters* **533**: 119–123.

Motiejūnaitė, R., Armalytė, J., Markuckas, A. & Sužiedėlienė, E., (2007) *Escherichia coli dinJ-yafQ* genes act as a toxin–antitoxin module. *FEMS microbiology letters* **268**: 112–119.

Mutschler, H., Gebhardt, M., Shoeman, R. L. & Meinhart, A., (2011) A Novel Mechanism of Programmed Cell Death in Bacteria by Toxin–Antitoxin Systems Corrupts Peptidoglycan Synthesis. *PLOS Biology* **9**: e1001033.

Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A. P., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Lee, P. C., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K. N., Düsterhöft, A., Tümmler, B. & Fraser, C. M., (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environmental microbiology* **4**: 799–808.

Neubauer, C., Gao, Y. G., Andersen, K. R., Dunham, C. M., Kelley, A. C., Hentschel, J., Gerdes, K., Ramakrishnan, V. & Brodersen, D. E., (2009) The structural basis for mRNA recognition and cleavage by the ribosome-dependent endonuclease RelE. *Cell* **139**: 1084–1095.

Norton, J. P. & Mulvey, M. A., (2012) Toxin-antitoxin systems are important for niche-specific colonization and stress resistance of uropathogenic *Escherichia coli*. *PLoS pathogens* **8**: e1002954.

Ogura, T. & Hiraga, S., (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **80**: 4784–4788.

Otsuka, Y. & Yonesaki, T., (2012) Dmd of bacteriophage T4 functions as an antitoxin against *Escherichia coli* LsoA and RnlA toxins. *Molecular microbiology* **83**: 669–681.

Page, R. & Peti, W., (2016) Toxin-antitoxin systems in bacterial growth arrest and persistence. *Nature chemical biology* **12**: 208–214.

Pandey, D. P. & Gerdes, K., (2005) Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic acids research* **33**: 966–976.

Pecota, D. C. & Wood, T. K., (1996) Exclusion of T4 phage by the *hok*/*sok* killer locus from plasmid R1. *Journal of bacteriology* **178**: 2044–2050.

Pedersen, K., Christensen, S. K. & Gerdes, K., (2002) Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Molecular microbiology* **45**: 501–510.

Pedersen, K., Zavialov, A. V., Pavlov, M. Y., Elf, J., Gerdes, K. & Ehrenberg, M., (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* **112**: 131–140.

Peil, L., Virumäe, K. & Remme, J., (2008) Ribosome assembly in *Escherichia coli* strains lacking the RNA helicase DeaD/CsdA or DbpA. *The FEBS journal* **275**: 3772–3782.

Prysak, M. H., Mozdzierz, C. J., Cook, A. M., Zhu, L., Zhang, Y., Inouye, M. & Woychik, N. A., (2009) Bacterial toxin YafQ is an endoribonuclease that associates with the ribosome and blocks translation elongation through sequence-specific and frame-dependent mRNA cleavage. *Molecular microbiology* **71**: 1071–1087.

Putrinš, M., Ainelo, A., Ilves, H. & Hõrak, R., (2011) The ColRS system is essential for the hunger response of glucose-growing *Pseudomonas putida*. *BMC microbiology* **11**: 170.

Ramisetty, B. C. M., Natarajan, B. & Santhosh, R. S., (2015) *mazEF*-mediated programmed cell death in bacteria: "What is this?". *Critical Reviews in Microbiology* **41**: 89–100.

Ramisetty, B. C. M., Raj, S. & Ghosh, D., (2016) *Escherichia coli* MazEF toxin-antitoxin system does not mediate programmed cell death. *Journal of Basic Microbiology* **56**: 1398–1402.

Ren, D., Bedzyk, L. A., Thomas, S. M., Ye, R. W. & Wood, T. K., (2004) Gene expression in *Escherichia coli* biofilms. *Applied Microbiology and Biotechnology* **64**: 515–524.

Rocker, A. & Meinhart, A., (2015) A *cis*-acting antitoxin domain within the chromosomal toxin-antitoxin module EzeT of *Escherichia coli* quenches toxin activity. *Molecular microbiology* **97**: 589–604.

