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I **Tamman H, Ainelo A, Ainsaar K, Hõrak R.** A moderate toxin, GraT, modulates growth rate and stress tolerance of *Pseudomonas putida*. Journal of bacteriology. 2014 Jan;196(1):157–69.

II **Tamman H, Ainelo A, Tagel M, Hõrak R.** Stability of the GraA antitoxin depends on the growth phase, ATP level and global regulator MexT. Journal of bacteriology. 2015 Dec 14.

III **Ainelo A, Tamman H, Leppik M, Remme J, Hõrak R.** The toxin GraT inhibits ribosome biogenesis. Molecular microbiology. 2016 May;100(4):719–34.

My contribution to the publications is following:

Ref I – I participated in planning the experiments, construction of plasmids and strains, performed the *in vivo* experiments, contributed to the writing of the manuscript and participated in its editing.

Ref II – I participated in planning the experiments, construction of plasmids and strains, conducted the experiments and wrote the manuscript.

Ref III – I participated in planning and performed the *in vivo* transposon mutagenesis experiments and DnaK pull-down assay, participated in the writing and editing of the manuscript.

ABBREVIATIONS

(p)ppGpp	guanosine penta- or tetraphosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
chr I	the larger chromosome of *Vibrio cholerae*, contains most of the essential genes (about 2.96 Mbp)
chr II	the smaller chromosome of *Vibrio cholerae*, contains very few essential genes (about 1.07 Mbp)
GTP	guanosine triphosphate
ITC	isothermal titration calorimetry
LB	lysogeny broth
PCD	programmed cell death
PI	propidium iodide, a DNA binding stain that can permeate only damaged membranes
PSK	post segregational killing
SD	Shine-Dalgarno sequence
TA	toxin-antitoxin
WT	wild-type

Origin of the names of type II TA systems mentioned in the thesis

ChpB	chromosomal homologue of Pem, ChpBK – toxin, ChpBI – cognate antitoxin.
CcdAB	coupled cell division. CcdB – toxin, CcdA – cognate antitoxin
DinJ/YafQ	damage-inducible, YafQ – systematic nomenclature, toxin, DinJ – cognate antitoxin.
GraTA	growth-rate-affecting, GraT – toxin, GraA – cognate antitoxin.
HipBA	high persistence protein, HipA – toxin, HipB – cognate antitoxin.
HigAB	host inhibition of growth; HigB – toxin, HigA – cognate antitoxin.
HicAB	hif contiguous (homologues of *Haemophilus influenza hif* locus); HicA – toxin, HicB – antitoxin.
Kis/Kid	Kid – Killing determinant, toxin, Kis – a suppressor of killer, cognate antitoxin.
MazEF	"ma-ze" means "What is it?" in Hebrew, MazF – toxin, MazE – cognate antitoxin.
MosAT	maintenance of SXT, MosT – toxin, MosA – cognate antitoxin.
MqsRA	MqsR – motility quorum-sensing regulator, toxin, MqsA – cognate antitoxin.
ParDE	derived from the word partition, ParE – toxin, ParD – cognate antitoxin.
PezAT	PezT – pneumococcal epsilon zeta toxin, PezA – cognate antitoxin.

Phd/Doc	Doc – death on curing, toxin, Phd – prevents host death, antitoxin.
RatA	ribosome association toxin.
RelBE	derived from the 'delayed-relaxed' phenotype (mutations in *relE* cause a lag before stable RNA synthesis continues after inducing amino acid starvation). RelE – toxin, RelB – cognate antitoxin.
RnlAB	RNase LS (late gene silencing in T4), RnlA – toxin, RnlB – cognate antitoxin.
TalAB	TA (toxin-antitoxin) of *Lxc* (*Leifsonia xyli* subsp. *cynodontis)*, TalB – toxin, TalA – cognate antitoxin.
VapBC	virulence associated protein, VapC – toxin; VapB – cognate antitoxin.
YafNO	systematic nomenclature. YafO – toxin, YafN – cognate antitoxin.
YefM/YoeB	systematic nomenclature. YoeB – toxin, YefM – cognate antitoxin.

INTRODUCTION

Life is full of stress. This is true for us, but even more so for unicellular organisms like bacteria. Bacteria inhabit a wide variety of different environments and often encounter very diverse stress conditions. During stress the most convenient thing for us sometimes seems to be curling up, doing nothing and waiting for things to get better. A similar strategy is also used by bacteria – upon stress conditions their growth rate decreases and dormancy is induced (Lewis, 2007). This helps bacteria to survive under unfavourable conditions and resume growth after the stress has passed (Tuomanen*, et al.*, 1986, Eng*, et al.*, 1991). Many different growth rate regulating mechanisms have evolved that help bacteria cope with stress (Starosta*, et al.*, 2014). In my thesis, I will focus on a recently recognized mechanism of stress tolerance – the potentially poisonous bacterial toxin-antitoxin (TA) systems.

As a general rule, these systems consist of two counterparts – a toxin that inhibits some major process or compartment of the cell, and an antitoxin that is able to counteract the harmful effect of the toxin (Buts*, et al.*, 2005). At first, these potentially noxious modules were discovered in plasmids, where they are important for maintaining the plasmid in the bacterial population (Gerdes*, et al.*, 1986a). Later on they were also found to be abundant in the bacterial chromosomes (Pandey & Gerdes, 2005). The importance of chromosomal TA modules has been a subject of debate ever since their discovery as it seems incomprehensible for a living organism to produce a self-poisoning protein. Yet, recent results have associated the chromosomal TA systems not only with growth and dormancy of bacteria, but also with other stress responses, suggesting their involvement in virulence modulation, phage protection, biofilm formation and general stress response (Gerdes*, et al.*, 2005).

The participation of TA systems in stress management was first proposed because of their uneven distribution among bacteria from different habitats. There are numerous TA systems in the chromosomes of environmental and pathogenic species, i.e. in bacteria that frequently encounter different stressful conditions during their life (Pandey & Gerdes, 2005). In intracellular bacteria, on the other hand, none or a few systems are present, suggesting that in stable environments TA systems lose their importance and respective genes are eliminated from the genome (Pandey & Gerdes, 2005). Yet, although in recent years the TA systems have been extensively studied, their biological importance and a precise role in bacterial stress tolerance has remained a subject of debate.

The natural habitat of the environmental bacterium *Pseudomonas putida* is very varying, necessitating the need for different mechanisms to survive the potentially encountered stress conditions (Silby*, et al.*, 2011). Therefore, it is not surprising that this bacterium encodes many TA modules in its chromosome (Shao*, et al.*, 2011). Yet, when I started my PhD studies, only limited knowledge about the TA systems in this bacterium was available. The intriguing finding that a disruption of a putative antitoxin gene PP1585 leads to the alleviation of

membrane stress of bacteria deficient in the ColRS two-component system (Putriņš*, et al.*, 2011) led us to study that TA system in *P. putida* more closely to determine its involvement in suppression of the membrane stress of *colR*-deficient bacteria as well as its overall role in the physiology and stress management of *P. putida*.

The first part of this thesis gives an overview of the current knowledge about the variability of different TA systems: their mechanisms of action as well as the involvement of the chromosomal TA modules in the physiology and stress regulation of different bacteria. The experimental part of the thesis focuses on the characterization of the growth-rate-affecting GraTA system, which is the first well-described TA system in *P. putida* and possesses several unusual features not present in other TA systems.

REVIEW OF LITERATURE

1. General traits of toxin-antitoxin systems

Toxin-antitoxin (TA) systems are widespread in the prokaryotic world (Pandey & Gerdes, 2005). In general, bacterial TA systems, as the name suggests, consist of two counterparts: a toxin and an antitoxin. The toxin influences cell growth by targeting major processes and compartments like translation, replication, cell wall or membrane. Under normal conditions the activity of the toxin is inhibited by the other component of the system – the antitoxin. There are also a few unconventional systems that are composed of three counterparts (Zielenkiewicz & Ceglowski, 2005, Hallez*, et al.*, 2010, Melnicakova*, et al.*, 2015) and so far just one TA module has been described that has both activities in one molecule (Rocker & Meinhart, 2015). The activation of a TA system requires either increased expression of the toxin or decreased ability of the antidote to inhibit the toxin. The latter is most often achieved by triggering the degradation of the antitoxin (Brzozowska & Zielenkiewicz, 2013). As a result, the stable toxin is released from the antitoxin's inhibition, and can exert the poisonous effect on its target(s) thereby influencing the life of the host bacterium.

TA systems can be encoded on plasmids as well as on chromosomes. At first these small modules were discovered from low-copy number plasmids where they function as plasmid maintenance systems through a mechanism known as post-segregational killing (PSK) (Ogura & Hiraga, 1983, Gerdes*, et al.*, 1986b, Gerdes*, et al.*, 1986a) (Fig 1). In a stable system, when bacterium harbours the TA-encoding plasmid, both the toxin and antitoxin are produced. The antitoxin prevents the activation of the toxin, for example, by complex formation. Although the antitoxin is labile, it can be produced from the plasmid. Yet, unequal distribution of plasmids during cell division may result in daughter cells without the plasmid and, thus, disability to produce either toxin or antitoxin. However, the TA proteins are inherited through the cytoplasm and as antitoxins are degraded faster than toxins, the toxins are freed from the antitoxin-mediated inhibition. Most toxins act in a bacteriostatic manner, but they can also have an irreversible effect and kill the cells. Nonetheless, irrespective of whether the toxin effect is bactericidal or bacteriostatic, the plasmid-free cells are eventually outcompeted from the population (Gerdes*, et al.*, 1986a) (Fig 1).

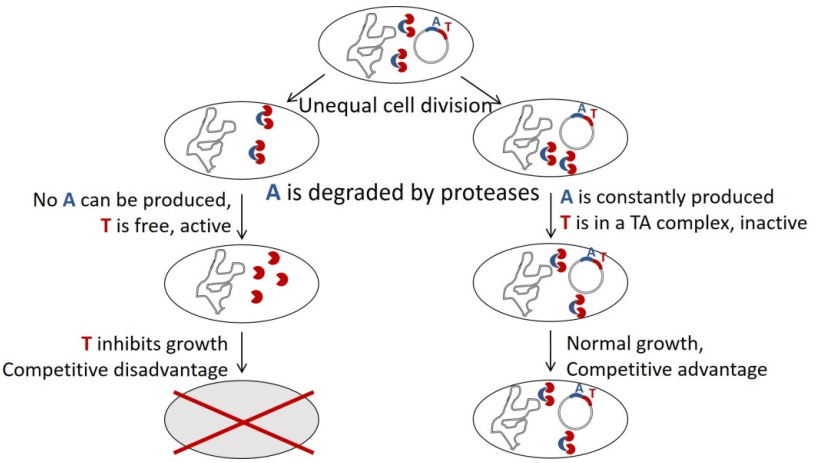


Figure 1. The PSK mechanism of plasmid-borne TA systems. Unequal cell-division leads to cells without the TA-encoding plasmid. TA proteins are still inherited via cytoplasm. Labile antitoxins are degraded and more stable toxins are activated and inhibit the growth of the cells (or kill them). Consequently, only the cells with the plasmid remain in the population. Antitoxin is marked with blue, toxin with red colour

2. Diversity of the TA systems

In recent years the research and knowledge on TA systems has largely expanded. For clarity, the TA systems have been divided into six types by the nature and inhibitory mechanism of the antitoxin (Page & Peti, 2016). The antitoxins may be either small RNAs (the TA types I and III) or proteins (types II, IV, V and VI), and the mechanisms of toxin inactivation vary largely as well. Antitoxins can inhibit toxins' synthesis (types I and V) (Fozo, *et al.*, 2008, Wang, *et al.*, 2012), activity (types II and III) (Marianovsky, *et al.*, 2001, Fineran, *et al.*, 2009), stability (type VI) (Aakre, *et al.*, 2013) or stabilize the toxins' target (type IV) (Masuda, *et al.*, 2012). The most prevalent and thoroughly studied are TA systems of type I and II, while others are represented by only a few examples. Still, more and more experimental data now emerge for other TA types as well.

2.1. Type I TA systems

In type I TA systems, the antitoxins are sRNAs that inhibit the translation of the toxin (Fig 2). The RNA antitoxins are complementary to the toxin mRNAs and by binding them, they prevent the toxin synthesis. Some RNA antitoxins, like IstR, inhibit ribosome binding to toxin mRNA (Darfeuille, *et al.*, 2007), but others, like RatA, induce the toxin's mRNA degradation (Silvaggi, *et al.*, 2005). There are also antitoxins, for example SprF1 and SR4 that act by combining

both mechanisms (Jahn & Brantl, 2013, Pinel-Marie, *et al.*, 2014). As the inhibition of toxins takes place before the protein production, this kind of toxin suppression could be beneficial in the case where toxin is harmful for bacteria already at a very low concentration.

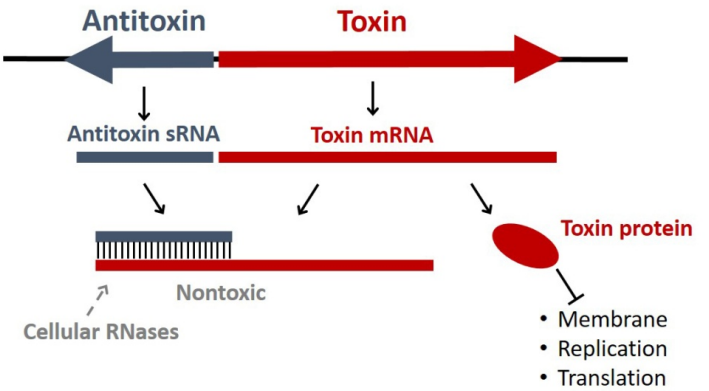


Figure 2. Type I TA system. The toxin protein, usually targeting the membrane, is inhibited at a pretranslational level by sRNA antitoxin that binds the toxin mRNA and (usually) promotes its degradation. The antitoxin is depicted in blue and the toxin in red.

Type I systems can be activated either by the antitoxin sRNA degradation (Jahn, *et al.*, 2012) or by the induction of toxin expression (13). For instance, the activity of the BsrG-SR4 system is regulated by the different stability of the counterparts (Jahn, *et al.*, 2012). TisB-IstR system, on the other hand, is under the control of a global regulator, the LexA repressor (Wagner & Unoson, 2012). During the DNA damage-induced SOS response when LexA is degraded, the rapid transcription of *tisB* mRNA outcompetes the IstR antitoxin and the TisB toxin is produced. The production of TisB decreases only after the SOS-conditions have passed (Wagner & Unoson, 2012).

Most commonly, the toxins of type I TA systems are small hydrophobic pore-forming peptides that damage the bacterial membrane. An illustrative representative of such toxins is the Hok toxin from the Hok-Sok system. This system was one of the first type I system to be discovered – it was initially found from *E. coli* R1 plasmid (Gerdes, *et al.*, 1986b, Gerdes, *et al.*, 1986a). Later, similar loci were identified in chromosomes of bacteria as well (Pedersen & Gerdes, 1999). The Hok toxin is a small peptide that targets the cell membrane and increases its permeability. As a result, the proton motive force required for ATP synthesis is decreased and the growth of bacteria inhibited (Gerdes, *et al.*, 1986b). Another type I TA module, that also encodes a pore-forming peptide, is the *tisB-istR* locus (Unoson & Wagner, 2008). As in case of the Hok toxin, the membrane becomes permeabilized and the cellular energy level decreased when TisB is produced (Gurnev, *et al.*, 2012).

Pore-forming is not the only way that type I systems use to damage the bacterial membrane. For example, the toxin BsrG of the *bsrG/*SR4 system disturbs the synthesis of the cell envelope, but causes neither membrane permeabilization nor energy starvation as the pore-forming toxins do (Jahn*, et al.*, 2015). The effects of type I toxins are even more diverse because some membrane-spanning toxins, e.g. Fst, have also been shown to cause nucleoid condensation (Kawano*, et al.*, 2002, Patel & Weaver, 2006, Weaver*, et al.*, 2009).

Recent studies have revealed that the type I toxins can be divided into two distinct groups according to their localisation and mechanisms of action (Brielle*, et al.*, 2016). While most of the described toxins are representatives of the membrane-associated proteins, the smaller group of toxins is comprised of cytosolic proteins that cleave nucleic acids. For instance, the toxin encoded by the *ralR-ralA* locus cleaves DNA (Guo*, et al.*, 2014). There is also one type I toxin, SymE, which resembles the MazF toxin from the type II TA system and acts as an endoribonuclease (Kawano*, et al.*, 2007). Therefore, although the majority of type I TA toxins disturb the bacterial cell membrane, a great variety in this type of TA systems exists and toxins may have very different targets and mechanisms of action.

2.2. Type II TA systems

An entirely different form of TA systems is the type II TA module in which both components are proteins. The type II systems are extremely widespread in the prokaryotic world and are the most thoroughly studied among the TA systems. Although the extensive research has revealed a striking diversity of type II systems, most of them still share several common features: (i) TA genes are adjacent and encoded by the same operon; (ii) both the toxin and antitoxin are small proteins; (iii) the toxin's activity is inhibited by the antitoxin binding; (iv) the antitoxin and/or the TA complex usually acts as an autorepressor of the operon; and (v) the mechanism of action of these systems is based on the higher stability of the toxin compared to its antidote (Gerdes & Maisonneuve, 2012). Figure 3 shows the mechanism of action of a typical type II TA system, yet many exceptions apply for distinct systems. As type II TA proteins are the main focus of this thesis, next chapters will describe this group of TA systems more comprehensively.

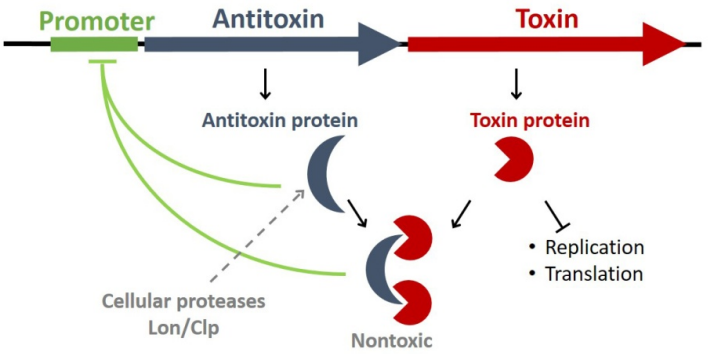


Figure 3. A typical type II TA system. The TA genes are coded by one locus, the antitoxin gene usually preceding the toxin gene. The operon is autorepressed by the antitoxin and/or the TA complex. In complex with the antitoxin, the toxin is inactive. The stable toxins will be activated when labile antitoxins are degraded by cellular proteases. Toxins inhibit major cellular processes such as translation and replication. The promoter is depicted in green, antitoxin in blue and toxin in red.

2.2.1. Regulation of Type II TA systems

Type II TA systems are regulated at two levels. Firstly, the production of both counterparts is regulated at transcriptional level. Secondly, the activity of the toxin is regulated post-translationally, by modulating the stability of the antitoxin.

