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LIST OF ORIGINAL PUBLICATIONS

I **Ainelo A, Porosk R, Kilk K, Rosendahl S, Remme J, Hõrak R**. *Pseudomonas putida* Responds to the Toxin GraT by Inducing Ribosome Biogenesis Factors and Repressing TCA Cycle Enzymes. Toxins (Basel). 2019. 11(2):103.

II **Rosendahl S, Ainelo A, Hõrak R**. The Disordered *C*-Terminus of the Chaperone DnaK Increases the Competitive Fitness of *Pseudomonas putida* and Facilitates the Toxicity of GraT. Microorganisms. 2021. 9(2):375.

III **Rosendahl S, Tamman H, Brauer A, Remm M, Hõrak R**. Chromosomal toxin-antitoxin systems in *Pseudomonas putida* are rather selfish than beneficial. Scientific Reports. 2020. 10(1):9230.

My contribution to the publications is the following:

Ref I – I participated in the construction of plasmids and strains and performed *in vivo* experiments.

Ref II – I participated in the construction of plasmids and strains, conducted most of the experiments and contributed to the manuscript writing and editing.

Ref III – I participated in the construction of plasmids and strains, conducted most of the experiments and contributed to the manuscript writing and editing.

ABBREVIATIONS

(p)ppGpp	guanosine penta- or tetraphosphate
Abi	abortive infection
AMP	adenosine monophosphate
Ara	arabinose
ATP	adenosine triphosphate
BACTH	bacterial adenylate cyclase two-hybrid system
Cas	CRISPR-associated protein
CRISPR	clustered regularly interspaced short palindromic repeats
GTP	guanosine triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
MOI	multiplicity of infection
NBD	nucleotide-binding domain
PCD	programmed cell death
ppApp	adenosine tetraphosphate
PSK	post-segregational killing
SAH	small alarmone hydrolase
SAS	small alarmone synthetase
SBD	substrate-binding domain
Sm	streptomycin
TA	toxin-antitoxin
TCA	tricarboxylic acid
wt	wild-type

1. INTRODUCTION

Toxin-antitoxin systems are fascinating genetic elements that generally consist of two counterparts – a noxious toxin that can impede essential cellular processes and an antitoxin that efficiently neutralizes the toxin. TA systems were first discovered about 40 years ago from plasmids, where their function is to stabilize the plasmid in the cells (Ogura & Hiraga, 1983, Gerdes *et al.*, 1986a). Extensive bacterial genome sequencing has revealed that TA systems are also extremely widespread in bacterial chromosomes and bacteria can have dozens of TA systems in their genome (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011). It is intriguing why bacteria encode so many genetic elements that can harm themselves and this should mean that TA loci carry benefits to their host. Since their discovery, chromosomal TA systems have been associated with stress response regulation (Gerdes *et al.*, 2005). It was proposed that stress liberates toxin from antitoxin's control, and since free toxin has been shown to inhibit bacterial growth (Gerdes *et al.*, 2005, Pedersen *et al.*, 2002, Budde *et al.*, 2007), then toxin-mediated growth rate modulation could help bacteria endure stressful times. In addition, some TA systems have been shown to stabilize mobile genomic DNA (Wozniak & Waldor, 2009, Yao *et al.*, 2015) and some protect bacteria during bacteriophage invasion (Fineran *et al.*, 2009, LeRoux *et al.*, 2022). However, there are also indications that TA systems are just selfish DNA elements that entail no importance to bacteria (Tsilibaris *et al.*, 2007, Goormaghtigh *et al.*, 2018). Since the results of different research groups are controversial, the significance of chromosomal TA systems has remained unclear and needs further investigation.

The common soil bacterium *Pseudomonas putida* has been predicted to encode 15 chromosomal TA systems (Xie *et al.*, 2018), but only four of them (GraTA, MqsRA, RES-Xre and MazEF) have been characterized in previous studies (Tamman *et al.*, 2014, Sun *et al.*, 2017, Skjerning *et al.*, 2019, Miyamoto *et al.*, 2016). The most thoroughly investigated is the GraTA system that stands out from other TA systems by its unusually stable and fully structured antitoxin GraA and its only mildly poisonous toxin GraT, which has an intrinsically disordered N-terminus that is of great importance to its toxicity (Tamman *et al.*, 2016, Talavera *et al.*, 2019, Tamman *et al.*, 2014). Like many other toxins, GraT is a ribosome-dependent mRNase (Talavera *et al.*, 2019). However, interestingly, GraT activity causes temperature-dependent growth and ribosome biogenesis defect and the chaperone protein DnaK is somehow involved in this (Tamman *et al.*, 2014, Ainelo *et al.*, 2016). The toxin GraT has been shown to have opposite effects on *P. putida* stress tolerance in the absence of the antitoxin GraA (Tamman *et al.*, 2014). Yet, the deletion of the whole *graTA* locus does not affect *P. putida* at all (Tamman *et al.*, 2014), raising the question of *graTA* locus and also other TA systems' importance to *P. putida*.

The first part of this thesis gives a general overview of TA systems, their potential functions in bacteria and of what is known about *P. putida* TA systems. The experimental part of the thesis describes how *P. putida* responds to the toxin GraT on cellular level, how the chaperone protein DnaK is involved in the toxin GraT activity, and what is the role of TA systems in *P. putida*.

2. REVIEW OF LITERATURE

2.1. Toxin-antitoxin systems

Toxin-antitoxin (TA) systems are genetic elements that generally consist of a noxious toxin gene and a counteracting antitoxin gene. The majority of toxins are proteins (Type I–VII), but RNA toxins have also been found recently (Type VIII) (Jurėnas *et al.*, 2022, Song & Wood, 2020b). Most often, toxins target translation, replication or cell wall integrity (Harms *et al.*, 2018). Antitoxins can be either proteins or RNAs that neutralize the toxin by inhibiting its translation, activity or destabilizing it (Jurėnas *et al.*, 2022, Song & Wood, 2020b).