Rocker, A., Peschke, M., Kittilä, T., Sakson, R., Brieke, C. & Meinhart, A., (2018) The ng_ζ1 toxin of the gonococcal epsilon/zeta toxin/antitoxin system drains precursors for cell wall synthesis. *Nature communications* **9**: 1686.

Sala, A., Calderon, V., Bordes, P. & Genevaux, P., (2013) TAC from *Mycobacterium tuberculosis*: a paradigm for stress-responsive toxin–antitoxin systems controlled by SecB-like chaperones. *Cell Stress and Chaperones* **18**: 129–135.

Samson, J. E., Spinelli, S., Cambillau, C. & Moineau, S., (2013) Structure and activity of AbiQ, a lactococcal endoribonuclease belonging to the type III toxin–antitoxin system. *Molecular microbiology* **87**: 756–768.

Sanwal, B. D. & Smando, R., (1969) Malic Enzyme of *Escherichia coli* diversity of the effectors controlling enzyme activity. *Journal of Biological Chemistry* **244**: 1817–1823.

Schifano, J. M., Cruz, J. W., Vvedenskaya, I. O., Edifor, R., Ouyang, M., Husson, R. N., Nickels, B. E. & Woychik, N. A., (2016) tRNA is a new target for cleavage by a MazF toxin. *Nucleic acids research* **44**: 1256–1270.

Schifano, J. M., Edifor, R., Sharp, J. D., Ouyang, M., Konkimalla, A., Husson, R. N. & Woychik, N. A., (2013) Mycobacterial toxin MazF-mt6 inhibits translation through cleavage of 23S rRNA at the ribosomal A site. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 8501–8506.

Schifano, J. M., Vvedenskaya, I. O., Knoblauch, J. G., Ouyang, M., Nickels, B. E. & Woychik, N. A., (2014) An RNA-seq method for defining endoribonuclease cleavage specificity identifies dual rRNA substrates for toxin MazF-mt3. *Nature communications* **5**: 3538.

Schmidt, O., Schuenemann, V. J., Hand, N. J., Silhavy, T. J., Martin, J., Lupas, A. N. & Djuranovic, S., (2007) *prlF* and *yhaV* encode a new toxin–antitoxin system in *Escherichia coli*. *Journal of molecular biology* **372**: 894–905.

Schumacher, M. A., Piro, K. M., Xu, W., Hansen, S., Lewis, K. & Brennan, R. G., (2009) Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. *Science* **323**: 396–401.

Schureck, M. A., Dunkle, J. A., Maehigashi, T., Miles, S. J. & Dunham, C. M., (2015) Defining the mRNA recognition signature of a bacterial toxin protein. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 13862–13867.

Schureck, M. A., Maehigashi, T., Miles, S. J., Marquez, J., Cho, S. E., Erdman, R. & Dunham, C. M., (2014) Structure of the *Proteus vulgaris* HigB-(HigA)2-HigB toxin-antitoxin complex. *The Journal of biological chemistry* **289**: 1060–1070.

Schureck, M. A., Maehigashi, T., Miles, S. J., Marquez, J. & Dunham, C. M., (2016a) mRNA bound to the 30S subunit is a HigB toxin substrate. *Rna* **22**: 1261–1270.

Schureck, M. A., Repack, A., Miles, S. J., Marquez, J. & Dunham, C. M., (2016b) Mechanism of endonuclease cleavage by the HigB toxin. *Nucleic acids research* **44**: 7944–7953.

Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K. & Lewis, K., (2006) Persisters: a distinct physiological state of *E. coli*. *BMC microbiology* **6**: 53.

Shajani, Z., Sykes, M. T. & Williamson, J. R., (2011) Assembly of bacterial ribosomes. *Annual review of biochemistry* **80**: 501–526.

Short, F. L., Pei, X. Y., Blower, T. R., Ong, S.-L., Fineran, P. C., Luisi, B. F. & Salmond, G. P. C., (2013) Selectivity and self-assembly in the control of a bacterial toxin by an antitoxic noncoding RNA pseudoknot. *Proceedings of the National Academy of Sciences* **110**: E241.