2.2.1.1. Transcriptional regulation

The toxin and antitoxin genes of a type II TA system belong to the same operon where the antitoxin gene is usually the first, followed by the toxin gene (Fig 3). This gene organization has been suggested to ensure a higher production of the antitoxin compared to the toxin (Gerdes, *et al.*, 2005). Still, some systems, like the HigBA (Christensen-Dalsgaard & Gerdes, 2006), HicAB (Jorgensen, *et al.*, 2009), MqsRA (Christensen-Dalsgaard, *et al.*, 2010, Kasari, *et al.*, 2010) and RnlAB (Otsuka, *et al.*, 2010), have a reversed order of genes, with the toxin gene preceding the antitoxin. The higher production of the antitoxin in these cases can be achieved with an additional promoter in front of the antitoxin gene, as has been suggested for *higA* (Tian, *et al.*, 1996b) from the Rts-1 plasmid of *Proteus vulgaris* and *rnlB* (Otsuka, *et al.*, 2010) from the *E. coli* chromosome. The additional promoter in front of the *higA* is weaker than the one in front of the *higBA* operon, but the *rnlB* promoter is about eightfold stronger than the promoter in front of the *rnlAB* operon (Otsuka, *et al.*, 2010), so the higher expression of the antitoxin would be guaranteed. For *mqsRA* and *hicAB* systems, no additional promoter in front of the antitoxin gene has been found (Jorgensen, *et al.*, 2009, Christensen-Dalsgaard, *et al.*, 2010, Bibi-Triki, *et al.*, 2014).

The antitoxins usually contain a DNA-binding domain and act as transcriptional regulators by repressing the expression of their own operon. Interestingly, not only the antitoxin alone but also the TA complex can act as an autorepressor of the TA genes (Fig 3). For many type II systems, a regulatory mechanism has been observed, where the effect of the TA proteins on the promoter depends on the ratio between the toxin and antitoxin. Such regulation is termed conditional cooperativity (Overgaard*, et al.*, 2008) as the transcription is cooperatively repressed by both TA proteins only under certain conditions. For example, repression of the *relBE* operon is the strongest, when the anti-toxin:toxin ratio is 2:1, whereas higher levels of the RelE toxin result in derepression of the TA promoter (Cataudella*, et al.*, 2012). A similar transcriptional regulation has been observed for many other type II TA systems as well: the strongest repression is usually achieved in the 1:1 ratio of the toxin and antitoxin, whereas derepression is seen when the proportion of the toxin increases over this level. Thus, the excess of the toxin results in higher expression of the TA operon and production of the antitoxin. When proteases are active, the produced antitoxin is degraded rapidly, but when the proteases become inactive, the antitoxin amount is quickly restored to the level needed to inhibit the toxin present in excess (Loris & Garcia-Pino, 2014).

Some type II TA systems have other mechanisms of transcription control. For example, the TalAB system of *Leifsonia xyli* subsp. *cynodontis* is regulated in an entirely opposite manner. For this system, the toxin is the protein with the DNA-binding domain (Cheng*, et al.*, 2008). However, without the antitoxin, the binding of the toxin to DNA is extremely weak, addition of the antitoxin enhances the protein's binding to the DNA, indicating that repression is actually carried out by the TA complex. Thus, this system represents an exceptional type of regulation with a reversed functionality of the two TA proteins (Cheng*, et al.*, 2008).

There are also other type II TA modules, where antitoxin and toxin do not exhibit cooperative binding to the promoter. For example, the transcription of *mqsRA* system of *E. coli* is autorepressed by the antitoxin MqsA only and the toxin acts as a derepressor, irrespective of the ratio between the two proteins (Brown*, et al.*, 2013). Also, the affinity of the DinJ/YafQ complex to the promoter DNA is similar to that of the antitoxin DinJ alone (Ruangprasert*, et al.*, 2014) indicating that the YafQ toxin does not affect the DinJ binding activity. For the HicAB system, it remains unclear whether the regulation resembles the MqsRA system or is mediated by conditional cooperativity (Bibi-Triki*, et al.*, 2014). There are also type II TA systems that are not autoregulated by TA proteins at all. For instance, the unconventional three-component system ω-ε-ζ is regulated neither by the epsilon antitoxin nor the zeta toxin, but by an omega protein, encoded in the same operon (de la Hoz*, et al.*, 2000). Neither is the expression of the *mazEF-Sa* locus from *Staphylococcus aureus* autoregulated by the TA proteins but rather controlled by transcription regulators SarA and σ^B (Donegan & Cheung, 2009). All in all, although

conditional cooperativity is the most common mechanism of type II TA operons' regulation, the high number of type II systems means that also many different regulation mechanisms are applied in their expression.

2.2.1.2. Post-translational regulation

Besides the transcriptional regulation, the activity of the toxin is controlled by the stability of the antitoxin protein. Activation of the type II toxins is mostly achieved by degradation of antitoxins by stress-induced proteases Lon or ClpP (Brzozowska & Zielenkiewicz, 2013). Commonly, the antitoxins are quite unstable proteins with half-lives mostly around 15 minutes (Christensen*, et al.*, 2001, Ning*, et al.*, 2011, Hansen*, et al.*, 2012). Therefore, the antidote proteins need to be constantly produced to ensure the inhibition of the toxin. Structural analysis of antitoxins has revealed that besides well-ordered DNA binding domains, many type II antitoxins, e.g. MazE, Kid and HipB, contain an unstructured toxin-binding domain (Loris*, et al.*, 2003, Kamphuis*, et al.*, 2007, Schumacher*, et al.*, 2009). While the disordered nature of the antidote proteins makes them available substrates for cellular proteases (Gazit & Sauer, 1999, Loris*, et al.*, 2003, Kamphuis*, et al.*, 2007, Hansen*, et al.*, 2012, Brzozowska & Zielenkiewicz, 2013), the TA complex formation usually stabilizes the antitoxins as the flexible parts form a more ordered structure (Kamada*, et al.*, 2003), or become masked from proteases by the toxins (Schumacher*, et al.*, 2009). In stressful conditions, when the activity of ATP-dependent proteases increases, the ratio of toxin:antitoxin changes in favour of the toxins. This results not only in toxin activation and growth suppression, but also in derepression of the TA operon. In this way a transient activation of the toxin is achieved, while the conditional cooperativity mechanism guarantees that in case of surplus accumulation of the toxin, the expression of the TA proteins will be increased again. Once the stress is over, the inactivation of proteases leads to the stabilization of the antitoxin, and eventually the excess toxin can be inactivated again by complex formation. When the toxin:antitoxin ratio becomes normalized, the production of TA proteins is further repressed at the transcriptional level (Loris & Garcia-Pino, 2014).

Bearing in mind the diversity of the type II TA systems, it is not surprising that some exceptions regarding antitoxin instability exist. For example, the antitoxin MqsA is a quite stable protein in growth-favouring conditions. This is probably due to its more structured nature compared to other type II antitoxins (Brown*, et al.*, 2009). Yet, under oxidative stress conditions, the degradation rate of MqsA accelerates, the half-life of the protein dropping from one hour to just one minute (Wang*, et al.*, 2011). Hence, this system represents another model of regulation, where the constant production of MqsA is not crucial and antitoxin's degradation is triggered only under distinct stress conditions. In compliance with that, as already mentioned, the regulation of *mqsRA* expression does not involve conditional cooperativity (Brown*, et al.*, 2013).

2.2.1.3. Activation by stress conditions

While the MqsRA in *E. coli* is activated upon oxidative stress (Kwan*, et al.*, 2015), many other systems are activated by a variety of signals. For example, nutrition starvation, oxidative stress, different antibiotics, attack by bacteriophages and other stress factors can trigger the activation of many TA systems (Hazan*, et al.*, 2004, Ramage*, et al.*, 2009, Christensen-Dalsgaard*, et al.*, 2010, Otsuka & Yonesaki, 2012). Considering that in *E. coli* most type II antitoxins are degraded by Lon or Clp proteases (Brzozowska & Zielenkiewicz, 2013), it is expected that conditions leading to the activation of these proteases also lead to the activation of many TA systems (Brzozowska & Zielenkiewicz, 2013). It has been shown that accumulation of the stringent response alarmone (p)ppGpp results in the activation of Lon and Clp (Germain*, et al.*, 2015). There are two enzymes that control (p)ppGpp production, RelA and SpoT. RelA induces the production of (p)ppGpp in response to amino acid starvation (Hauryliuk*, et al.*, 2015) whereas SpoT is probably more important in many other kinds of stresses, like carbon, iron or fatty acid starvation (Xiao*, et al.*, 1991, Seyfzadeh*, et al.*, 1993, Vinella*, et al.*, 2005). Thus, the levels of (p)ppGpp in cells increase in stressful conditions and, at least in *E. coli*, lead to the activation of Lon and Clp. Thereby antitoxin degradation is increased, toxins are liberated and TA operon transcription is derepreressed (Germain*, et al.*, 2015) (see also chapter 4.2.3.).

Besides already mentioned stress signals, TA systems are also activated in other stress conditions. The SOS response has been shown to trigger several type II TA systems, e.g. YafNO, YafQ/DinJ and MazEF (Hazan*, et al.*, 2004, Prysak*, et al.*, 2009, Christensen-Dalsgaard*, et al.*, 2010). Also, exposure to different antibiotics activates certain TA systems such as YafNO, HigBA, HicAB, RelBE and YoeB/YefM (Sat*, et al.*, 2001, Kohanski*, et al.*, 2007, Jorgensen*, et al.*, 2009, Christensen-Dalsgaard*, et al.*, 2010). As normal functioning of a type II TA system needs constant production of the antitoxin, it is easily comprehensible that inhibition of protein synthesis in general leads to the decrease in antitoxin levels and therefore also to derepression of TA operons and activation of the toxins. As described in the next chapter, many type II toxins inhibit translation. Thus, activation of one TA system can contribute to the activation of others.

In *E. coli*, the stress-induced regulation has been extensively studied and well characterized, but several studies also show the activation of TA systems by stressful conditions in other bacteria. For example, in *Xylella fastidiosa*, the MqsRA system is activated upon copper stress (Merfa*, et al.*, 2016). Moreover, several TA systems of *Salmonella enterica* are triggered during exposure to erythromycin and tetracycline (Donegan & Cheung, 2009). Therefore, although the research on TA systems' activating conditions in other bacteria has not been as systematic as in *E. coli*, the activation upon different stresses seems to be a universal trait.

2.2.2. Targets of type II TA systems' toxins

The activation of TA systems triggers the toxin proteins. As briefly mentioned above, the toxins of type II systems most commonly target translation. Most of them inhibit protein synthesis by cutting mRNA, but some also cleave rRNA, tRNA or tmRNA (Winther & Gerdes, 2011, Schuessler*, et al.*, 2013, Schifano & Woychik, 2014).

The mechanisms of action also vary within the groups of toxins with a similar target. Many toxins that cleave mRNA, e.g. RelE, YafQ, and YoeB of *E. coli*, need to be bound to ribosome to exert their nuclease effect (Pedersen*, et al.*, 2003, Christensen-Dalsgaard & Gerdes, 2008, Prysak*, et al.*, 2009, Zhang*, et al.*, 2009). Other toxins with the mRNA target, e.g. *E. coli* HicA and MazF, cleave mRNA independent of the ribosome (Zhang*, et al.*, 2003, Jorgensen*, et al.*, 2009). The mRNase toxins also differ by their recognition sequence. Some of them have short target sequences that occur often in different mRNAs. For example, the ribosome-dependent YafQ toxin from *E. coli* cleaves the AAA codons that are followed by G or A nucleotide (Prysak*, et al.*, 2009). The HigB toxin from the Rts-1 plasmid also cuts the AAA or ACA codons, whereas an A in the third position of the codon ensures an effective cleavage (Hurley & Woychik, 2009, Schureck*, et al.*, 2014). The ribosome independent MazF toxin from *E. coli* cleaves mRNAs at ACA sites (Zhang*, et al.*, 2003). These short recognition sites probably explain why these toxins rapidly decompose the cellular mRNAs and have a drastic effect on bacterial physiology (Zhang*, et al.*, 2003). Still, some MazF family toxins recognise much longer sequences. For example, MazF homologues from pathogenic species *Clostridium difficile*, *S. aureus* and *Mycobacterium tuberculosis* cleave either the five-base sequence UACAU (MazF-cd and MazF-Sa) or UUCCU/CUCCU (MazF-mt3), or UCGCU (MazF-mt7) and thus target just a specific set of mRNAs (Zhu*, et al.*, 2008, Zhu*, et al.*, 2009, Rothenbacher*, et al.*, 2012, Williams & Hergenrother, 2013). These sequences have an uneven distribution among cellular RNAs, being especially over- (Zhu*, et al.*, 2009, Rothenbacher*, et al.*, 2012) or under-represented (Zhu*, et al.*, 2008, Williams & Hergenrother, 2013) in pathogenicity factors. It has been suggested that these more specific toxins comprise a distinct group, which is important in the pathogenicity of bacteria rather than in growth regulation (Zhu*, et al.*, 2009).

In addition to the variable sequence recognition by MazF toxins in different bacteria, some of the members of this toxin family do not cleave mRNA. So, *M. tuberculosis* encodes at least nine MazF family toxins (Schifano*, et al.*, 2013), but rather than targeting mRNA, several of them cleave other types of RNA. For example, MazF-mt6 and MazF-mt3 (in addition to mRNA) target ribosomal RNA (Schifano*, et al.*, 2013, Schifano & Woychik, 2014, Schifano*, et al.*, 2014), and MazF-mt9 cuts tRNA (Schifano*, et al.*, 2016). tRNA is also cleaved by a mycobacterial VapC4 toxin (Cruz*, et al.*, 2015), whilst another VapC homolog in *M. tuberculosis*, the VapC20, cleaves 23S ribosomal RNA instead (Winther*, et al.*, 2013). Even tmRNA has been shown to be cleaved by a

type II toxin, the HigB of *M. tuberculosis* (Schuessler, *et al.*, 2013). Thus, all the types of RNA can be targeted by type II TA toxins. Translation can also be inhibited by using other ways as exemplified by the bacteriophage P1-encoded Doc and the *E. coli* HipA toxins, which phosphorylate the translation factor EF-Tu (Castro-Roa, *et al.*, 2013) and the glutamyl-tRNA-synthetase GltX (Germain, *et al.*, 2013, Kaspy, *et al.*, 2013), respectively. The RatA toxin, also known as PasT, interferes with translation by binding to the ribosomal 50S subunit and inhibiting the assembly of 70S ribosomes (Zhang & Inouye, 2011).

Although a vast majority of type II toxins inhibit translation, some of them affect other major processes. For example, the CcdB and ParE toxins inhibit replication by binding the DNA gyrase, an enzyme required to relieve supercoiling in front of the replication fork (Cozzarelli, 1980, Yuan, *et al.*, 2010). The PezT toxin of *Streptococcus pneumoniae* phosphorylates peptido-glycan precursors and thereby inhibits cell wall synthesis (Mutschler, *et al.*, 2010). Summing up, just like type I systems that have mostly membrane-damaging toxins, but have other targets as well, the type II toxins mostly inhibit translation but also have some alternative targets.

2.3. Type III TA systems

Analogously to the type I TA systems, the antidote role of type III TA modules is carried out by a small RNA. However, the mechanism of inhibition of a toxin is entirely different. The type III antitoxin is an sRNA composed of nucleotide direct repeats, which fold into hairpin-like structures and inactivate the toxic protein by binding it through RNA-protein interactions (Fig 4). Two TA systems belonging to the type III family have been characterized so far: ToxIN and AbiQ-antiQ. In both systems, the toxins elicit their poisonous effect through ribonucleolytic cleavage and induction of growth arrest, which can be reversed by increased production of cognate antitoxins (Blower, *et al.*, 2011, Samson, *et al.*, 2013, Short, *et al.*, 2013).

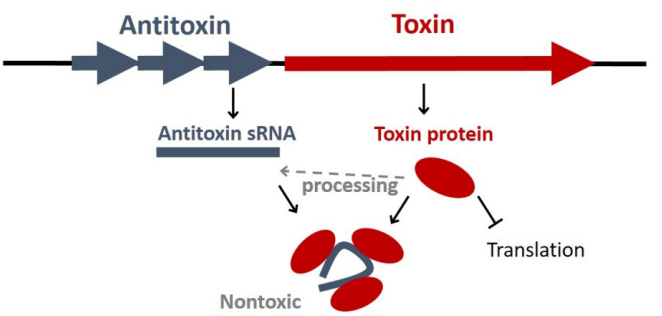


Figure 4. Type III TA system. The sRNA antitoxins fold into hairpin like structures, the toxic proteins bind the antitoxin RNA, which results in their inactivation. Free toxins have ribonucleolytic activity and they inhibit translation. The antitoxin is depicted in blue and the toxin in red colour.

How the activity of type III TA toxins is triggered has remained ambiguous as both the toxins and the antitoxins are continuously expressed throughout the growth (Fineran, *et al.*, 2009, Samson, *et al.*, 2013). For the activation of ToxN toxin, it has been hypothesized that upon phage infection the toxin:antitoxin ratio in the cell is altered due to the suppression of transcription and translation or the degradation of the host DNA (Fineran, *et al.*, 2009). For the AbiQ-antiQ system, there are no remarkable changes in the levels of the two counterparts during phage infection, so it is more likely that the toxin is activated by some other mechanism of switching the toxin into an active form which still needs to be discovered (Samson, *et al.*, 2013).

2.4. Type IV TA systems

Type IV group of TA systems is comprised of very unconventional systems because the antitoxin does not inhibit the toxin itself but rather counteracts the toxin's effect by stabilizing the target of the toxin (Masuda, *et al.*, 2012) (Fig 5). For example, the CbeA antitoxin binds to and promotes bundling of FtsZ and MreB filaments and thereby counteracts the CbtA toxin's effect on the inhibition of cytoskeleton (Masuda, *et al.*, 2012). Moreover, the antitoxin CbeA has been shown to protect bacteria from other cytoskeleton inhibiting proteins as well (Masuda, *et al.*, 2012). Considering that this antitoxin does not inactivate solely its toxin, but also other FtsZ or MreB inhibitors like SulA and A22 (Masuda, *et al.*, 2012), classification of the type IV module within the TA systems can even be considered questionable. As there is no direct interaction between the two counterparts, one could count any harmful protein and its repressor a TA system.

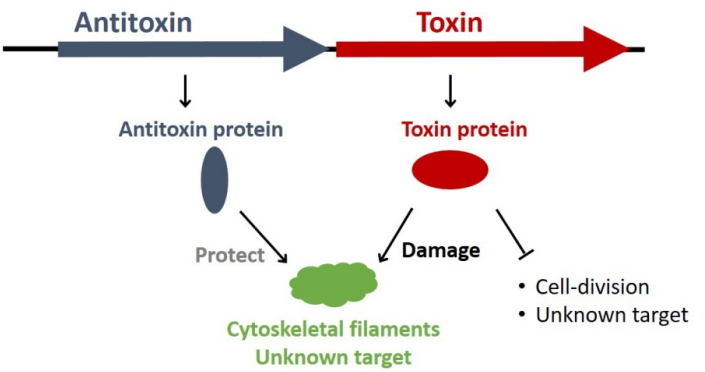


Figure 5. Type IV TA system. The TA proteins do not interact directly; the antitoxin counteracts the toxin by stabilizing its target. The antitoxin is blue and the toxin red.

However, another TA system, AbiE from *Lactococcus lactis*, has been recently described to function through a type IV suppression mechanism as well. Ectopic overexpression of AbiEii toxin was shown to be counteracted by its antitoxin

AbiEi without complex formation between the two proteins (Dy, *et al.*, 2014). The mechanism of action of this system is currently not clear. However, as the toxin AbiEii was predicted to belong to a nucleotidyl transferase superfamily and it specifically binds GTP, it is proposed that AbiEii inactivates its so far unknown target by guanylylation (Dy, *et al.*, 2014). The counteraction of the antitoxin AbiEi might be removing the toxin-added GMP (Dy, *et al.*, 2014), but this has yet to be experimentally confirmed as well. The regulation of the AbiE operon resembles to that of the type II systems, as the transcription of the AbiE is autorepressed by the antitoxin. Since the proteins do not interact and the AbiEii toxin does not act as an autorepressor, naturally, no conditional cooperativity in the regulation can occur (Dy, *et al.*, 2014).

2.5. Type V TA system

Only one type V TA system has been described to date. The system is named GhoST as the toxin GhoT interferes with the inner membrane integrity and results in lysed cells with damaged membranes (ghost cells) (Wang, *et al.*, 2012). GhoST system has features similar to type I systems as the membrane damaging toxin GhoT is also inhibited by the cognate antitoxin prior to the synthesis of the toxin protein. However, the inhibition is achieved by cleavage of the GhoT mRNA by proteic GhoS that has endoribonucleolytic activity (Wang, *et al.*, 2012) (Fig 6). Differently from other proteinaceous systems, GhoS antitoxin is not degraded during stress and neither does it possess a DNA binding ability. The regulation of the system instead involves another TA system. It has been shown that the activation of the type II MqsRA system increases the mRNA ratio of GhoT to GhoS, as the toxin MqsR preferentially degrades the mRNA of GhoS antitoxin (Wang, *et al.*, 2013). Hence, upon stress, when MqsR is activated, the GhoS mRNA levels drop and the GhoT toxin can be produced.

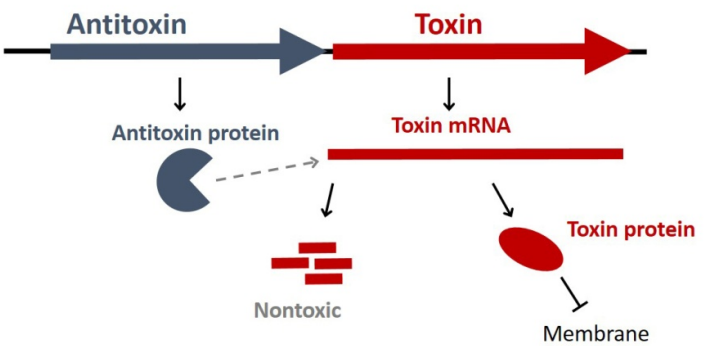


Figure 6. Type V TA system. The protein antitoxin (GhoS) degrades the toxin (GhoT) mRNA. The toxic protein damages the bacterial inner membrane. The antitoxin is blue and the toxin red.

The type I and V toxins are mostly membrane-spanning, pore-forming peptides and all of them are inhibited before their synthesis. Therefore, it is tempting to speculate that toxins of this nature have to be inhibited before translation because once in the membrane, they already exert their toxic effect and are thus hard to counteract.

2.6. Type VI TA system

Recently, a new type of TA system has been described in *Caulobacter crescentus* (Aakre*, et al.*, 2013). The type VI SocAB system differs from all other TA pairs, as the antitoxin SocA is a destabilizer of the toxin SocB (Fig 7). While usually the toxins are stable proteins, SocB is labile and degraded by ClpXP protease. SocA antitoxin is required as an adaptor and its binding to the toxin is needed for the degradation to occur. In the absence of the antitoxin, the toxin is not degraded and replication elongation is inhibited by SocB binding to β-sliding clamp and outcompeting other clamp-binding factors (Aakre*, et al.*, 2013).

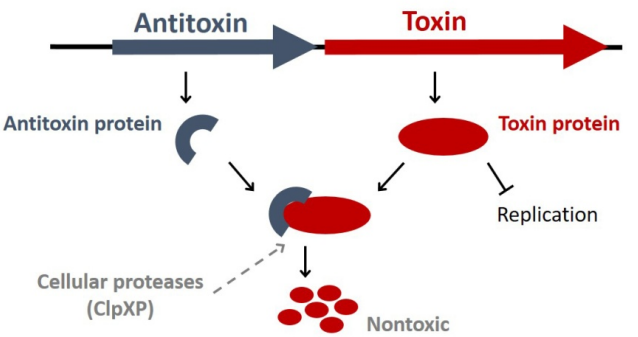


Figure 7. Type VI system. The antitoxin (SocA) is a proteolytic adaptor. It binds the toxin (SocB) and promotes its degradation. The antitoxin is blue and the toxin red.

Categorising the TA systems into different groups can be considered a violent and artificial approach. Nevertheless, to create more clarity into the diverse world of TA systems, it seems to be a reasonable practice. The different inactivation ways of toxins may have a meaning in understanding the systems. For example, as exemplified by the type I and V systems, in some cases it may be better not to allow the toxin to be synthesised at all. On the other hand, the advantage of the type II systems is that their activation can be achieved quicker as the protein toxin is already present in the cell in a harmless form of a TA complex. Thus, no protein production is required for toxin to be able to influence the physiology of bacteria. The most recently described TA types (IV–VI) broaden the world of TA systems and suggest that many more different TA types could possibly exist that still remain to be discovered.

3. The abundance and potential cross-talk between multiple TA systems

The genes for TA systems are widespread in the chromosomes of bacteria, particularly in the pathogenic and free-living species that encounter many different stresses during their life (Pandey & Gerdes, 2005). The chromosomal distribution of TA loci in bacteria is well correlated with the diversity of the environment they inhabit. So, obligate intracellular parasites that live in stable and predictable conditions, harbour only few or none TA systems. For example, obligate intracellular parasites such as different *Chlamydiacea* spp. have no chromosomal TA systems (Leplae*, et al.*, 2011). This suggests that in unchanging, stable conditions the TA systems lose their beneficial effects and respective genes are eliminated from the genome. On the contrary, in fluctuating environments, they seem to confer an advantage and have thus been maintained throughout evolution (Pandey & Gerdes, 2005). For example, a stark contrast can be seen in the genus *Mycobacteria*, where intracellular *M. leprae* encodes only one set of TA genes (Leplae*, et al.*, 2011), whereas *M. tuberculosis* encodes no less than 88 TA systems in its chromosome, of which at least 63 have been functionally tested in *E. coli* and/or *M. smegmatis* with 37 proven active under at least one tested condition (Gupta, 2009, Ramage*, et al.*, 2009, Huang & He, 2010, Singh*, et al.*, 2010, Zhu*, et al.*, 2010, Ahidjo*, et al.*, 2011, Sala*, et al.*, 2014). This indicates both the benefits as well as the costs of the TA systems: a high number of functional systems suggests that these TA loci confer beneficial effects on *M. tuberculosis* survival; on the other hand, as many mycobacterial TA systems have lost their functionality (Sala*, et al.*, 2014), the TA loci seem to be under strong selection.

The high number of TA modules in one bacterium raises many intriguing questions. Firstly, what could be the relationship between the proteins from different systems and secondly, if there is regulatory cross-activation between the systems? These questions seem particularly relevant when considering that 47 (about half have been confirmed to have active toxins in *M. smegmatis*) of *M. tuberculosis* TA systems are of the VapBC type (Ramage*, et al.*, 2009, Ahidjo*, et al.*, 2011). One would expect that similar TA proteins of the same family could interact with one another. Nevertheless, analysis of four heterologous VapBC toxins and antitoxins showed that the antitoxins could inhibit only their cognate toxin (Ramage*, et al.*, 2009, Ahidjo*, et al.*, 2011). Although not all the systems were tested, it clearly indicates a high specificity for at least the VapBC systems in *M. tuberculosis*. Several other studies also indicate that most TA proteins are very specific to interact with only their cognate partners (Masuda*, et al.*, 1993, Fiebig*, et al.*, 2010, Nolle*, et al.*, 2013). Considering that maintaining the toxin:antitoxin ratio is crucial for a bacterium, it is comprehensible why the proteins of one system do not interact with those of other systems. Yet, there are some indications of complex interactions between the members of different systems. For example, the three RelBE family systems

in *M. tuberculosis* are able to form heterologous TA complexes where RelB antitoxins also neutralize other RelE toxins besides their own partners (Yang*, et al.*, 2010). Interaction between the proteins from different systems has also been recorded for two *M. tuberculosis* MazEF (mt1 and mt3) systems. Moreover, the proteins of these systems could also interact with proteins of two VapBC systems of this bacterium (Zhu*, et al.*, 2010). Yet, the opposite results have been obtained by others, as no cross-interaction between three mycobacterial MazEF systems (MazEF-mt3, mt6 and mt9) was seen (Tiwari*, et al.*, 2015a). The discrepancy between different studies may originate from different experimental design. In their TA protein interaction experiments Zhu et al (Zhu*, et al.*, 2010) expressed the toxins from arabinose-inducible P_{BAD} promoter and antitoxins from an IPTG-inducible P_{tac} promoter. However, as IPTG directly inhibits the P_{BAD} promoter, the experiments by Zhu et al could have given artificial results (Kasari*, et al.*, 2013). Thus for truthful results, the promoters being used for the expression of TA proteins should be carefully considered or direct interactions between the TA proteins confirmed experimentally.

The regulatory cross-activation between different chromosomal TA systems has also been observed. Moreover, this cross-regulation can include members of different types of TA systems. In a recently published study, the adjacently located type II MazEF system and type I TxpA/ratA of *Enterococcus faecalis* were shown to activate each other (Wessner*, et al.*, 2015). The regulatory interplay relied on the ability of the MazEF complex to not only autorepress its own transcription, but also to activate the expression of the neighbouring *ratA* gene. The binding of MazEF complex on the *ratA* promoter decreased the amount of MazEF proteins that could otherwise repress the *mazEF* locus, thus leading to the activation of the *mazEF* transcription as well (Wessner*, et al.*, 2015). As already mentioned in chapter 2.5, the type II TA system MqsRA regulates the type V GhoST system in *E. coli* (Wang*, et al.*, 2013). The toxin MqsR preferentially cleaves the GhoS mRNA, thus decreasing its levels and inducing the GhoT toxin's activation. Cross-activation between other type II TA systems in *E. coli* has also been proposed (Kasari*, et al.*, 2013) and the cumulative effect of type II TA systems' deletions on persister cell formation (Maisonneuve*, et al.*, 2011) suggests that different TA systems may constitute a coordinately acting regulatory network (see also chapter 4.2.3.). The fact that some systems regulate or interact with each other, whereas other systems do not, indicates that these systems could have different roles in bacterial physiology. Some situations likely require more holistic TA systems' action, whereas others may need a more specific approach by individual TA systems.

4. Functions of chromosomal TA systems

Considering that TA toxins from all types can have many different targets and they inhibit vital processes and structures of bacteria, an intriguing question to ask is: what could be the benefit of these noxious systems? This question became increasingly attractive when aside of plasmids, the TA systems were found to be abundant in bacterial chromosomes (Pandey & Gerdes, 2005). While the importance of the plasmid-borne TA systems in plasmid stabilization is quite straightforward (Fig. 1), the importance of such systems in chromosomes is much more difficult to understand.

The high number of TA loci in bacterial chromosomes (Pandey & Gerdes, 2005) suggests that they play important role(s) in the life of bacteria. Although some TA systems are inactive and probably just remnants of mobile genetic elements (Mine*, et al.*, 2009, Ramage*, et al.*, 2009), others seem to be very important for bacteria. Throughout the years of the TA studies, there has been much debate about the functions of the chromosomal TA systems. The roles proposed for genomic TA modules include maintenance of genetic islands, protection against bacteriophages and increasing amount of data suggest that they play an important role in bacterial stress management (Unoson & Wagner, 2008, Wang*, et al.*, 2011). All these options are discussed more thoroughly in the next few chapters.

4.1. Protection from DNA loss

TA modules can be often found in the mobile parts of the chromosomes such as superintegrons (Rowe-Magnus*, et al.*, 2003) or pathogenicity islands (Ma*, et al.*, 2013). In resemblance to the plasmid-borne TA systems that contribute to the maintenance of plasmids, several chromosomal TA pairs can also hinder large-scale deletions of otherwise disposable genomic regions. *Vibrio cholerae*, a bacterium that has two chromosomes (Heidelberg*, et al.*, 2000), portrays a good example for such a role of chromosomal TA pairs. The larger chromosome (chr I) encodes most of the vital genes, whereas the second one (chr II) is smaller and has only very few essential genes. On the other hand, 17 out of the 18 TA systems of *V. cholera* are encoded on chr II, within one superintegron (Iqbal*, et al.*, 2015). It has been shown that ParDE modules of *V. cholerae* control the missegregation of chr II and thus, contribute to its stabilization. Upon the loss of the smaller chromosome, the toxins are liberated from the antitoxin-mediated control and degrade the essential chr I, thereby killing the cell (Yuan*, et al.*, 2011). The MosAT system of *V. cholerae* has also been shown to have a similar function in maintaining an integrative and conjugative element SXT (Wozniak & Waldor, 2009). In order to undergo conjugation, the element has to be excised from the chromosome and circulate. Yet, under these conditions the MosAT system, encoded on the element, is activated and prevents the loss of SXT, probably by PSK mechanism (Wozniak & Waldor, 2009). The mechanism

of action of MosT is still not clear and the possibility of MosT acting prior to the loss of the SXT cannot be ruled out either. In that case, MosT would inhibit the growth of bacteria to increase the possibility of reintegrating the SXT element into the chromosome (Wozniak & Waldor, 2009). While these few examples illustrate the chromosomal TA systems acting similar to the plasmidic ones, most of the chromosomal systems have other functions.

4.2. TA systems in stress management

Increasing amount of data associates TA modules with survival under stress. The involvement of TA systems in stress response is suggested by the high number of systems that are activated in stressful conditions (chapter 2.2.1.3). Additionally, many TA systems are integrated into global stress-response pathways, e.g. SOS-response induces YafQ-DinJ, YafON and TisB-IstR systems (Vogel*, et al.*, 2004, Prysak*, et al.*, 2009, Singletary*, et al.*, 2009, Dörr*, et al.*, 2010); a number of TA systems are activated by the stringent response, induced by (p)ppGpp (Germain*, et al.*, 2015). The antitoxin MqsA can even regulate the RpoS-controlled general stress response in *E. coli* as it directly represses the expression of the *rpoS* gene (Wang*, et al.*, 2011). So the production of the stress-specific sigma factor is increased upon oxidative stress, when the amount of MqsA decreases (Wang*, et al.*, 2011, Wang & Wood, 2011) (chapter 2.2.1.2).

Below, I give a short overview on TA systems functioning in: (i) protection against bacteriophages; (ii) biofilm production; (iii) regulation of growth and dormancy; and (iv) survival in the host organism upon infection.

4.2.1. Protection against bacteriophages

As in case of humans who are stressed when they become ill, the attack by bacteriophages inflicts a great stress to bacteria and can trigger various stress responses. Recent findings suggest that several chromosomal TA systems play an important role in protecting bacterial host from phage infection (Hazan & Engelberg-Kulka, 2004, Fineran*, et al.*, 2009, Samson*, et al.*, 2013). Phage invasion causes stress leading to increased degradation of antitoxins and consecutive activation of toxins. For example, RnlAB system from *E. coli*, is considered to be a phage-defence module: phage infection triggers the degradation of the antitoxin RnlB and the activation of the RnlA toxin resulting in phage mRNA degradation and the blocking of the expression of phage genes (Koga*, et al.*, 2011). A bacteriostatic effect upon phage invasion has also been observed for the type III ToxIN and type IV AbiE systems, where phage invasion triggers toxin activation and reversible inhibition of growth, which is restored after the stress has passed (Fineran*, et al.*, 2009, Dy*, et al.*, 2014).

The other strategy of protecting bacterial population from spread of phages is much more severe and involves death of phage-infected bacteria. This kind of

phage defence has been reported for *E. coli* MazEF system, which is the first chromosomal TA system to be confirmed to have a function in protection against phages. The MazEF is among the most studied TA systems and represents a chromosomal module that has been suggested to trigger programmed cell death (PCD) of bacteria (Sat*, et al.*, 2001, Engelberg-Kulka*, et al.*, 2005). As proposed, the MazEF-mediated PCD functions as a defence mechanism of a bacterial culture against P1 phage spread (Hazan & Engelberg-Kulka, 2004). Dying of individual cells has been considered as an altruistic mechanism to improve the survival of the remaining population (Hazan & Engelberg-Kulka, 2004). However, the PCD mechanism of MazEF system in phage defence has been questioned by various researchers (Pedersen*, et al.*, 2002, Yamaguchi & Inouye, 2011, Ramisetty*, et al.*, 2016). Indeed, PCD induced by the MazEF system has only been shown by one research group for one certain *E. coli* strain MC4100, which is reported to be *relA*$^+$ (Hazan*, et al.*, 2001, Sat*, et al.*, 2001, Hazan & Engelberg-Kulka, 2004, Hazan*, et al.*, 2004, Kolodkin-Gal & Engelberg-Kulka, 2006). However, this strain actually has a mutation in the *relA* gene (Tsilibaris*, et al.*, 2007) and, moreover, its ΔmazEF derivative additionally has a partial deletion of *mazG* gene, making the wild-type strain *relA*$^-$ and the ΔmazEF derivative *relA*$^-$*mazEFG*$^-$ (Tsilibaris*, et al.*, 2007). For other *E. coli* strains, including MG1655, MC1000 and MC4100 *relA*$^+$, the PCD mechanism seems to not occur (Christensen*, et al.*, 2003, Tsilibaris*, et al.*, 2007). Moreover, even for the very same *E. coli* strain used by the Engelberg-Kulka research group who obtained the MazF-mediated PCD results, other researchers could not obtain the results to confirm the mechanism (Ramisetty*, et al.*, 2016).

Still, the MazEF system protects against phage attack. This is also supported by the interactions between the chromosomal TA system and phages. Specifically, the MazEF system suppresses the growth of the T4 phage (Alawneh*, et al.*, 2016). Interestingly, T4 has a special way of escaping the anti-phage effects of the MazF toxin. It encodes three ADP-ribosyltransferases, and one of them, Alt, modifies MazF to decrease its toxicity and inactivate it upon phage infection (Alawneh*, et al.*, 2016). The T4 phage also encodes a Dmd protein that acts as an antitoxin against the RnlA toxin from the RnlAB system of *E. coli* K-12. The Dmd antitoxin enables the phage to escape the anti-phage activity of RnlAB (Naka*, et al.*, 2014). Moreover, the phage-encoded antitoxin can also inactivate the toxin LsoA from the LsoAB system encoded by a plasmid (Naka*, et al.*, 2014). This indicates that TA proteins are used as tools in the evolutionary race between bacteria and phages.