Siibak, T. & Remme, J., (2010) Subribosomal particle analysis reveals the stages of bacterial ribosome assembly at which rRNA nucleotides are modified. *Rna* **16**: 2023–2032.

Silvaggi, J. M., Perkins, J. B. & Losick, R., (2005) Small untranslated RNA antitoxin in *Bacillus subtilis*. *Journal of bacteriology* **187**: 6641–6650.

Smith, A. S. & Rawlings, D. E., (1997) The poison–antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. *Molecular microbiology* **26**: 961–970.

Smith, B. A., Gupta, N., Denny, K. & Culver, G. M., (2018) Characterization of 16S rRNA Processing with Pre-30S Subunit Assembly Intermediates from *E. coli*. *Journal of molecular biology* **430**: 1745–1759.

Smock, R. G., Blackburn, M. E. & Gierasch, L. M., (2011) Conserved, disordered C terminus of DnaK enhances cellular survival upon stress and DnaK in vitro chaperone activity. *The Journal of biological chemistry* **286**: 31821–31829.

Song, S. & Wood, T. K., (2018) Post-segregational Killing and Phage Inhibition Are Not Mediated by Cell Death Through Toxin/Antitoxin Systems. *Frontiers in Microbiology* **9**.

Srivastava, A. K. & Schlessinger, D., (1988) Coregulation of processing and translation: mature 5' termini of *Escherichia coli* 23S ribosomal RNA form in polysomes. *Proceedings of the National Academy of Sciences of the United States of America* **85**: 7144–7148.

Szekeres, S., Dauti, M., Wilde, C., Mazel, D. & Rowe-Magnus, D. A., (2007) Chromosomal toxin–antitoxin loci can diminish large-scale genome reductions in the absence of selection. *Molecular microbiology* **63**: 1588–1605.

Sun, C., Guo, Y., Tang, K., Wen, Z., Li, B., Zeng, Z. & Wang, X., (2017) MqsR/MqsA Toxin/Antitoxin System Regulates Persistence and Biofilm Formation in *Pseudomonas putida* KT2440. *Frontiers in Microbiology* **8**.

Suzuki, M., Mao, L. & Inouye, M., (2007) Single protein production (SPP) system in *Escherichia coli*. *Nature protocols* **2**: 1802.

Zhang, J., Zhang, Y. & Inouye, M., (2003a) Characterization of the interactions within the mazEF addiction module of *Escherichia coli*. *The Journal of biological chemistry* **278**: 32300–32306.

Zhang, Y. & Inouye, M., (2009) The inhibitory mechanism of protein synthesis by YoeB, an *Escherichia coli* toxin. *The Journal of biological chemistry* **284**: 6627–6638.

Zhang, Y. & Inouye, M., (2011) RatA (YfjG), an *Escherichia coli* toxin, inhibits 70S ribosome association to block translation initiation. *Molecular microbiology* **79**: 1418–1429.

Zhang, Y., Zhang, J., Hoeflich, K. P., Ikura, M., Qing, G. & Inouye, M., (2003b) MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Molecular cell* **12**: 913–923.

Zhang, Y., Yamaguchi, Y. & Inouye, M., (2009) Characterization of YafO, an *Escherichia coli* toxin. *The Journal of biological chemistry* **284**: 25522–25531.

Zhao, Y., McAnulty, M. J. & Wood, T. K., (2016) Toxin YafQ Reduces *Escherichia coli* Growth at Low Temperatures. *PloS one* **11**: e0161577.

Zhu, L., Zhang, Y., Teh, J.-S., Zhang, J., Connell, N., Rubin, H. & Inouye, M., (2006) Characterization of mRNA Interferases from *Mycobacterium tuberculosis*. *Journal of Biological Chemistry* **281**: 18638–18643.

Zielenkiewicz, U. & Ceglowski, P., (2005) The toxin-antitoxin system of the streptococcal plasmid pSM19035. *Journal of bacteriology* **187**: 6094–6105.