4.2.2. Production of biofilm

Biofilm production is of great importance for bacteria to tackle different stress conditions. For example, pathogenic bacteria constantly face stressful conditions created by the host organism or antibacterial treatments. Biofilm can protect

bacteria against these threats. The TA systems are activated in biofilms, and not only in clinical strains (Mitchell, *et al.*, 2010, Hemati, *et al.*, 2014), but also in non-pathogenic bacteria (Ren, *et al.*, 2004). Although the deletion of one TA locus usually has no major effect on biofilm formation (Lemos, *et al.*, 2005), the successive deletion of five TA modules from *E. coli* strongly influenced biofilm formation so that it was decreased at 8 hours of growth, yet, increased after 24 h due to the reduced dispersal (Kim, *et al.*, 2009). In *V. cholera* three of the six active *relBE* loci were reported to contribute to biofilm formation. Two of them increased biofilm production and one contributed to its maturation (Wang, *et al.*, 2015).

It has been proposed that TA systems could constitute a switch from planktonic growth to biofilm formation upon stress (Wang & Wood, 2011). However, different TA modules influence biofilm formation in different ways (Hu, *et al.*, 2010). Some of the TA systems in *E. coli* (MazEF, RelBE, ChpB, YefM/YoeB, DinJ/YafQ, MqsRA) control biofilm formation through influencing the expression of curli or fimbriae (Kim, *et al.*, 2009, Soo & Wood, 2013), whereas the HipA toxin positively influenced biofilm formation by promoting cell lysis and increasing the amount of extracellular DNA (Zhao, *et al.*, 2013). The effect of MazF and YafQ on biofilm formation has also been shown to be linked to their ability to kill cells, yet their involvement is probably not connected with the release of DNA (Kolodkin-Gal, *et al.*, 2009). Thus, many TA systems play a role in biofilm development, but the mechanisms employed by different systems seem to vary widely.

4.2.3. Growth regulation and persister cell formation

Given that toxins target essential cellular processes and structures the general outcome of the TA system activation is the slowdown of growth (Schuessler, *et al.*, 2013). Importantly, decreasing growth rate is a common strategy to increase stress tolerance (Tuomanen, *et al.*, 1986, Eng, *et al.*, 1991). Therefore, it is not surprizing that the TA systems-caused growth cessation contributes to the tolerance of a wide variety of stresses including exposure to antibiotics (Keren, *et al.*, 2004), heavy metals (Harrison, *et al.*, 2005), oxidative stress (Wu, *et al.*, 2011, Frampton, *et al.*, 2012), UV irradiation (Wu, *et al.*, 2011) and high temperatures (Maezato, *et al.*, 2011, Wu, *et al.*, 2011, Frampton, *et al.*, 2012). The growth rate inhibition can be quite strong and in many cases, the toxins are so severe that the cell growth is ceased entirely (Pedersen, *et al.*, 2002).

The toxin-mediated growth cessation has been associated with the formation of metabolically inactive and dormant persister cells. Multi-drug tolerant persister cells of clinical isolates pose a high risk in medicine as persister formation is considered a major reason of relapsing infections after antibiotic treatment (Conlon, 2014). To tackle the problem of persistent bacteria, the molecular mechanisms of their emergence and the involvement of TA systems

in persistence have been studied extensively (Lewis, 2010, Maisonneuve & Gerdes, 2014, Page & Peti, 2016). It has frequently been shown that the effect of any particular TA module on persistence is minor: the deletion of a single system usually has no effect on the number of dormant cells (Maisonneuve*, et al.*, 2011). However, bacteria usually possess multiple TA systems and for example in *E. coli* a combined deletion of at least five TA systems decreased the number of persisters significantly, whereas the deletion of 10 TA loci decreased the amount of persisters by more than two orders of magnitude (Maisonneuve*, et al.*, 2011). Yet, persister formation between bacterial species differ. In *Salmonella* Typhimurium the deletion of each single TA module already decreased persister formation (Helaine*, et al.*, 2014). Moreover, it has been shown that three RelE homologues in *M. tuberculosis* induce growth stasis, but rather than contributing to overall persistence, each toxin increases the tolerance to distinct antibiotics (Singh*, et al.*, 2010).

The persistence induced by TA toxins is a subpopulation phenotype, meaning that only a small number of cells in the population become persisters. In *E. coli*, persister formation is shown to originate from the activation of TA systems in response to stochastic increase of (p)ppGpp concentration (Maisonneuve*, et al.*, 2013). The synthesis of this stringent response alarmone is usually induced by nutritional stress [(Chatterji & Ojha, 2001), see also chapter 2.2.1.3.]. Yet, (p)ppGpp levels can occasionally rise above a certain threshold in sporadic cells also in nutrient-rich conditions, as has been recently shown by Maisonneuve and colleagues (Maisonneuve*, et al.*, 2013). Moreover, one of *E. coli* TA toxins can enhance the (p)ppGpp production and persister cell formation. The active toxin HipA phosphorylates the glutamyl-tRNA synthetase GltX (Germain*, et al.*, 2013) that leads to accumulation of uncharged $tRNA^{Glu}$ and stalls ribosomes, which in turn activates the RelA-dependent production of (p)ppGpp (Germain*, et al.*, 2015) (Fig 8). In the cells with increased alarmone molecule levels, the cellular proteases Lon and Clp are activated, TA antitoxins are degraded, and the liberated toxins will suppress cell growth and induce persistence (Maisonneuve*, et al.*, 2013) (Fig 8). An analogous (p)ppGpp-dependent activation pathway has also been suggested for the type I *hokB-sokB* system (Verstraeten*, et al.*, 2015). Thus, a well-ordered regulatory pathway for the activation of different TA systems has been proposed in *E. coli* (Germain*, et al.*, 2015, Verstraeten*, et al.*, 2015).

Yet, the results of the T. K. Wood group reveal that the (p)ppGpp-dependent pathway cannot explain all aspects of persister formation in *E. coli* as TA-mediated persistence also occurs in the absence of (p)ppGpp (Chowdhury*, et al.*, 2016). Moreover, what is true for *E. coli* is not necessarily true for all other bacteria. For example, (p)ppGpp-mediated stringent response and TA modules do not control persister formation in *S. aureus* (Conlon*, et al.*, 2016). The regulation of persister cell formation in *S. aureus* rather involves decreased levels of ATP, which leads to the expression of stationary-phase markers, genes *arcA* and *cap5A*, and induces dormancy (Conlon*, et al.*, 2016). Nevertheless, in

clinical strains of *M. tuberculosis* numerous TA systems are upregulated in mutants with high persister frequency (Torrey, *et al.*, 2016) and most characterized TA toxins can cause reversible growth arrest and dormancy (Pedersen, *et al.*, 2002, Schmidt, *et al.*, 2007, Kasari, *et al.*, 2010). Thus, the majority of TA systems in various bacterial species are involved in growth regulation and persistence, but possibly in a different manner than described for *E. coli*.

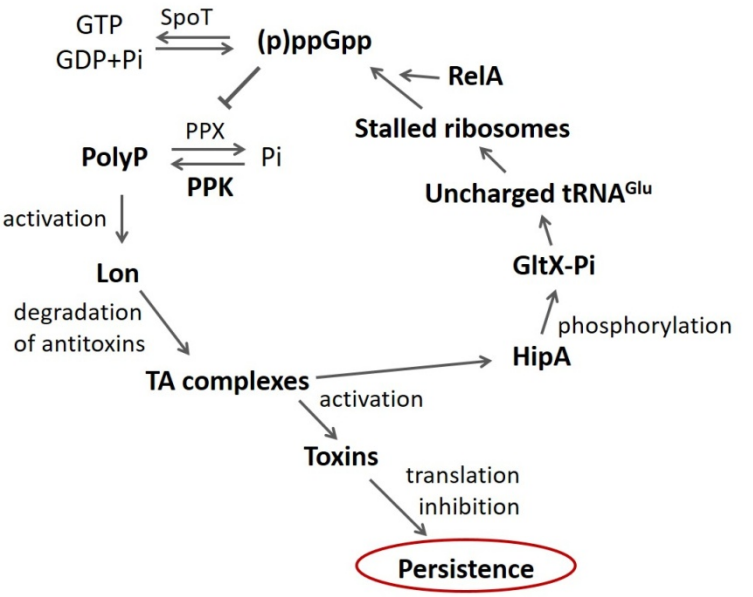


Figure 8. The (p)ppGpp-dependent pathway of induction of persister formation in *E. coli*. Active HipA toxin phosphorylates GltX, which leads to the accumulation of uncharged $tRNA^{Glu}$ that stalls ribosomes. This triggers RelA-dependent (p)ppGpp synthesis. Increased levels of (p)ppGpp inhibit PolyP phosphatase PPX, which leads to accumulation of PolyP produced by polyphosphate kinase PPK. As a result, Lon protease is activated, TA antitoxins degraded and the TA toxins (including HipA) activated. The mRNAse activity of toxins finally leads to induction of persistence [adapted from (Germain, *et al.*, 2015)].

4.2.4. Contributors to virulence

Previously described functions of TA systems in biofilm and persister cell formation were shown to be beneficial for pathogenic bacteria as they help to survive under different stresses the bacteria encounter upon infecting their host organism (Stallings & Glickman, 2010, Wang, *et al.*, 2015). The importance of TA systems in virulence was first suggested by their great prevalence in infectious bacteria, but there are also many experimental evidences to support their requirement for pathogenic microbes. For instance, the TA systems-caused

persistence and biofilm formation help pathogenic species to overcome both immune responses as well as antibiotic treatments (Ceri, *et al.*, 1999, Kedzierska & Hayes, 2016). In uropathogenic strains of *E. coli* and pathogenic *Haemophilus influenzae* they promote survival and also colonization ability in animal organs (Norton & Mulvey, 2012, Ren, *et al.*, 2012, Ren, *et al.*, 2014). The same effect has been observed for *E. faecalis*, where the deletion of the antitoxin of the Fst toxin caused hypervirulence and improved colonization ability of the mutant compared to the wild-type bacteria (Michaux, *et al.*, 2014). Also, deleting three MazF-mt genes from the *M. tuberculosis* chromosome decreased its ability to colonize and damage infected tissues (Tiwari, *et al.*, 2015b, Tiwari, *et al.*, 2015a). The MazF-mt systems increase the tolerance of bacteria to different stress conditions that can occur during infection and contribute to the survival in macrophages (Tiwari, *et al.*, 2015a). The triple-deletion of MazF-mt toxins decreases persister cell formation (Tiwari, *et al.*, 2015a), so the influence on virulence by these systems can probably be attributed to increased level of persisters. The association of TA modules with virulence has also been observed for the cholera pathogen *V. cholerae*. Two RelBE systems in this bacterium contribute to colonization both *in vitro* and *in vivo* mice models, although the mechanism of action of these systems is not clear (Wang, *et al.*, 2015). They affect neither the tolerance of *V. cholerae* to bile or reactive oxygen species, which are frequently encountered by bacteria upon infection, nor does their deletion change the expression of virulence factors (Wang, *et al.*, 2015). TA systems are also widespread and actively transcribed in clinical isolates of *Pseudomonas aeruginosa* (Williams, *et al.*, 2011), *S. aureus* (Williams, *et al.*, 2011), *Enterococcus faecium* and *E. faecalis* (Komi, *et al.*, 2015), although the involvement of the systems in the virulence of these bacteria remains a subject of future research.

Increasing data support the involvement of TA systems in virulence, whereas the ways how TA systems influence pathogenicity are complex and diverse (De la Cruz, *et al.*, 2013) and for many systems the exact mechanism has not been confirmed yet. Nevertheless, the main strategy of TA toxins in supporting the virulence is probably to promote the stress tolerance of a pathogen by increasing the number of persistent cells in a population.

5. TA systems in *Pseudomonas* spp.

TA systems are most thoroughly studied in *E. coli,* but also in species of *Staphylococcus* (Bukowski, *et al.*, 2013, Nolle, *et al.*, 2013, Williams & Hergenrother, 2013, Schuster, *et al.*, 2015), *Bacillus* (Silvaggi, *et al.*, 2005, Fico & Mahillon, 2006, Wu, *et al.*, 2011, Jahn, *et al.*, 2012), *Salmonella* (De la Cruz, *et al.*, 2013, Helaine, *et al.*, 2014) and *Mycobacterium* (Singh, *et al.*, 2010, Frampton, *et al.*, 2012, Tiwari, *et al.*, 2015a). Interestingly, at the time I started my PhD studies, hardly any data about TA systems in pseudomonads were

available, although the members of this genus have been of great interest to numerous researchers. *P. aeruginosa* is the most studied species in this genus due to its pathogenicity to human. On the other hand, research on *P. putida* is mostly instigated by its remarkable metabolic capabilities that allow a high tolerance to different stresses and potential use in bioremediation (Belda*, et al.*, 2016). All in all, the genus *Pseudomonas* inhabits a large variety of environments and includes plant growth promoting soil bacteria (*P. putida, P. fluorescens*) as well as plant pathogens (*P. syringae*) and human pathogens (*P. aeruginosa*). Yet, when I started my PhD studies, there were only some bioinformatic predictions about the distribution of TA systems in these bacteria (Pandey & Gerdes, 2005, Makarova*, et al.*, 2009, Shao*, et al.*, 2011), and just one study that confirmed active transcription of three TA systems in clinical isolates of *P. aeruginosa* (Williams*, et al.*, 2011). No confirmation about TA systems' functionality in this genus had been published.

The unsteady environments inhabited by *Pseudomonas* species (Silby*, et al.*, 2011) suggest the importance of TA systems in this genus. Indeed, for each fully sequenced *Pseudomonas* strain, at least a few TA systems have been predicted. The number of the systems varies from only four in *P. aeruginosa* PAO1 to 19 in *P. syringae* pv. tomato DC3000 (Makarova*, et al.*, 2009, Shao*, et al.*, 2011). Given that TA genes are small and not easily recognized, it is not surprising that the number of TA systems for each species varies in different predictions. For example, for *P. putida* KT2440, the pet bacterium of our laboratory, the results vary from 8 (Pandey & Gerdes, 2005) to 12 (Makarova*, et al.*, 2009) and 15 (Shao*, et al.*, 2011). One recent study showed that in *P. putida,* the number of different TA systems is higher for the environmental than for clinical strains (Molina*, et al.*, 2016), whereas the TA systems can be grouped into environmental, clinical, and universal clusters (Molina*, et al.*, 2016). This suggests that certain TA systems may play distinct roles in bacterial physiology at specific situations, although the experimental data on this subject is yet scarce. Still, the number of type II TA modules in pseudomonads has been found to be positively correlated with the persistence to antibiotics as more persistent *Pseudomonas* species possessed more type II TA systems in their chromosomes (Vogwill*, et al.*, 2016).

During the four years of my studies, the research about these fascinating systems has advanced and some functional studies of TA systems in pseudomonads have been published by now. For example, the functional analysis of the HicAB system of *P. aeruginosa* showed that the overexpression of the HicA toxin in *E. coli* suppresses growth and causes cell aggregation. Yet, the deletion of the entire *hicAB* locus from *P. aeruginosa* had no effect neither on biofilm formation nor virulence in mice (Li*, et al.*, 2016). The result is actually not surprising considering that single TA deletion mutants mostly show no recognizable phenotype (Maisonneuve*, et al.*, 2011).

Another TA system with assayed functionality is HigBA system of *P. aeruginosa*. The authors demonstrated that the HigB of *P. aeruginosa* has an

mRNase activity just like previously described toxins from this family (Hurley & Woychik, 2009), yet, differently from the HigBA system of *V. cholerae* (Budde*, et al.*, 2007), the antitoxin gene *higA* could be disrupted in the chromosome of *P. aeruginosa* (Wood & Wood, 2016), which suggests a more moderate toxicity for the HigB toxin. Surprisingly, the experiments with a transposon mutant of *higA* antitoxin of *P. aeruginosa* PA14 indicate that the HigB toxin has a negative effect on biofilm formation (Wood & Wood, 2016). Moreover, the levels of virulence factors pyochelin and pyocyanin were reduced at least two-fold in the antitoxin knockout strain (Wood & Wood, 2016). This indicates that TA systems may act very differently in the genus *Pseudomonas* compared to other bacteria as most TA toxins have been shown to increase virulence and biofilm production (see chapters 4.2.2. and 4.2.4.). Data on HigBA system in *P. aeruginosa* suggests an entirely opposite effects: the toxin proposedly decreases virulence of bacteria. However, as the cited work (Wood & Wood, 2016), did not include measurement of virulence itself, no clear conclusions can be made about the effects of the HigBA on regulation of pathogenicity in *P. aeruginosa*.

Mostly, the studies published on TA systems of *Pseudomonas* spp. have focused only on the phenotypic effects and the mechanisms of respective toxins were not addressed. In *P. putida* KT2440 only one thorough analysis of the mechanism of a toxin is on the MazEF-pp system. This work revealed that similarly to well-studied *E. coli* MazEF system, the toxin MazF-pp is an mRNAase, which recognises the sequence UAC (instead of ACA of *E. coli*) (Miyamoto*, et al.*, 2016). Yet, as for this system no physiological data are available, no final conclusions can be made about its importance to *P. putida*.

THE AIM OF THE THESIS

This work was initiated by a finding that a transpositional interruption of a putative antitoxin gene PP1585 suppressed the glucose-specific lysis of *P. putida* PaW85 deficient in the ColRS signal transduction system (Putrinš*, et al.*, 2011). Given that the *colR*-deficient bacteria have problems with maintaining membrane integrity (Kivistik*, et al.*, 2006, Putrinš*, et al.*, 2008, Putrinš*, et al.*, 2010), the antitoxin inactivation probably alleviated the membrane stress. This seemed highly intriguing because inactivation of the PP1585, which is the second gene in a putative TA system of the PP1586-PP1585 operon, should result in the activation of the toxin PP1586. The ability to disrupt the antitoxin gene is in itself quite uncommon among TA systems, as it normally leads to toxin-mediated growth inability. Yet, in the case of PP1586-PP1585, it was not only possible to disrupt the antitoxin gene without significant growth impairment, but the antitoxin inactivation was even beneficial for the *colR*-deficient strain (Putrinš*, et al.*, 2011). This observation can have two possible explanations. Firstly, assuming that the PP1586-encoded toxin is inactive, the stress relief could result from antitoxin absence. Bearing in mind that many antitoxins are transcription factors, it is possible that PP1585 is a regulator of some unknown stress factor. Secondly, PP1586-PP1585 could be a bona fide TA system and the disruption of the antitoxin could result in toxin activation and relieve the stress the *colR*$^{-}$ strain experiences. This possibility would suggest an uncommonly mild toxicity of the toxin as growth rate of bacteria was only slightly reduced after the disruption of the antitoxin gene. Based on these intriguing possibilities we hypothesised that:

- the genes PP1585 and PP1586 encode an active TA system
- PP1586 codes for an unusually mild toxin able to contribute to the stress tolerance of *P. putida.*

To control these hypotheses, the functionality and regulation of PP1586-PP1585 operon-encoded TA system was analysed.

RESULTS AND DISCUSSION

1. PP1586-PP1585 codes for a functional TA system widespread in pseudomonads (Ref I)

The disruption of an antitoxin gene of a TA system is often impossible (Shah*, et al.*, 2006, Budde*, et al.*, 2007) as it results in uncontrolled activation of the toxin and growth inhibition. Considering that the transposon-disrupted PP1585 was only predicted to be an antitoxin gene, my first goal was to confirm that the PP1586-PP1585 two-gene operon codes for a functional TA system.

1.1. The HigBA-resembling PP1586-PP1585 module is widespread in the genomes of *Pseudomonas* spp.

To get the first insight into the newly discovered system, I started my studies with a bioinformatic analysis of PP1586-PP1585 genes. Using the NCBI BLAST tool, I searched for putative PP1586-PP1585 homologs in the chromosomes of other bacteria and found that the most similar protein to PP1585 was a predicted HigA family antitoxin from *Nitrosococcus halophilus* Nc4 (57% identity), and the most similar protein to PP1586 was a putative killer protein from *Delftia* sp. Cs1–4 (71% identity). The closest chromosome-encoded proteins that had already been experimentally described were also proteins of the HigBA system: a chromosomal TA system from *V. cholerae* (identity 37% between PP1586 and the HigB toxin and 36% between PP1585 and the HigA antitoxin) and a TA system from the Rts-1 plasmid of *P. vulgaris* (identities 23% and 26% for toxins and antitoxins, respectively). Figure 9 shows the alignment of these three proteins created by ClustalW2 (Thompson*, et al.*, 1994).

The structure of the PP1586-PP1585 locus differs from the majority of type II TA systems by the arrangement of the two genes. Usually the longer antitoxin gene precedes the toxin gene, but in case of PP1586-1585, the order and sizes of the partner genes is reversed as is typical also for *higBA* loci (Ref I, Fig 1A). Thus, both the sequence similarity and gene arrangement suggest that the locus PP1586-1585 does code for a HigBA family TA system, although the sequence identity of PP1586-1585-encoded proteins with previously studied HigBA proteins is quite modest (Fig 9).

To get insight into the system's distribution, the fully sequenced species of the genus *Pseudomonas* were scanned for orthologues of PP1586-PP1585 genes. It turned out that the system is quite conserved in the strains of the variable genus *Pseudomonas* with 30 out of 48 completely sequenced pseudomonads containing a TA pair orthologous to PP1586-PP1585 (Ref I, Table 2S). The most similar proteins were encoded in the chromosomes of other *P. putida* species, F1 and NBRC 14164, with identities over 97% and 92% for the toxin and 99% and 97% for the antitoxin. Besides *P. putida* strains, the locus is also

present in two *P. fluorescens* strains and one *P. stutzeri, P. syringae* and *P. denitrificans* strain. Moreover, the locus was found to be present and extremely conserved (identity in case of the toxins over 96% and in case of the antitoxins over 97%) in all of the 13 fully sequenced *P. aeruginosa* strains. Identity of these orthologues to PP1586 and PP1585 was about 68–70% and 48–56%, respectively (Ref I, Table 2S). The high conservation of the system reflects its possible physiological importance for these bacteria. Considering that the orthologues of PP1586-PP1585 were present in all 42 *P. aeruginosa* clinical isolates obtained from 20 cystic fibrosis patients and were actively transcribed in all tested strains (Williams*, et al.*, 2011), the system could be involved in the virulence of this opportunistic pathogen.

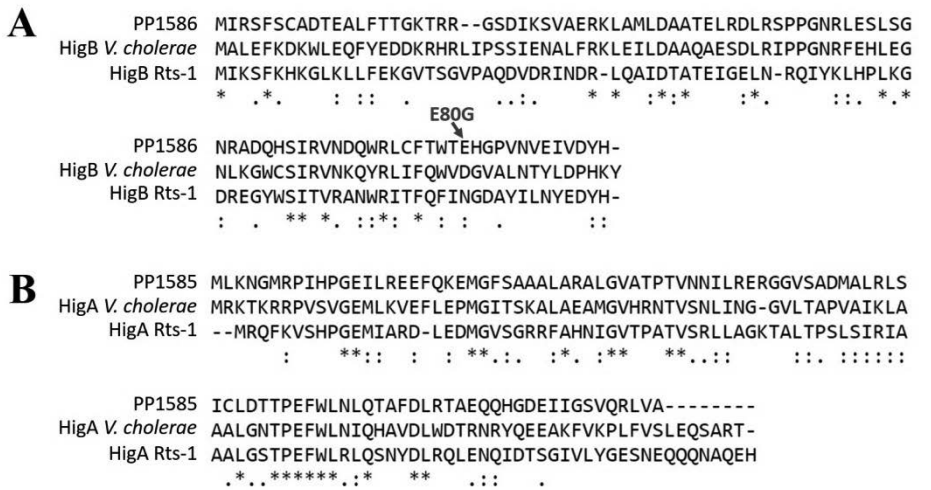


Figure 9. Alignment of PP1586 and PP1585 proteins with chromosomal (from *V. cholerae*) and plasmid-borne (from Rts1 of *P. vulgaris*) HigBA. A: PP1586 and HigB (toxins), B: PP1585 and HigA (antitoxins).
* indicates fully conserved residues
: indicates residues belonging to the same 'strong' group
. indicates residues belonging to the same 'weak' group
The arrow indicates the position of the point mutation E80G in the PP1586 toxin

1.2. The PP1586-PP1585 codes for a growth-rate-affecting TA system GraTA

To analyse the functionality of the PP1585-PP1586 system, we deleted the genes from the wild-type *P. putida* PaW85 chromosome individually (antitoxin PP1585 – ΔgraA or toxin PP1586 – ΔgraT) as well as knocked out the entire operon (PP158-PP1585 – ΔgraTA) (Ref I, Fig 1A). To find out whether the previously described lysis suppression of the *colR*$^{-}$ mutant was either due to the

loss of the antitoxin or the activation of the toxin, we constructed respective deletion mutants also in the *colR*-defective strain. By accidental PCR error, we obtained an antitoxin deletion mutant with a point-mutation in the toxin gene, resulting in replacement of glutamic acid with glycine at the 80th position ($\Delta graA_{T(E80G)}$) (Ref I, Fig 1A). This deletion strain was also included in the experiments to investigate the effect of the mutation on the toxin.

To evaluate the influence of the PP1585-PP1586 genes on the cell envelope stress phenotype of the *colR*-deficient strain, I compared the *colR*$^{-}$ strain with its TA-deletion derivatives using flow cytometry that enables to study the bacterial populations at single cell level. The staining of the cells with two DNA-binding stains, Syto 9 and propidium iodide (PI), differentiates subpopulations of intact, damaged and dead cells. We found that the subgroup of dead cells disappeared from the population of *colR*-deficient bacteria where only the PP1585 gene (the antitoxin) had been deleted, yet the deletion of the entire locus showed no effect (Ref I, Fig 5A). Therefore, this analysis confirmed that the stress-relieving factor was not the PP1585 disruption itself, but rather the presence of PP1586 in the absence of PP1585. This strongly suggested that PP1586 codes for a functional toxin, which has a stress-relieving effect. Considering that the deletion of the antitoxin gene also resulted in a slight growth rate reduction (Ref I, Fig 1B), the PP1586-PP1585-encoded TA system was named GraTA for the toxin's growth-rate-affecting ability.

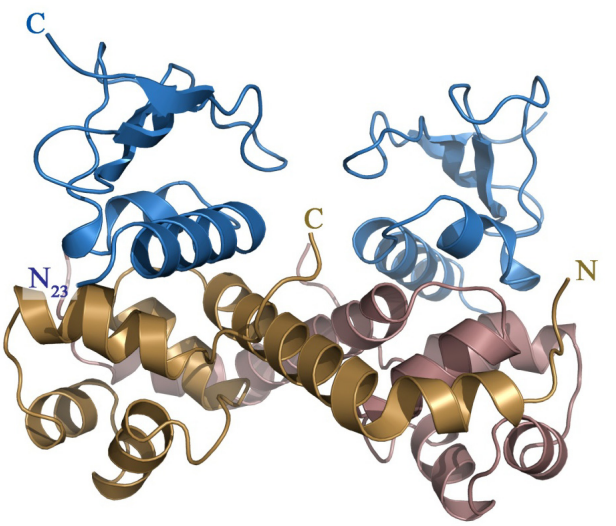
The fact that antitoxin deletion allowed the cells to grow quite well necessitated a confirmation of the toxin's activity. Thus, I overexpressed the toxin in the wild-type *P. putida* and its deletion derivatives of the *graTA* system (ΔgraA, ΔgraT, and ΔgraTA) (Ref I, Fig 1A). The ectopic overexpression of GraT confirmed its toxicity as it resulted in severe growth defects of bacteria lacking the GraA antitoxin (Ref I, Fig 2A). Surprisingly, no apparent effect on growth was observed in wild-type bacteria (Ref I, Fig 2A). Thus, the innate antitoxin should very efficiently inactivate the overexpressed toxin. To finally confirm the counteracting ability of the antitoxin, I overexpressed the GraA antitoxin in the same mutants. As the ectopically expressed antitoxin could relieve the growth rate reduction caused by the innate toxin (Ref I, Fig 2B), we concluded that the *graTA* codes for a functional TA system.

The toxin GraT is easily inactivated by the antitoxin GraA. Furthermore, even in the absence of the antitoxin, the GraT showed only a moderate effect on growth rate of bacteria in optimal conditions (rich broth, 30 °C) (Ref I, Fig 1B). Yet, observing these results, one has to bear in mind that usually the effects of toxins are studied in overexpression conditions (Gotfredsen & Gerdes, 1998, Zhang*, et al.*, 2003, Christensen-Dalsgaard*, et al.*, 2010, Li*, et al.*, 2016), which can be quite different from natural ones. Considering that several other chromosomal antitoxins can be disrupted without severe effects on bacterial growth, similarly to GraTA system (Jorgensen*, et al.*, 2009, Daimon*, et al.*, 2015), it is tempting to hypothesize that the toxins expressed from a single chromosomal gene copy would not be so noxious as usually presumed from the over-

expression experiments. Therefore, I would like to emphasize that all the effects of the GraT toxin presented in the current thesis are observed in the antitoxin deletion strain, which represents the maximum effect that the toxin can have for the bacterium in natural situations.

To find out if the effects of GraT on bacterial growth could be more severe in some other growth situations, we tested deletion mutants in non-optimal conditions, including various minimal media with different carbon sources and at lower (20 and 25 °C) and higher (37 °C) temperatures. These experiments revealed that the effect of GraT on growth rate is much more pronounced at temperatures below the optimal and the defect of growth disappeared at 37 degrees (Ref I, Fig 1B), which shows a temperature dependency of the GraT's toxicity.

The GraTA system most closely resembles the HigBA of the type II TA systems. These systems consist of two proteins that interact directly to form a complex in which the toxin is inactive (Review of literature, chapter 2.2). To test if GraTA shares these features, we co-purified His-tagged GraT with untagged GraA. The co-purification resulted in approximately equal amounts of both proteins, which shows that a ratio of GraT and GraA proteins in the complex is 1:1 (Ref I, Fig 3). In collaboration with R. Loris group, the crystal structure of the GraTA complex was recently solved proving that the proteins form a tetrameric complex of a GraA dimer and two GraT monomers (Fig 10). Interestingly, despite the modest similarity on the sequence level, the structures of GraT and GraA proteins are structurally very similar to HigBA proteins from the *P. vulgaris* plasmid-borne system (Schureck*, et al.*, 2014).



Fgure 10. Crystal structure of GraTA complex. The GraTA complex consists of a GraA dimer bound to two GraT monomers. GraT molecules are blue, one GraA monomer is brown and the other one light purple. Amino-acids 23 (N_{23}) to 92 (C) of GraT and 1 (N) to 99 (C) of GraA can be seen in the structure (Talavera, Tamman *et al*., unpublished).

Taken together, the first few experiments with *graT* and *graA* deletion strains as well as the overexpression of GraT and GraA in *P. putida* verified that GraTA is a functional TA system. However, it has many uncommon traits: (i) the toxic effect of GraT is probably much milder compared to most TA toxins as it allows disruption of the antitoxin gene; (ii) GraT toxin causes a cold-sensitive growth inhibition, which has not been described for other type II TA toxins; (iii) the innate GraA antitoxin is expected to be a very efficient antidote for GraT as the overexpression of the toxin has no observable effects on wild-type *P. putida*.

2. Regulation of the GraTA system (Refs I and II)

Antitoxins of type II TA systems are usually very labile proteins (Brzozowska & Zielenkiewicz, 2013) and need constant production to inhibit the toxin (Review of literature, chapter 2.2.1.). To address the unexpected result of the uncommon efficiency of GraA to inactivate the overexpressed GraT, it was important to understand the regulation of the system. The high efficacy of GraA could be achieved by two possible ways: (i) efficient synthesis of the antitoxin could guarantee its excess amount over the toxin, (ii) a high stability of the antitoxin could ensure efficient inhibition of the ectopically expressed toxin.

2.1. *graTA* is repressed by GraA and derepressed by GraT

Transcriptional regulation of type II TA systems is usually achieved by auto-repression of the operon by the antitoxin and/or the TA complex (Loris & Garcia-Pino, 2014). TA complex has generally a higher affinity to promoter DNA than the antitoxin alone and derepression happens only when the amount of toxin exceeds that of the antitoxin. Thus, the expression of a TA operon is typically controlled by conditional cooperativity, where the lower toxin concentrations enhance and higher concentrations decrease the binding of the antitoxin to the promoter (Loris & Garcia-Pino, 2014).

To study transcriptional regulation of the *graTA* system, I measured the *graTA* promoter activity by using a reporter gene *lacZ.* As a significant increase in the expression from the *graTA* promoter was seen in the ΔgraA strain (Ref I, Fig 4A), the GraA protein was confirmed to act as an autorepressor. DNase I Footprint analysis indicated that the regulation was achieved by direct binding of GraA to the *graTA* promoter DNA (Ref I, Fig 4C). The absence of GraT resulted in slightly stronger repression of transcription compared to wild-type, suggesting that GraT might act as a derepressor of the operon (Ref I, Fig 4A). Yet, the DNase I Footprint analysis did not support this idea, as the antitoxin and the TA complex were needed in almost similar amounts to produce the DNase I-protected area on the operator DNA (Ref I, Fig 4C).

To figure out the action of the toxin in the regulation of *graTA* expression, the binding of GraA and the GraTA complex to DNA was analysed by

isothermal titration calorimetry (ITC). The results showed a high affinity for the binding between the GraA and DNA (K_D=200 nM) (Fig 11A), which is quite similar to some other TA antitoxins (Wen, *et al.*, 2014). Yet, determining the thermodynamic parameters of GraA binding to *graTA* operator region showed also an unexpected result. Commonly, one type II antitoxin dimer binds to one palindromic sequence of a DNA molecule (Brown, *et al.*, 2011, Wen, *et al.*, 2014, Zorzini, *et al.*, 2015), and we had found one palindromic sequence from the operator region of *graTA* (Ref I, Fig 4B). Thus, we expected to see that one GraA dimer binds to the promoter. However, ITC results showed that two GraA dimers bind to the *graTA* operator DNA to repress the expression from the *graTA* locus (Fig 11A).

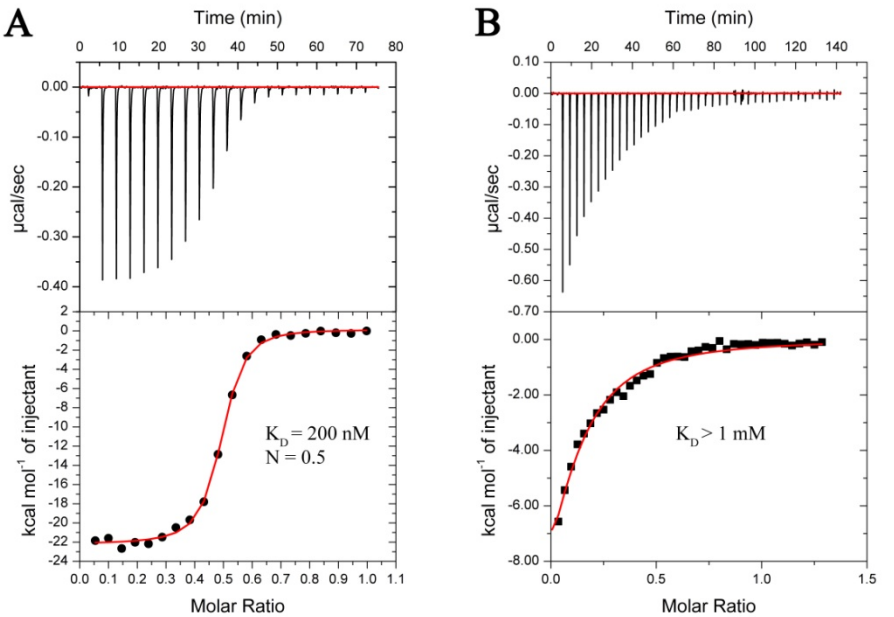


Figure 11. The ITC curves of GraA and GraTA binding to DNA. A: 90 µM of GraTA operator DNA was titrated into 20 µM of GraA. B: 989 µM GraTA operator DNA was titrated into 165 µM of GraTA. The upper panels show the raw injection heats, the lower panels, the corresponding binding isotherms. The dissociation constants and molar ratio of the binding of the proteins and DNA are indicated (Talavera, Tamman *et al.*, unpublished).

From the footprinting data we had presumed that GraT does not essentially influence the DNA-binding ability of the antitoxin (Ref I, Fig 4C). Yet, the thermodynamic parameters of the binding between GraTA complex and DNA surprisingly showed that the affinity of the GraTA complex to DNA is over three orders of magnitude lower than for GraA antitoxin alone (Fig 11B). Moreover, the binding of GraTA complex to DNA is so low that the precise

binding affinity cannot be determined. Thus, the ITC data showed that the toxin inhibits the DNA binding of GraA (Fig 11B), indicating that GraT acts as a derepressor in *graTA* regulation. The discrepancy between the GraTA complex binding ability in footprinting and ITC analysis is not easy to explain. Still, one may speculate that DNA cleavage by DNase I may somehow stabilize the GraTA binding to the operator in the footprint experiment (Ref I, Fig 4C). In spite of this discrepancy, both *in vitro* analyses show that, differently from what is reported on many TA systems, *graTA* is not regulated by conditional cooperativity. Rather GraA alone is a repressor of *graTA* expression while the toxin acts as a derepressor only.