Zuo, Z., Gong, T., Che, Y., Liu, R., Xu, P., Jiang, H., Qiao, C., Song, C. & Yang, C., (2015) Engineering *Pseudomonas putida* KT2440 for simultaneous degradation of organophosphates and pyrethroids and its application in bioremediation of soil. *Biodegradation* **26**: 223–233.

Talavera, A., Tamman, H., Ainelo, A., Hadži, S., Garcia-Pino, A., Hõrak, R., Konijnenberg, A. & Loris, R., (2017) Production, biophysical characterization and crystallization of *Pseudomonas putida* GraA and its complexes with GraT and the *graTA* operator. *Acta Crystallographica Section F: Structural Biology Communications* **73**.

Tam, J. E. & Kline, B. C., (1989) The F plasmid *ccd* autorepressor is a complex of CcdA and CcdB proteins. *Molecular and General Genetics MGG* **219**: 26–32.

Tamman, H., Ainelo, A., Ainsaar, K. & Hõrak, R., (2014) A moderate toxin, GraT, modulates growth rate and stress tolerance of *Pseudomonas putida*. *Journal of bacteriology* **196**: 157–169.

Tamman, H., Ainelo, A., Tagel, M. & Hõrak, R., (2016) Stability of the GraA Antitoxin Depends on Growth Phase, ATP Level, and Global Regulator MexT. *Journal of bacteriology* **198**: 787–796.

Tan, Q., Awano, N. & Inouye, M., (2011) YeeV is an *Escherichia coli* toxin that inhibits cell division by targeting the cytoskeleton proteins, FtsZ and MreB. *Molecular microbiology* **79**: 109–118.

The UniProt Consortium, (2017) UniProt: the universal protein knowledgebase. *Nucleic acids research* **45**: D158–D169.

Tian, Q. B., Hayashi, T., Murata, T. & Terawaki, Y., (1996a) Gene product identification and promoter analysis of *hig* locus of plasmid Rts1. *Biochemical and biophysical research communications* **225**: 679–684.

Tian, Q. B., Ohnishi, M., Tabuchi, A. & Terawaki, Y., (1996b) A New Plasmid-Encoded Proteic Killer Gene System: Cloning, Sequencing, and Analyzing *hig* Locus of Plasmid Rts1. *Biochemical and biophysical research communications* **220**: 280–284.

Tsilibaris, V., Maenhaut-Michel, G., Mine, N. & Van Melderen, L., (2007) What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? *Journal of bacteriology* **189**: 6101–6108.

Tsuchimoto, S., Nishimura, Y. & Ohtsubo, E., (1992) The stable maintenance system pem of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. *Journal of bacteriology* **174**: 4205–4211.

Tuomanen, E., Cozens, R., Tosch, W., Zak, O. & Tomasz, A., (1986) The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. *Microbiology* **132**: 1297–1304.

Unoson, C. & Wagner, E. G. H., (2008) A small SOS□induced toxin is targeted against the inner membrane in *Escherichia coli*. *Molecular microbiology* **70**: 258–270.

Van Melderen, L., (2010) Toxin–antitoxin systems: why so many, what for? *Current Opinion in Microbiology* **13**: 781–785.

Van Melderen, L., Bernard, P. & Couturier, M., (1994) Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. *Molecular microbiology* **11**: 1151–1157.

Van Melderen, L. & Saavedra De Bast, M., (2009) Bacterial toxin-antitoxin systems: more than selfish entities? *PLoS genetics* **5**: e1000437.

Wang, X., Kim, Y., Hong, S. H., Ma, Q., Brown, B. L., Pu, M., Tarone, A. M., Benedik, M. J., Peti, W., Page, R. & Wood, T. K., (2011) Antitoxin MqsA helps mediate the bacterial general stress response. *Nature chemical biology* **7**: 359–366.

Wang, X., Lord, D. M., Cheng, H. Y., Osbourne, D. O., Hong, S. H., Sanchez-Torres, V., Quiroga, C., Zheng, K., Herrmann, T., Peti, W., Benedik, M. J., Page, R. & Wood, T. K., (2012) A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nature chemical biology* **8**: 855–861.