Although the *in vivo* transcriptional regulation measurements in *P. putida* also indicated that the toxin might be a derepressor, there was only a slight difference in β-galactosidase activities between the wild-type and the ΔgraT derivative (Ref I, Fig. 4A). Thus, to further analyse the effect of the GraTA proteins on their promoter activity *in vivo*, I performed the β-galactosidase activity measurements in *E. coli*, so that the chromosomal *graTA* genes could not influence the obtained results. I used a two-plasmid system: the *graTA-lacZ* transcriptional fusion was encoded by the plasmid p9TT1586 (as in previous experiments in *P. putida*; Ref I, Fig 4A), and the GraA or GraTA proteins were expressed from the IPTG-inducible promoter in plasmids pBBR_GraA and pBBR_GraTA, respectively. In good accordance with the ITC data of GraA having a higher affinity to DNA than the GraTA complex, I found that GraA repressed the *graTA* promoter strongly, whereas almost no repression was caused by the complex (Fig 12A).

Thus, our results suggest a mechanism for *graTA* transcriptional regulation, which differs from conditional cooperativity, where toxin can act both as corepressor and derepressor (Overgaard*, et al.*, 2008, Garcia-Pino*, et al.*, 2010, Cataudella*, et al.*, 2012). In the regulation of *graTA* expression, GraT toxin is a derepressor only, resembling that of the MqsRA system, as the MqsR toxin also inhibits the antitoxin binding to *mqsRA* operator (Brown*, et al.*, 2013). If GraA is in excess, it binds to the operator and efficiently represses the transcription from the *graTA* promoter. However, when the abundance of GraT increases and equals with that of the GraA, the GraTA complex is formed and due to its low DNA binding affinity, expression from the *graTA* is elevated. Thus, increased amount of toxin leads to a stronger expression of the *graTA* locus, including an increase in the synthesis of the antitoxin. Note, that when compared to conditional cooperativity, the outcome in response to toxin excess is quite similar: the *graTA* genes are derepressed and enhanced expression of the locus guarantees that antitoxin levels increase again and silence the toxin.

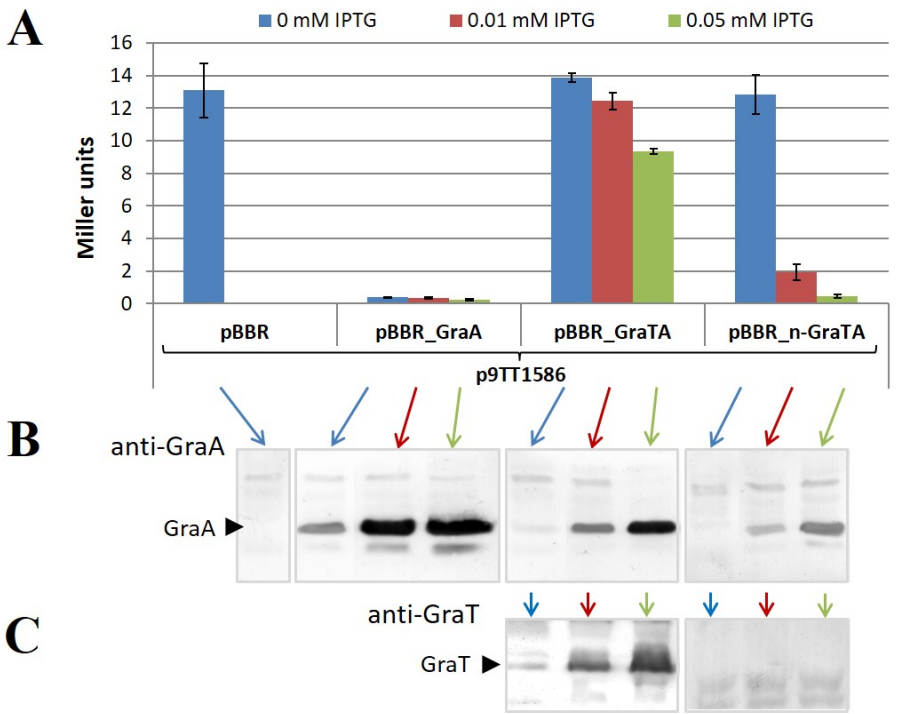


Figure 12. *graTA* operon is repressed only by GraA. A: Measurements of β-galactosidase activity in *E. coli* containing the plasmids p9TT1586 with *graTA-lacZ* transcriptional fusion and pBBR enabling the overexpression of GraTA proteins. The first bar represents the β-galactosidase activities in the presence of an empty pBBR vector. Second and third clusters of bars show the effects of GraA and GraTA (respectively) expressed from genes preceded by a strong Shine-Dalgarno (SD) from pET11c vector. The last cluster of bars represents the effect of GraTA expressed from its native SD (n-GraTA). B and C: Western blots using anti-GraA (B) or anti-GraT (C) antibodies to estimate the amount of expressed GraA or GraT, respectively. Coloured arrows show the bands of the Western blot corresponding to bars of the diagram.

2.2. GraA is translated more efficiently than GraT

In order for the TA system not to poison the cell, the antitoxin must be produced in a higher amount than the toxin. For most type II TA systems the antitoxin gene precedes the toxin gene in the operon, which is thought to result in a higher production of the antitoxin compared to the toxin. In the TA families with reversed operon structure, different options may be applied to achieve antitoxin's efficient production. For example, some systems have an additional promoter inside the operon, in front of the antitoxin gene (Tian*, et al.*, 1996b, Otsuka*, et al.*, 2010). For *graA* an additional promoter could not be identified

with 5'-RACE analysis (Ref I). Thus, there should be another explanation for the efficient production of the antitoxin.

Studying the transcriptional regulation of the *graTA* system in *E. coli*, I used a construct where the original Shine-Dalgarno (SD) sequence of the *graTA* genes was replaced with a strong SD from a pET plasmid (pBBR_GraTA and pBBR_GraA). This resulted in high expression of both proteins (Fig 12B and C) However, for expression of the GraTA complex I also used another plasmid, pBBR_n-GraTA, where the *graTA* genes retained their original upstream region, i.e. translation of both GraT and GraA originated from their native SD. Measuring the *graTA* promoter activity in the presence of pBBR_n-GraTA revealed largely different results from data obtained with pBBR_GraTA. While the expression of GraTA complex from the pBBR-GraTA resulted in clear derepression of transcription, the pBBR_n-GraTA construct allowed only partial activation of the *graTA* promoter (Fig 12A). To figure out what could be the reason for these conflicting results, the amount of the proteins was estimated by Western analysis using antibodies against the GraA or GraT proteins. Both proteins were detectable in high amounts when they were expressed from pBBR_GraTA (Fig 12B and C). However, when the GraTA proteins were expressed from pBBR_n-GraTA, I could only detect the antitoxin, but not the toxin on Western blot membranes (Fig 12B and C). For translation initiation, the ribosome binds to the SD sequence, commonly located about eight bases upstream of the start codon. The efficiency of protein synthesis largely depends on the ability of the SD of a particular gene to bind to the complementary 3' end of 16S rRNA (Shine & Dalgarno, 1974). As our results show that the *graTA* promoter repression observed with the GraTA complex expressed from the native SD sequence resulted from a high amount of free antitoxin in the cells (Fig 12B and C), the SD sequence preceding *graA* must be stronger than that in front of *graT* in the native *graTA* locus. Thus, our data suggest that the higher antitoxin production is regulated not at the level of transcription, but rather at the translational level. These data are in line with the results reported for the HigBA system from the Rts-1 plasmid – the authors claim that when expressing the *higBA* genes in their native arrangement from the T7 promoter, only the HigA protein was detected (Tian*, et al.*, 1996a). This kind of difference between the translation efficiency of the two TA proteins seems meaningful for TA systems with a reversed gene order, as it ensures increased production of the antitoxin over the toxin. The higher translation rate of the antitoxin would suffice to keep the antitoxin amount high enough for inactivation of the toxin even without the additional promoter for the antitoxin gene (Review of literature, chapter 2.2.1.1.).

2.3. GraA is an exceptionally stable protein (Ref II)

Most type II antitoxins are labile proteins with half-lives of only around 15 minutes (Review of literature, chapter 2.2.1.2). Considering the uncommonly efficient inactivation of the overexpressed GraT by the GraA antitoxin (Ref I, Fig 2B), it was tempting to speculate that GraA could be a quite stable protein. To control this hypothesis, the degradation rate of GraA was determined by incubating the purified antitoxin in the cell lysate of *P. putida* and monitoring the amount of GraA using Western blotting. The results showed that the degradation of GraA is very slow in cell lysates from *P. putida* grown at optimal growth conditions (rich broth, 30 °C) (Ref II, Fig 1). However, study of GraA degradation rate throughout the bacterial growth revealed that the stability of GraA decreases at the transition from exponential to stationary phase (Ref II, Fig 1) when bacteria adapt to lower nutrient levels and other disfavoured conditions. Although the stability of GraA decreased markedly, the half-life of the antitoxin still remained around one hour, which is roughly about four times higher compared to most type II antitoxins (Brzozowska & Zielenkiewicz, 2013). Also, the energetic status of the cell is probably an important factor influencing the GraA degradation rate as addition of ATP to the degradation mixture stabilized GraA remarkably (Ref II, Fig 2A).

The rapid degradation of antitoxins is usually instigated by the unstructured and flexible C-terminal part of the protein and carried out by major cellular stress-induced proteases Lon or ClpP (Brzozowska & Zielenkiewicz, 2013). These proteases recognize the unstructured part of the antitoxins and degrade them to oligopeptides (Sauer & Baker, 2011). Notably, GraA seems to have a more fixed structure (Fig 13). Also, the antitoxin stability was not altered by the deletion of either *lon* or *clpP* from *P. putida* genome (Ref II, Fig 2A). These results strongly suggest an entirely different regulation of GraA stability. Indeed, GraA degradation seems to be triggered by an endoprotease that cuts the antitoxin into two unequal parts at about the 80th position. These parts are afterwards probably individually directed to final degradation. The cleavage site was initially suggested by a small peptide appearing on Western blot membranes during the GraA degradation (Ref II, Fig 1B and D) and further supported by site-directed mutagenesis. We mutated several amino acids in the vicinity of the 80th position and noticed that GraA(R80A) was more stable and GraA(L79A) more labile compared to wild-type GraA (Ref II, Fig 3).

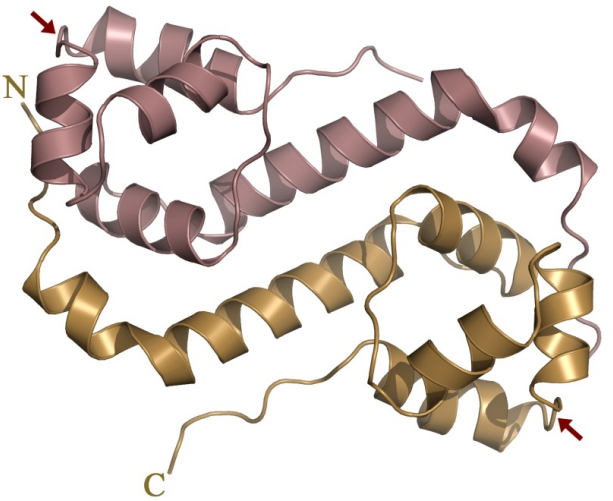


Figure 13. The crystal structure of GraA dimer. One monomer is in purple, the other one in brown colour. The approximate position of the 80th amino-acid is marked with red arrows. The N- and C-terminus are marked on the brown monomer (Talavera, Tamman *et al*., unpublished).

The increased lability of GraA(L79A) allowed us to carry out a transposon mutagenesis to search for regulators of GraA stability. We expressed the GraA(L79A) protein in the ΔgraA mutant at such a low level that its degradation rate exceeded its synthesis rate. Thus, it could not complement the lack of GraA unless it was stabilized by a transpositional disruption of a protease gene. The inability of ΔgraA bacteria to form colonies at 20 °C, was a convenient phenotype in the screen for the GraA-stabilizing mutants. Unexpectedly, the transposon mutant screen did not lead to the discovery of the GraA-degrading protease. Instead, the disruption of *mexT*, which codes for a LysR type transcriptional regulator, allowed colony formation of ΔgraA bacteria at 20 °C. To test if MexT is indeed involved in the stability of GraA, the degradation dynamics of GraA were analysed in the cell lysate of a *mexT* deletion strain. Given that the absence of MexT regulator increases the stability of GraA more than threefold (Ref II, Fig 4), MexT somehow contributes to GraA degradation. MexT has been extensively studied in *P. aeruginosa*, where it modulates virulence and tolerance to antibiotics (Tian*, et al.*, 2009b, Fargier*, et al.*, 2012). MexT of *P. putida* PaW85 is over 80% identical to its orthologue in *P. aeruginosa* (Winsor*, et al.*, 2009), which suggests that the regulator has similar functions in these bacteria. Indeed, it has been shown that in both bacteria, MexT activates the expression of an efflux pump MexEF-OprN (Tian*, et al.*, 2009a, Herrera*, et al.*, 2010). Considering that MexT has many other target genes with still unknown functions (Tian*, et al.*, 2009a), it is possible that the same conditions that activate MexT could trigger the degradation of GraA.

MexT has been shown to be activated under oxidative stress (Fargier*, et al.*, 2012), and indeed, the *mexT* deletion decreased the tolerance of *P. putida* to diamide, known to cause oxidative stress (Fig 14). Yet, when the *graTA* genes were deleted from the genome either together or individually, the diamide tolerance was not decreased compared to the wild-type (Fig 14). Therefore, the GraTA system is not important for oxidative stress tolerance in *P. putida*, at least under tested laboratory conditions.

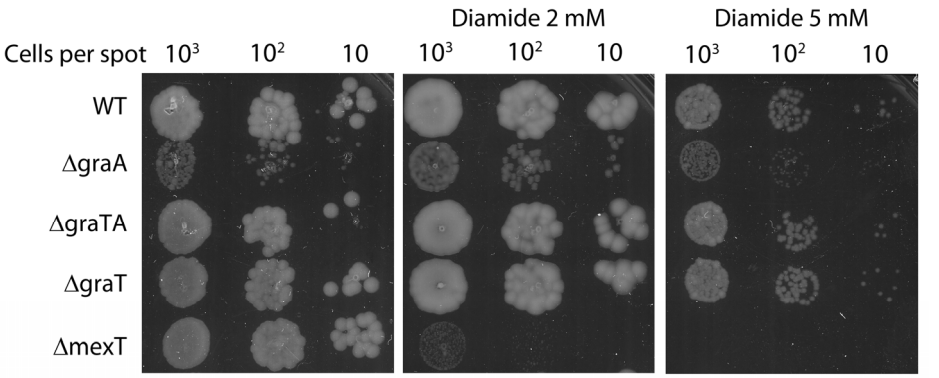


Figure 14. Oxidative stress tolerance of *P. putida* PaW85 (WT) and its *graTA* (ΔgraA, ΔgraTA and ΔgraT) or *mexT* (ΔmexT) deletion derivatives. Overnight cultures were serially diluted and spotted on LB plates containing 2 mM or 5 mM diamide and incubated for 44 h at 25 °C. Numbers above the pictures show the approximate amount of cells in each spot.

2.4. A proposed model for the regulation of GraTA system

The current study revealed several regulatory mechanisms that guarantee the silencing of GraT toxin under normal growth conditions. We propose the following regulation of the GraTA system in *P. putida* (Fig 15). Firstly, the expression of *graTA* genes is repressed in the excess amount of the antitoxin over the toxin (Ref I Fig 4A; Fig 12). Differently from several other TA systems, the GraT toxin acts as a derepressor only and no conditional cooperativity is involved in the *graTA* regulation. Secondly, though the antitoxin gene is the second one in the operon, the production of GraA is higher than that of the toxin, due to increased translation efficiency of the GraA-specific mRNA (Fig 12). Thirdly, GraA is an unusually stable antitoxin, though several factors, including the growth phase, ATP levels, an unknown endoprotease and MexT regulator can affect its stability (Ref II).

The most interesting question regarding the regulation of any TA system is how the activation of the toxin is achieved. Considering the high stability of the antitoxin GraA, we assume that the activation is caused by the degradation of GraA triggered at conditions that remain to be discovered.

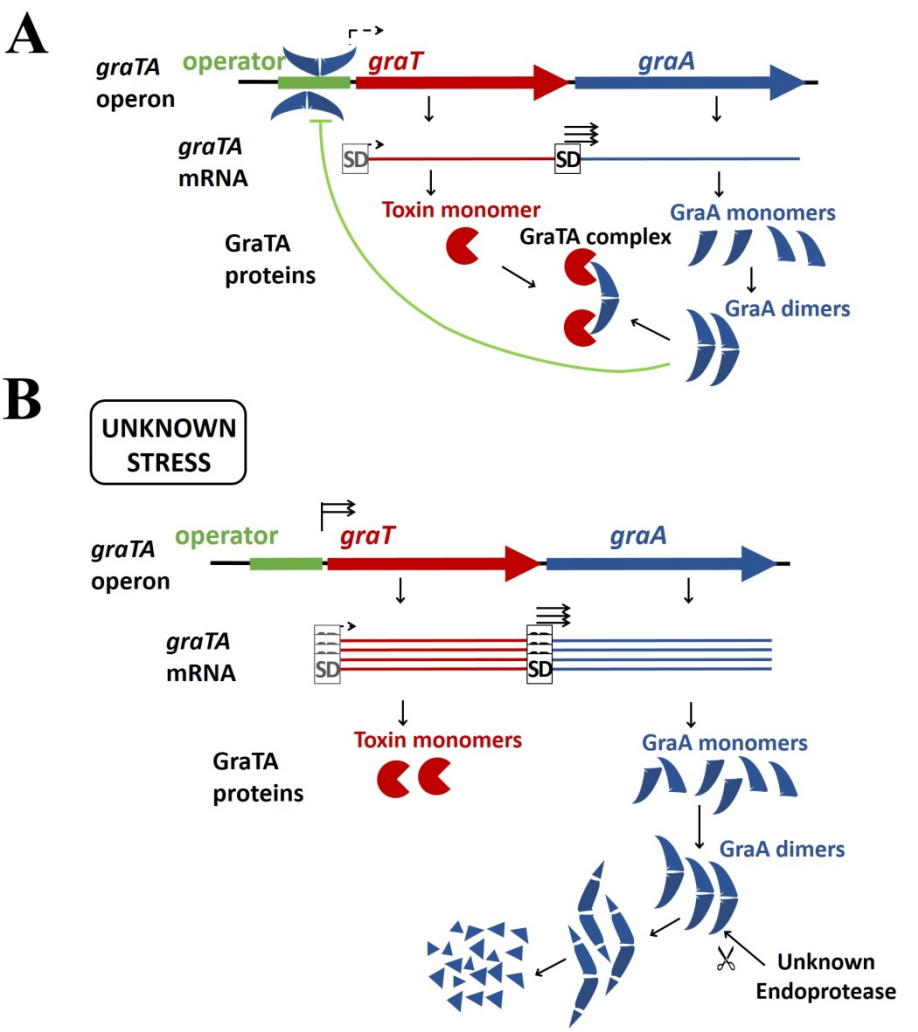


Figure 15. Schematic representation of the regulation of the GraTA system. A: In unstressed conditions the expression of *graTA* genes is repressed by the antitoxin. From the small amount of mRNA that is still produced, the GraA antitoxin is translated more efficiently than the GraT toxin. GraA forms into a dimer and inhibits the toxin by binding two GraT monomers. B: Unknown stress conditions activate an unknown endoprotease that cuts GraA near the 80th position (marked with scissors), resulting in the degradation of GraA. In the lack of the antitoxin, GraT is free to attack its cellular target and the operon is derepressed. The operator region is green, toxin red and antitoxin blue. Transcription/translation is marked with an arrow (dotted line for weak and solid for strong efficiency).

In several regulatory aspects the GraTA system resembles the MqsRA system of *E. coli*. Just like for GraTA, the regulation of MqsRA does not include conditional cooperativity and the toxin MqsR acts solely as a derepressor of the TA operon (Brown*, et al.*, 2013). Moreover, in the *mqsRA* locus, the toxin gene precedes the antitoxin gene and no additional promoter for antitoxin expression has been determined (Christensen-Dalsgaard*, et al.*, 2010). Notably, both MqsA and GraA are quite stable antitoxins. One can hypothesize that to achieve an efficient amount of the MqsA antitoxin, it also needs higher translation efficiency as observed for GraA, but sadly, this has remained unstudied to date. Alike other TA systems, the trigger of MqsRA and GraTA systems, considering the liberation of MqsR or GraT toxins, is the increase in the degradation rate of antitoxins. Degradation of MqsA was shown to accelerate upon oxidative stress resulting in the activation of the system (Wang*, et al.*, 2011). Unfortunately, the stress conditions that would lead to the destabilization of GraA and, thus, activation of GraT have not been determined. Nevertheless, stabilizing effect of ATP and involvement of MexT in GraA degradation could give decisive leads to ascertain the conditions of GraT activation (Ref II, Fig 6).

3. The effect of GraT on translation involves the DnaK chaperone

3.1. The GraT toxin inhibits ribosome biogenesis (Ref III)

Temperature dependence is hardly ever associated with TA toxins. Only a few examples can be found: heat shock destabilizes the toxin mRNA of a type I system BsrG/SR4 (Jahn*, et al.*, 2012) and activates the type II toxin YoeB (Janssen*, et al.*, 2015). GraT, however, causes severe growth defects at reduced temperature. It can be that susceptibility of the GraT target is increased at lower temperature. As cold sensitivity of bacteria can be associated with disturbed ribosome biogenesis (Shajani*, et al.*, 2011), and a majority of type II TA toxins target translation (Guglielmini & Van Melderen, 2011), the starting point for discovering the target of GraT was analysing the ribosome profiles of wild-type and ΔgraA strains of *P. putida*. The finding that bacteria with the active toxin (ΔgraA strain) contained a remarkably increased amount of free ribosomal subunits (Ref III, Fig 1) indicated that the toxin had disturbed the translational machinery. In accordance with the cold sensitivity of the ΔgraA strain, the accumulation of free subunits was more pronounced at low temperatures (Ref III, Fig 1). Further analysis of accumulating subunits by mapping the 5’ ends of rRNAs and studying nucleotide modifications, revealed that the subunits lacked the final processing (Ref III, Fig 2) and late-assembly specific modifications (Ref III, Fig 3), thereby proving that subunits were immature. These results show that GraT inflicts a ribosome biogenesis defect, which well coincides with the cold sensitivity of the ΔgraA strain. Considering that the cold sensitivity

often accompanies the defects in the ribosome biogenesis process, the increased effect of GraT at reduced temperature is probably explained by the higher vulnerability of the process it targets. Still, we cannot exclude the possibility that the GraT amount in cells is increased at reduced temperature, but as the antibodies against GraT are not sensitive enough, we have not been able to test it.

What we have discovered about the toxin GraT is different from what is known about its closest relatives – the ribosome-dependent HigB mRNases of *V. cholera* and *P. vulgaris*. Firstly, GraT of *P. putida* is less toxic than chromosomally encoded HigB of *V. cholera,* because *graA* can be deleted, but respective gene (*higA*) from *V. cholerae* cannot (Budde*, et al.*, 2007). Secondly, GraT causes accumulation of free ribosomal subunits, while overexpression of *P. vulgaris* HigB does not result in this phenotype, rather the relative abundance of 70S monosomes increases (Hurley & Woychik, 2009). Third, in spite of several attempts, we have not been able to detect GraT on ribosomes, while HigB of *P. vulgaris* has been shown to associate with the ribosomal 50S subunit (Hurley & Woychik, 2009). Although these data suggest significant differences in the mechanism of action for distinct proteins of the same toxin family, we still wanted to find out if GraT could also be an mRNase similarly to its closest relatives. Unfortunately, we did not succeed in purifying the toxin for *in vitro* analysis of mRNase activity due to rapid precipitation of the GraT protein. We also do not have a stable plasmidial overexpression system for *P. putida* that would allow to study the *in vivo* effects of overexpressed toxin. Thus, we set out to find the target and precise mechanism of GraT's action using a different approach.

3.2. The toxicity of GraT is suppressed by the deletion of DnaK C-terminus (Ref III)

To get insight into the mechanism of GraT and identify factors involved in its toxicity, I carried out one more transposon mutagenesis experiment. This time I used a transposon containing a lacItac cassette with the IPTG-inducible tac-promoter directed towards the end of the transposon. This allows the transposon to be used for insertional activation as well as for inactivation of genes. The library of ΔgraA lacItac-transposon mutants was again screened for the ability of cells to form colonies at 20 °C. The plates contained IPTG to find genes that's disruption or overexpression could suppress the growth defect of ΔgraA strain. We supposed that if a factor involved in GraT toxicity was disrupted or the target of the toxin induced, the cold-sensitive phenotype of cells would disappear and the colonies could form at 20 °C. Surprisingly, we could not find any mutants with their growth dependent on IPTG. Thus, rather the disruption and not the overexpression of some gene suppressed the growth inability of the ΔgraA strain. Closer analysis showed that the cold-sensitivity of growth inflicted by the GraT toxin was relieved by disruption of the most distal C-terminal part

of the molecular chaperone DnaK (Ref III, Fig 5A and B). Analysing the ribosome profile of these bacteria showed that ribosome biogenesis defect was suppressed as well (Ref III, Fig 5C). Considering that DnaK assists the late stages of ribosome assembly especially at suboptimal conditions (e.g. at high or low temperatures) (Maki*, et al.*, 2002, Rene & Alix, 2011), these results further suggested that ribosome biogenesis is the process targeted by GraT.

Next, we aimed to determine if GraT may directly interact with DnaK. The fact that His-tagged DnaK could pull down the tagless GraT from *E. coli* lysate confirmed a direct interaction between the two proteins (Ref III, Fig 6). Although the deletion of the C-terminus of DnaK suppressed GraT toxicity, the interaction between the proteins occurred regardless of the presence of the C-terminal part of DnaK. These data are in line with previous studies, showing that the truncation of the distal part of the C-terminal lid-domain of DnaK does not influence its protein-binding ability (Swain*, et al.*, 2006). However, as the pull-down assay is not a quantitative method for detecting the binding between two proteins, we cannot draw conclusions about the possible differences in the binding affinities of GraT and C-terminally truncated DnaK compared to GraT and intact DnaK.

The direct interaction between GraT and DnaK suggests an important role of DnaK in GraT toxicity. Although we cannot completely exclude the possibility of GraT just out-titrating DnaK and causing chaperone limitation that inhibits ribosome biogenesis, this possibility seems highly unlikely. First, DnaK is abundant protein in bacteria (Calloni*, et al.*, 2012) and it is not likely that GraT can be present in levels high enough to just titrate out the chaperone. As already mentioned before, we cannot detect native levels of GraT by Western analysis indicating that the GraT concentration in *P. putida* is low. Also, as a single point mutation in the toxin gene can abolish the GraT toxicity (Ref I, Fig 2C), it is not likely that this mutation can also abolish binding to DnaK (though this has not been tested experimentally).

A much more plausible explanation is that the binding of GraT inhibits the chaperone activity of DnaK in facilitating ribosome biogenesis (Ref III, Fig 7A). If DnaK was the direct target of GraT, the C-terminal deletion of DnaK should enhance its chaperone activity. This possibility would be in accordance with other studies that have suggested an increased ATPase activity (Slepenkov*, et al.*, 2003) and refolding ability (Aponte*, et al.*, 2010) for the C-terminally truncated DnaK. In this case, the deletion of the C-terminus of DnaK could possibly alleviate the effect of GraT by increasing the efficiency of the chaperone.

On the other hand, considering that DnaK is a protein folding factor, the GraT-DnaK interaction might be important for the toxin to attain its active form and enable it to inflict its poisonous effect on its so far unknown target (Ref III, Fig 7B). Although Aponte *et al*. showed an increase in DnaK refolding ability upon C-terminal truncation (Aponte*, et al.*, 2010), another study shows the opposite results (Smock*, et al.*, 2011). Here, the authors suggest that the C-terminal tail either contributes to the repeated binding of a peptide or facilitates

local unfolding of a misfolded substrate. Thus, if this was the case, the C-terminal part of DnaK would improve GraT's folding, and upon its deletion, GraT may remain inactive. In this option, the direct target of GraT remains unclear. Still, the unpublished data from our laboratory suggest that GraT, alike other HigB family toxins, may act as an RNase (A. Ainelo, personal communication). In accordance with that, it was recently reported that the GraT orthologue in *P. aeruginosa* PA14 has an RNase activity upon overexpression in *E. coli* (Wood & Wood, 2016). Thus, the possibility of GraT's ability to cleave RNA is quite probable, but considering GraT's moderate toxicity and other abovementioned differences between HigB and GraT, the set of targeted RNAs might be restricted to only a few, possibly of some ribosome-biogenesis factors, instead of a global mRNA cleavage as carried out by HigB (Hurley & Woychik, 2009).

This would not be the first report of proteins from one family having distinct targets. The proteins belonging to the MazF toxin family have been shown to have several different ways of inhibiting translation. They can carry out universal mRNA degradation by having a short recognition sequence (Zhang*, et al.*, 2003) or longer recognition sites, resulting in cleavage of more specific set of mRNAs (Zhu*, et al.*, 2009, Rothenbacher*, et al.*, 2012) or even cut tRNA or rRNA (Schifano & Woychik, 2014, Schifano*, et al.*, 2016) (Review of literature, chapter 2.2.2.). Therefore, it is not surprizing if GraT has a different and maybe more specific target(s) compared to the quite non-specific HigB mRNase (Hurley & Woychik, 2009).

4. The importance of the GraTA system for *P. putida* (Ref I)

The wide distribution of GraTA orthologues in *Pseudomonas* spp. suggests the importance of this TA system for these bacteria. Yet, the conditions promoting antitoxin's degradation and subsequent system activation have remained ambiguous. Considering the involvement of TA systems in stress management (Gerdes*, et al.*, 2005, Starosta*, et al.*, 2014) (Review of literature, chapter 4.2.), the abundance of GraTA orthologues in genomes of pseudomonads may indicate its contribution to coping with different stresses. This led us to study the role of GraT toxin in the stress tolerance of *P. putida*.

4.1. GraT plays a controversial role in the stress management of *P. putida*

4.1.1. GraT suppresses membrane stress of colR*-deficient bacteria (Ref I)*

The stress-relieving effect of GraT was first suggested by the mutant screening where the GraTA system was initially identified. The antitoxin gene was isolated as a gene that's disruption led to the suppression of defects caused by ColR deficiency (Putrinš*, et al.*, 2011). Lack of ColR decreases membrane integrity of bacteria, resulting in the lysis of a subpopulation on glucose medium. The latter phenotype can be determined by the leakage of DNA or β-galactosidase, the binding of Congo Red dye and studying the population structure with flow cytometry (Putrinš*, et al.*, 2008, Putrinš*, et al.*, 2010). Moreover, the *colR*-deficient bacteria also have a reduced tolerance to phenol (Kivistik*, et al.*, 2006) and to high concentration of various metals (Ainsaar*, et al.*, 2014).

The ability of *graA* disruption to suppress the ColR deficiency was first identified by reduced Congo Red binding and decreased leakage of β-galactosidase (Putrinš*, et al.*, 2011). and additionally confirmed with flow cytometry of the deletion mutants of the *graTA* system (Ref I, Fig 5A and B). To study if the deletion of the antitoxin suppressed other *colR*-deficiency-caused effects as well, we tested if the antitoxin deletion alleviated the *colR* mutant's sensitivity to phenol (Kivistik*, et al.*, 2006) (Ref I, Fig 6). Single cell analysis showed that the disruption of *graA* abolished cell death, and moreover, it also suppressed the phenol-sensitivity caused by the lack of ColR. These results illustrate nicely the stress-relieving effect of the toxin (Ref I, Fig 5A and Fig 6). GraT reduces the growth rate of bacteria and slower growth is known to support bacterial stress tolerance (Tuomanen*, et al.*, 1986, Eng*, et al.*, 1991). Thus, it seems logical to consider the growth rate reduction to be the main reason for the improved stress tolerance of the *colR*-deficient *P. putida*.

The discovery of GraTA system closely resembles another study that reported a mutational inactivation of an antitoxin gene *hicB* leading to the suppression of lethality caused by the deficiency of *rpoE* (Button*, et al.*, 2007, Daimon*, et al.*, 2015). The *rpoE* gene codes for an alternative sigma factor σ^E that regulates a set of genes required to overcome membrane stress and ensure proper folding of membrane proteins (Erickson & Gross, 1989). In *E. coli*, the *rpoE* gene cannot be deleted, but inactivation of the *hicB* antitoxin gene suppressed the σ^E-essentiality (Daimon*, et al.*, 2015). As was the case with GraTA, the reason of the suppression was the activation of HicA toxin. Moreover, one genomic copy of *hicA* did not inflict any significant growth impairment (Daimon*, et al.*, 2015) meaning that the beneficial effects of HicA and GraT toxins on bacteria can be seen even upon a slight decrease in growth rate. The envelope stress of σ^E defective cells was also alleviated by expression of HigB and YafQ toxins, but the inactivation of the cognate antitoxins did not

have the same effect. The authors proposed that the effect of these chromosomal toxins were too weak (Daimon, *et al.*, 2015), which suggests that these toxins are even less toxic than GraT. Nevertheless, these data highlight the potential of TA systems to contribute to cell envelope stress management of bacteria.

4.1.2. GraT has a potential to defend P. putida *against different stress inducers (Ref I)*

The effect of GraT under different stress conditions was tested by analysing the growth ability of *P. putida* wild-type and its *graTA* deletion derivatives on agar plates containing different antimicrobials (Ref I, Fig 7). ΔgraA cells grew better than the wild-type in the presence of various stress-inducers, which shows that GraT increases the tolerance to several of the tested chemicals. For example, the tolerance to translation inhibitors streptomycin and kanamycin and DNA damaging agents such as ciprofloxacin and mitomycin C is increased by the GraT toxin (Ref I, Fig 7).

Many TA loci increase the number of persisters, and thus, also the ability of bacteria to survive the lethal effect of antibiotics (Dörr, *et al.*, 2010, Maisonneuve, *et al.*, 2011). In order to test if GraT can increase the survival of bacteria during such treatment, the exponentially growing cultures of wild-type *P. putida* and the ΔgraA derivative were treated with a lethal concentration of streptomycin. Considering that the effects of GraT are cold-sensitive, the experiment was performed with bacteria grown at different temperatures. The killing curves clearly showed that lethality of streptomycin was reduced for ΔgraA mutant at 25 °C (Ref I, Fig 8A), thus the amount of persister cells was increased by GraT. In good agreement with the cold-sensitive growth suppression by GraT, the increased persistence of ΔgraA strain was observable only at temperatures below the optimal 30 °C (Ref I, Fig 8B). We suggest that GraT-caused growth reduction could be responsible for the improved persistence of ΔgraA strain to antibiotic treatment.

4.1.3. GraT increases biofilm formation

Several TA systems are suggested to increase stress tolerance of bacteria by facilitating biofilm formation (Wang & Wood, 2011, Thomason, *et al.*, 2012) whereas respective mechanisms are quite different (Review of literature, chapter 4.2.2.). As GraT influenced the stress tolerance of *P. putida,* the involvement of the GraTA system in biofilm formation was studied as well. Quantification of biofilm at different temperatures revealed that at 25 °C the ΔgraA strain produced significantly more biofilm than the wild-type or ΔgraTA strain (Fig 16). In accordance with the cold sensitivity of the GraT-caused effects, the difference between the wild-type and the ΔgraA in biofilm formation disappeared at temperatures above 25 °C. These data show that GraT increases biofilm production in *P. putida* in a temperature-dependent manner.

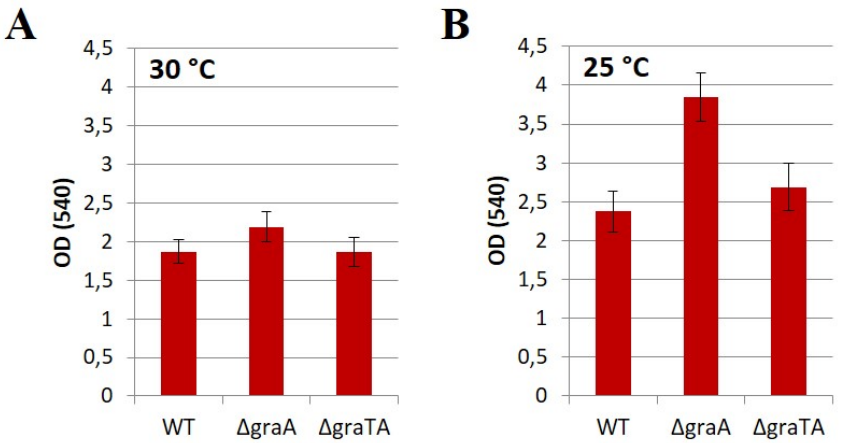


Figure 16. Biofilm formation of *P. putida* wild-type (WT) and its *graA* and *graTA* deletion derivatives (ΔgraA and ΔgraTA). Biofilm formation at A: 30 °C, B: 25 °C. Cells were grown statically in LB for 48 h. Error bars show the 95% confidence intervals.

The positive impact of GraT on biofilm formation is in accordance with the results reported for many other TA systems (Kim, *et al.*, 2009). However, conflicting results regarding biofilm formation were reported for an orthologue of the GraTA system of *P. aeruginosa* PA14, named HigBA (Wood & Wood, 2016). If the antitoxin gene *higA* was deleted from *P. aeruginosa*, the HigB toxin was seen to reduce *P. aeruginosa* biofilm more than 10-fold (Wood & Wood, 2016). One possibility for this discrepancy may be that regardless of the similarity of these two TA systems [69.6 % of identity between the toxins GraT and its *P. aeruginosa* orthologue HigB (Ref I, Table S2)], they are not identical and thus could have disparate ways of influencing biofilm formation. Alternatively, the opposite results could have been obtained due to somewhat different experimental conditions: in our experiment, the temperature was 25 and 30 °C instead of 37 °C that was used for *P. aeruginosa*. Moreover, we used lysogeny broth (LB), whereas Wood *et al*. measured biofilm in M9 medium with added amino acids (Wood & Wood, 2016). Although growth conditions can essentially affect the biofilm formation (Uhlich, *et al.*, 2014), it is still remarkable that two homologous TA systems would influence the formation of biofilm in entirely opposite directions. However, the biofilm formation of *P. putida* and *P. aeruginosa* is regulated in quite different ways (Fazli, *et al.*, 2014). For example, the supposedly most important protein in biofilm formation of *P. putida,* the large adhesion protein LapA (Gjermansen, *et al.*, 2010), is absent from *P. aeruginosa* (Duque, *et al.*, 2013). Conversely, quorum sensing controlled by LasIR and RhlRI systems plays an important role in the biofilm formation of *P. aeruginosa* (Davies, *et al.*, 1998), but not in *P. putida* (Fazli, *et al.*, 2014). Furthermore, the overall involvement of TA systems in biofilm formation is quite complex and a straightforward view of the role that the toxins

play has not been determined (Review of literature, chapter 4.2.2.). Thus, it can indeed be possible that similar TA systems in similar (but not identical) conditions could have opposite effects.