Wang, X., Lord, D. M., Hong, S. H., Peti, W., Benedik, M. J., Page, R. & Wood, T. K., (2013) Type II toxin/antitoxin MqsR/MqsA controls type V toxin/antitoxin GhoT/GhoS. *Environmental microbiology* **15**: 1734–1744.

Wang, X. & Wood, T. K., (2011) Toxin/Antitoxin Systems Influence Biofilm and Persister Cell Formation and the General Stress Response. *Applied and Environmental Microbiology*.

Verstraeten, N., Knapen, W. J., Kint, C. I., Liebens, V., Van den Bergh, B., Dewachter, L., Michiels, J. E., Fu, Q., David, C. C., Fierro, A. C., Marchal, K., Beirlant, J., Versees, W., Hofkens, J., Jansen, M., Fauvart, M. & Michiels, J., (2015) Obg and Membrane Depolarization Are Part of a Microbial Bet-Hedging Strategy that Leads to Antibiotic Tolerance. *Molecular cell* **59**: 9–21.

Vesper, O., Amitai, S., Belitsky, M., Byrgazov, K., Kaberdina, A. C., Engelberg-Kulka, H. & Moll, I., (2011) Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in *Escherichia coli*. *Cell* **147**: 147–157.

Winther, K., Tree, J. J., Tollervey, D. & Gerdes, K., (2016) VapCs of *Mycobacterium tuberculosis* cleave RNAs essential for translation. *Nucleic acids research* **44**: 9860–9871.

Winther, K. S., Brodersen, D. E., Brown, A. K. & Gerdes, K., (2013) VapC20 of *Mycobacterium tuberculosis* cleaves the sarcin-ricin loop of 23S rRNA. *Nature communications* **4**: 2796.

Winther, K. S. & Gerdes, K., (2011) Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 7403–7407.

Wozniak, R. A. F. & Waldor, M. K., (2009) A Toxin–Antitoxin System Promotes the Maintenance of an Integrative Conjugative Element. *PLoS genetics* **5**: e1000439.

Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C., Deng, Z. & Ou, H.-Y., (2018) TADB 2.0: an updated database of bacterial type II toxin–antitoxin loci. *Nucleic acids research* **46**: D749–D753.

Yamaguchi, Y. & Inouye, M., (2013) Type II Toxin-Antitoxin Loci: The *mazEF* Family. In: Prokaryotic Toxin-Antitoxins. K. Gerdes (ed). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 107–136.

Yamaguchi, Y., Nariya, H., Park, J.-H. & Inouye, M., (2012) Inhibition of specific gene expressions by protein-mediated mRNA interference. *Nature communications* **3**: 607.

Yamaguchi, Y., Park, J. H. & Inouye, M., (2009) MqsR, a crucial regulator for quorum sensing and biofilm formation, is a GCU-specific mRNA interferase in *Escherichia coli*. *The Journal of biological chemistry* **284**: 28746–28753.

Yamaguchi, Y., Park, J. H. & Inouye, M., (2011) Toxin-antitoxin systems in bacteria and archaea. *Annual review of genetics* **45**: 61–79.

Yang, J., Zhou, K., Liu, P., Dong, Y., Gao, Z., Zhang, J. & Liu, Q., (2016) Structural insight into the *E. coli* HigBA complex. *Biochemical and biophysical research communications* **478**: 1521–1527.

Yarbrough, M. L., Li, Y., Kinch, L. N., Grishin, N. V., Ball, H. L. & Orth, K., (2009) AMPylation of Rho GTPases by *Vibrio* VopS Disrupts Effector Binding and Downstream Signaling. *Science* **323**: 269.

Yuan, J., Sterckx, Y., Mitchenall, L. A., Maxwell, A., Loris, R. & Waldor, M. K., (2010) *Vibrio cholerae* ParE2 poisons DNA gyrase via a mechanism distinct from other gyrase inhibitors. *Journal of Biological Chemistry* **285**: 40397–40408.

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PUBLICATIONS

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List of publications:

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