4.1.4. Costs of activation of GraT (Ref I)

While many results have indicated that GraT is able to contribute to the stress tolerance of *P. putida*, several other data suggest that the GraT-mediated stress protection is costly and has harmful side effects. Analysis of the population structure of *graTA* deletion mutants at a single cell level resulted in an unexpected outcome. Although the deletion of the antitoxin gene suppressed the lysis of the *colR* mutant, the membrane permeability of ΔgraA cells was remarkably increased in both wild-type and *colR*-deficient backgrounds. The membrane damage caused by the absence of ColR is glucose-specific (Putrinš*, et al.*, 2008), yet GraT increases membrane permeability regardless of the growth medium (Ref I, Fig 5A and B). These data show that the active GraT also causes membrane defects, yet, can still eliminate the cell death caused by the absence of ColR.

The other indication that the activation of GraT can be costly for bacteria, comes from the experiments conducted to evaluate the stress-relieving ability of GraT. The obtained data showed that although GraT increased the tolerance to many stress inducers (Ref I, Fig 7), it sensitized them to others. For example, ΔgraA mutant is more sensitive to osmotic stress caused by excess of NaCl (Ref I, Fig 7). The decreased tolerance to osmotic stress is a clear indication of impaired membrane functions. The other antimicrobials that had a more severe effect on ΔgraA than on wild-type were tetracycline and paraquat. Tetracycline targets the 30S subunit of the ribosome and paraquat causes oxidative stress and damages DNA. The increased susceptibility of ΔgraA cells to these distinct compounds could be caused by their easier entry into the cells, resulting from the damaged membrane.

Similar dual effect of TA toxins has been observed by others as well. RelE family toxins from *Salmonella* Typhimurium increased bacterial tolerance to ciprofloxacin (Silva-Herzog*, et al.*, 2015), but at the same time decreased tolerance to acidic conditions (pH 3) (Silva-Herzog*, et al.*, 2015). Also, the deletion of toxin *ndoA* gene from *Bacillus subtilis* rendered the cells more susceptible to high temperatures and nutrient limitation, and conversely increased the tolerance to different antibiotics (kanamycin and moxifloxacin) and hydrogen peroxide (Wu*, et al.*, 2011). Moreover, opposite effects of NdoA toxin absence has been observed under UV irradiation: lack of the toxin decreased the tolerance upon weak, but increased the tolerance upon stronger UV stress (Wu*, et al.*, 2011).

Bearing in mind the controversial effects of TA toxins, it seems very important that their activation should occur under certain conditions only, as the

uncontrolled activation of the toxins can be costly and in case of GraT, for example, result in membrane defects and decreased tolerance to several stress conditions.

4.2. The possible physiological role of the GraTA system for *P. putida*

Although GraT has a potential to influence the stress tolerance of *P. putida,* we have not detected physiological effects after the removal of the entire *graTA* locus: neither biofilm formation (Fig 16) nor growth under different stress conditions (Ref I, Fig 7) was affected. This suggests a strict control of the toxin's activation in wild-type cells. Nevertheless, the effect of the deletion of the *graTA* system could be only subtle and may therefore remain unnoticed. In order to test if the GraTA system could give any growth advantages (or disadvantages) to *P. putida*, I analysed the competitiveness of the ΔgraTA strain by co-cultivating this strain with the wild-type. The cultures were mixed in 1:1 ratio and re-inoculated into a fresh medium after 24 h of growth. Before each reinoculation a sample from the mixed culture was taken and the changes in the ratio of the two subpopulations were determined by locus-specific PCR. As neither of the two subpopulations could outcompete the other during seven days of experiment (Fig 17), we conclude that the system does not incur fitness benefits or costs for *P. putida* in growth-favouring conditions. As TA systems are considered to be more important under non-optimal and growth-limiting conditions, we also tested the competing ability of the ΔgraTA strain at different temperatures (20, 25, 37 °C) and in minimal media. Again, we saw no effect of GraTA system deletion on the competing ability of the bacteria in any of the tested conditions (data not shown).

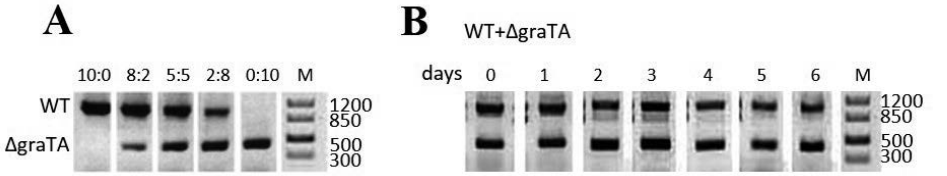


Figure 17. Deletion of the GraTA system does not affect the competitiveness of *P. putida* under optimal growth conditions. A: Calibration of PCR on different mixtures of *P. putida* wild-type (WT) and its *graTA* deletion derivative (ΔgraTA). The numbers above each panel indicate the ratio of two strains (WT:ΔgraTA) in the bacterial mixtures. PCR was carried out from the mixtures with primers specific for the *graTA* locus. B: Co-cultivation of *P. putida* wild-type and ΔgraTA strains (LB, 30 °C). The ratio of each subpopulation was determined by *graTA* locus-specific PCR every day (indicated by numbers above the panel) for a week. A representative picture of more than three experiments is shown. M represents the DNA size marker and numbers to the right of both figures show the sizes of the marker bands (bp).

It is quite common for bacteria with numerous TA systems to disclose no observable effects upon a deletion of a single TA locus (Tsilibaris*, et al.*, 2007, Maisonneuve*, et al.*, 2011). Yet, even if the absence of one system displays no effects, deleting multiple TA systems consecutively has increasingly stronger effects on persister formation in *E. coli* (Maisonneuve*, et al.*, 2011). Our preliminary results do not confirm the same trend for *P. putida.* Deletion of 9 out of the 15 predicted TA systems did not influence persister formation or competitiveness of *P. putida* under the conditions tested (similar as described above, data not shown). Thus, in *P. putida* the redundancy between the TA systems is probably not the reason for the lack of phenotypic effects in the *graTA* deletion strain.

The other possible explanation could be that the *graTA* system has not been studied in appropriate conditions to reveal the effects on fitness of *P. putida*. In wild-type *P. putida* the GraA antitoxin can very efficiently avoid the toxin activation under the conditions we have applied. Thus, the absence of effects of the *graTA* deletion can be merely blamed upon our insufficient knowledge about the exact activating conditions of GraA degradation and, thus, the activation of the GraTA system.

Gene expression of *P. putida* differs markedly between laboratory and environmental conditions (Morimoto*, et al.*, 2016), where bacteria face constant stress and nutrient limitation. Even if we use suboptimal conditions in the laboratory, they still remain quite growth-favouring compared to the environmental ones. Moreover, in microbial communities consisting of bacteria from very different species there is a constant need to compete with other bacterial species inhabiting the same niche (Foster & Bell, 2012), which is also a factor influencing the physiology of environmental bacteria (Morris*, et al.*, 2008, Stewart, 2012). Taken together, the set of conditions in natural habitats are very difficult to replicate in the laboratory. Nevertheless, the discovery of the conditions that could trigger the degradation of GraA should be the main goal in the future studies. This could eventually lead to discovering the activating conditions of GraTA system and thus shed more light to the importance of this quite uncommon TA system in the physiology of *P. putida*.

CONCLUSIONS

The involvement of bacterial TA systems in stress survival has made them a popular topic of research. In recent years, an increasing number of chromosomal TA systems are characterized in various bacterial species, yet the research on these systems in the highly diverse *Pseudomonas* genus has remained scarce. Thus, the current work set out to describe the first TA system in this genus of bacteria. Respective *P. putida* system was named GraTA for the growth-rate-affecting ability of the toxin. The main features of the system can be summarized as follows:

- The toxin GraT has moderate effects on bacterial growth at optimal growth conditions, yet causes a severe growth defect at reduced temperatures. GraT inhibits ribosome biogenesis and its toxicity needs the full-length DnaK chaperone.
- The GraT toxin has a potential to contribute to the stress tolerance of *P. putida*, as it
  - increases the tolerance to several antimicrobials,
  - decreases lethal effects of antibiotics,
  - increases the biofilm formation.
- Besides beneficial effects on stress tolerance, the activity of GraT has harmful side effects as it causes membrane impairment and decreased survival under certain stress conditions.
- The potentially harmful toxin is under a strict control of the antitoxin GraA:
  - GraA autorepresses the *graTA* operon, whereas the toxin GraT acts as a derepressor when bound to GraA.
  - GraA counteracts GraT by forming a protein complex, where the toxin is inactive.
  - GraA is an unusually stable antitoxin, degradation of which is probably initiated by an endoproteolytic cleavage. The degradation rate of GraA depends on the growth phase of bacteria, energetic status of the cell and the transcription regulator MexT.
- GraT is most probably activated by the accelerated degradation of GraA antitoxin in response to certain (yet unknown) stress conditions.

The results of the current thesis show that the GraT toxin can both increase and decrease the stress tolerance of *P. putida*. However, GraT is only conditionally toxic and is kept under an uncommonly strict control by the highly stable GraA antitoxin. One may hypothesize that evolution has shaped the chromosomal toxins to be less toxic and increased the inhibiting ability of the antitoxins to reduce the possible harmful effects of the toxins to the host bacterium. Also, it seems that the activation of the potentially damaging GraTA system is needed only in a narrow range of conditions. Identification of these conditions is crucial to understand the importance of GraTA system in the physiology and stress tolerance of *P. putida.*

SUMMARY IN ESTONIAN

Pseudomonas putida toksiin-antitoksiin süsteem GraTA: regulatsioon ja osalus stressitaluvuses

Bakteriaalsed toksiin-antitoksiin (TA) süsteemid koosnevad reeglina kahest osapoolest – raku elutegevust pärssivast toksiinist ja teda neutraliseerivast antitoksiinist. Kui toksiin on alati valk, siis antitoksiini funktsiooni võib täita nii valk kui ka sRNA. Antitoksiin võib takistada nii toksiini ekspressiooni, aktiivsust kui ka stabiilsust ning leidub ka selliseid antitoksiine, mis kaitsevad toksiini rakulist märklauda (Page & Peti, 2016). Käesolev töö käsitleb põhjalikumalt II tüüpi TA-süsteeme, milles nii toksiin kui antitoksiin on valgud ja toksiini inaktiveerimine toimub kahe valgu omavahelisel seondumisel (Gerdes*, et al.*, 2005).

Kuna TA-süsteemid kodeerivad rakule toksilist valku, siis pole sugugi üllatav, et neid süsteeme reguleeritakse mitmel eri tasandil. TA-operoni ekspressiooni autoreguleerib enamasti antitoksiin ja/või TA-kompleks. Toksiin võib, olenevalt tema suhtelisest hulgast antitoksiini suhtes, kas suurendada või vähendada TA-kompleksi seondumist TA-geenide operaatorpiirkonnale ja sel viisil põhjustada nii TA-geenide repressiooni kui ka derepressiooni (Loris & Garcia-Pino, 2014). TA-süsteemide II tüübi puhul on teiseks regulatsiooniastmeks antitoksiini stabiilsuse reguleerimine. Antitoksiinid on tavaliselt väga labiilsed valgud (nende pooleluiga on harilikult ligikaudu 15 minutit), mille lagundajateks on enamasti rakulised Lon või Clp proteaasid (Kedzierska & Hayes, 2016). Samas on antitoksiinide lagundamise regulatsioon TA-süsteemide aktiveerumisel oluline etapp, kuna ainult seeläbi saab toimuda toksiini valgu aktiveerumine.

Esmalt avastati TA-süsteemid väikese koopiaarvuga plasmiididest, kus nad on olulised tagamaks plasmiidi säilimist bakteripopulatsioonis (Gerdes*, et al.*, 1986a). Plasmiidiga rakus toodetakse nii toksiini kui antitoksiini, mistõttu toksiin on vaigistatud. Kuigi antitoksiini lagunemine on kiire, toodetakse seda plasmiidilt juurde ja toksiin jääb inaktiivsesse olekusse. Kui aga raku jagunemisel ei peaks tütarrakku plasmiidi kanduma, katkeb antitoksiini juurdesüntees, mis viib tsütoplasmas leiduva toksiini aktiveerumisele ja bakteri kasvu pärssimisele. See annab kasvueelise plasmiidi sisaldavatele bakteritele ja plasmiidi kaotanud rakud kõrvaldatakse populatsioonist.

Seoses mikroobide täisgenoomide sekveneerimisega ilmnes, et TA-süsteemid on üllatavalt laialt levinud ka paljude bakterite kromosoomides. Kui plasmiidsete TA-süsteemide funktsioon on üsna üheselt mõistetav, siis bakteri kromosoomis leiduvate potentsiaalselt toksilisi valke kodeerivate geenide olemasolu põhjustas arusaadavalt poleemikat nende geenide rollist bakteri bioloogias. Viimase aja uuringud on aga tuvastanud kromosomaalsete TA-süsteemide seotuse bakterite stressivastusega (Gerdes*, et al.*, 2005). Seda näitab nii TA-süsteemide otsene osalus üldistes stressivastustes kui ka nende olulisus faagivastases kaitses, biofilmi moodustumises, persistorrakkude tekkes ja

kasvuregulatsioonis. Kromosomaalsete TA-süsteemide olulisust bakteri stressitaluvuses peegeldab ka nende ebaühtlane jaotus erinevaid nišše asustavate bakterite genoomides. TA-süsteeme on väga palju muutlikke ja stressirohkeid keskkondi asustavatel bakteritel, samas kui rakusiseste parasiitide genoomis ei ole sageli ühtegi TA-süsteemi (Pandey & Gerdes, 2005, Leplae*, et al.*, 2011). Arvatakse, et stabiilsetes tingimustes kaotavad need süsteemid oma tähtsuse ja neid kodeerivad geenid kaovad kromosoomist.

Palju erinevaid keskkondi asustava *Pseudomonas*'e perekonna bakterites ei ole neid stressi talumises olulisi süsteeme väga põhjalikult uuritud, kuigi kõik selle perekonna bakterid kodeerivad ennustuse järgi vähemalt paari TA-süsteemi (Shao*, et al.*, 2011). Käesoleva töö eesmärk oli iseloomustada keskkonnabakter *Pseudomonas putida* oletatavat TA-süsteemi ja uurida selle TA-mooduli rolli bakteri stressitaluvuses. Eelnev teadmine, et selle TA-süsteemi antitoksiini geeni (PP1585) katkestus leevendab *P. putida* membraanistressi (Putrinš*, et al.*, 2011), tekitas küsimuse, kas stressi leevenemise põhjuseks on geeni PP1585 katkestus või selle tulemusel aktiveeruv toksiin (PP1586). Selgus, et membraanistressi vähendas aktiveerunud toksiin ja et PP1586-PP1585 lookus kodeerib funktsionaalset TA süsteemi, mille nimetasime kasvukiiruse regulatsioonis osalemise tõttu GraTA (*growth-rate-affecting*) süsteemiks. GraTA süsteemi peamised omadused saab kokku võtta järgnevalt:

- Kui tavatingimustel on GraT toksiin üsna mõõduka toksilisusega, siis madalal temperatuuril aeglustab toksiin tugevalt bakteri kasvu, kuna inhibeerib ribosoomi biogeneesi. GraT toksilisuse avaldumiseks on vajalik täispika DnaK šaperoni olemasolu.
- GraT toetab *P. putida* stressitaluvusest, sest ta:
  - suurendab tolerantsust erinevate kemikaalide suhtes,
  - vähendab antibiootikumide surmavat toimet,
  - suurendab *P. putida* biofilmi tootmist.
- Kuigi GraT on mitmetes stressitingimustes kasulik, võib selle toksiini kontrollimatu aktivatsioon olla kahjulik, sest GraT kahjustab bakterimembraani ja muudab bakterid mitme stressi suhtes tundlikumaks.
- Antitoksiin GraA vaigistab efektiivselt potentsiaalselt kahjulikku GraT toksiini:
  - GraA represseerib *graTA* operoni avaldumist, kusjuures GraT pärsib GraA seondumist DNAle.
  - GraA dimeer moodustab kahe toksiini monomeeriga kompleksi, milles GraT on inaktiivne.
  - GraA on ebaharilikult stabiilne valk, mille lagunemise algatab arvatavasti endoproteolüütiline lõikus 80. aminohappe juurest. GraA lagunemiskiirus sõltub bakteri kasvufaasist, kuid ka raku energeetilisest staatusest ja transkriptsiooniregulaatorist MexT.
- Oletatavalt on GraTA süsteemi päästikuks GraA kiirenenud lagunemine seni teadmata tingimustel.

Käesoleva töö tulemused näitavad, et GraT osaleb *P. putida* stressivastuses, kuid sõltuvalt tingimustest võib toksiini mõju olla bakterile nii kasulik kui ka kahjulik. Seda arvestades ei ole üllatav, et GraA on ebatavaliselt stabiilne ja efektiivne toksiini neutraliseerimisel. Kromosomaalsete toksiinide aktivatsiooni potentsiaalset kahjulikkust silmas pidades võib oletada, et TA toksiinid evolutsioneeruvadki mõõdukuse ja antitoksiinid tõhususe ja stabiilsuse suunas, et toksiinide võimalikku kahju minimeerida. Töös esitatud tulemustele tuginedes võib arvata, et tingimused, milles GraT vabaneb GraA neutraliseeriva mõju alt, on üsna spetsiifilised. Nende edaspidine tuvastamine on oluline, et mõista GraTA süsteemi olulisust keskkonnabakteri *P. putida* füsioloogias ja stressitaluvuses.

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PUBLICATIONS

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2. Tamman H, Ainelo A, Tagel M & Horak R. Stability of the GraA Antitoxin Depends on Growth Phase, ATP Level, and Global Regulator MexT. Journal of bacteriology. 2016; 198: 787–796.
3. Ainelo A, Tamman H, Leppik M, Remme J, Hõrak R. The toxin GraT inhibits ribosome biogenesis. Molecular microbiology. 2016 May; 100(4): 719–34.
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2. Tamman H, Ainelo A, Tagel M & Horak R. Stability of the GraA Antitoxin Depends on Growth Phase, ATP Level, and Global Regulator MexT. Journal of bacteriology. 2016; 198: 787–796.
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