The first two TA systems were discovered already in the 1980s from plasmids, *ccdAB* system from mini-F plasmid (Ogura & Hiraga, 1983) and *hok/sok* system from R1 plasmid (Gerdes *et al.*, 1986b). Both of these TA systems were shown to be important in plasmid maintenance in the cells (Ogura & Hiraga, 1983, Gerdes *et al.*, 1986b). Based on characterization of these two systems, a post-segregational killing (PSK) mechanism (Fig 1) was proposed for plasmid maintenance (Gerdes *et al.*, 1986b). This means that bacterial cells with the plasmid produce both toxin and antitoxin, and antitoxin keeps the toxin inactive by either forming a complex with the toxin (*ccdAB*, antitoxin is a protein) or by binding to toxin mRNA (*hok/sok*, antitoxin is an RNA). But upon plasmid loss, toxin is freed from the antitoxin's control as antitoxin is less stable than the toxin and therefore degraded first, and there are no means to make more antitoxin to silence the toxin.

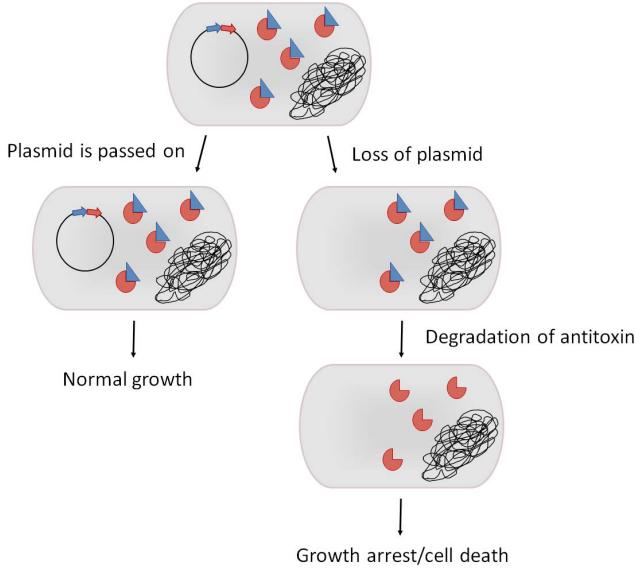

Figure 1. Plasmid maintenance via TA system-dependent post-segregational killing mechanism. Upon plasmid loss, no new antitoxin (blue) can be produced. Due to antitoxin's lower stability, it is degraded first, and free toxin (red) can affect its target, causing growth arrest of the bacterium, or cell death.

Free toxin can now affect its target, leading to either growth inhibition of the cell and plasmid-free cells get outcompeted from the population, or even cell death (Fig 1) (Gerdes *et al.*, 1986a). In addition to plasmid maintenance, TA systems harbored in plasmids can also be important in plasmid-plasmid competition. It was shown that TA systems found in plasmids ensure the fitness of their host plasmid during competition between plasmids from the same incompatibility group as daughter cells that do not inherit the TA encoded plasmid will be killed (Cooper & Heinemann, 2000).

Generally, TA systems consist of two components – a toxin and an antitoxin. But there can also be found three-component TA systems or TA systems which have the toxin and the antitoxin fused together. For instance, in addition to toxin ParE and antitoxin PaaA, the *Escherichia coli* Type II TA locus *paaR–paaA–parE* encodes a transcription regulator PaaR that is needed to regulate the TA operon expression (Hallez *et al.*, 2010). *Mycobacterium tuberculosis* also encodes a tripartite TA system, which consists of the toxin HigB1, the antitoxin HigA1 and the chaperone SecB. The chaperone is required for proper antitoxin folding and protects the antitoxin from degradation, thus it is crucial for the antitoxin to be able to neutralize the toxin (Bordes *et al.*, 2011, Bordes *et al.*, 2016). Interestingly, a couple of TA systems have been found that have the toxin and the antitoxin encoded as one polypeptide chain. In the case of *E. coli* TA system EzeT, the C-terminal part of the protein encodes the toxin and it is neutralized by the N-terminal antitoxin domain (Rocker & Meinhart, 2015). Unfortunately, there are no conditions detected which would lead to the toxin activation. But another fused TA system encoded by *Salmonella* phage SJ46 called CapRel, consisting of N-terminal toxin and C-terminal antitoxin domain, was shown to be activated during phage attack to stop the spread of the phage in the population (Zhang *et al.*, 2022).

2.1.1. Classification of TA systems

TA systems are not unique to plasmids and they have also been found in chromosomes. In fact, they are very widespread in chromosomes as most bacteria and many archaea encode them and often have multiple different TA systems in their genome (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011, Xie *et al.*, 2018). New TA systems are being discovered constantly and to date they have been classified into eight different groups based on the nature of antitoxin and its mode of action (Fig 2) (Jurėnas *et al.*, 2022, Song & Wood, 2020b). Antitoxin is an RNA in Type I, III and VIII TA systems and a protein in all the others.

In Type I TA systems, antitoxin is an antisense RNA that binds to the toxin mRNA and inhibits toxin's translation (Fig 2A). Many Type I toxins are small hydrophobic peptides, e.g. *E. coli* toxins Hok of *hok/sok* system and TisB of *istR/tisB* system, and upon production destabilize the cell by forming pores in the cell membrane (Gerdes *et al.*, 1986a, Unoson & Wagner, 2008). Type I toxins can also target nucleic acids as *E. coli* toxin SymE of *symER* system is an RNase (Kawano *et al.*, 2007) and RalR of *ralRA* system is a DNase (Guo *et al.*, 2014).

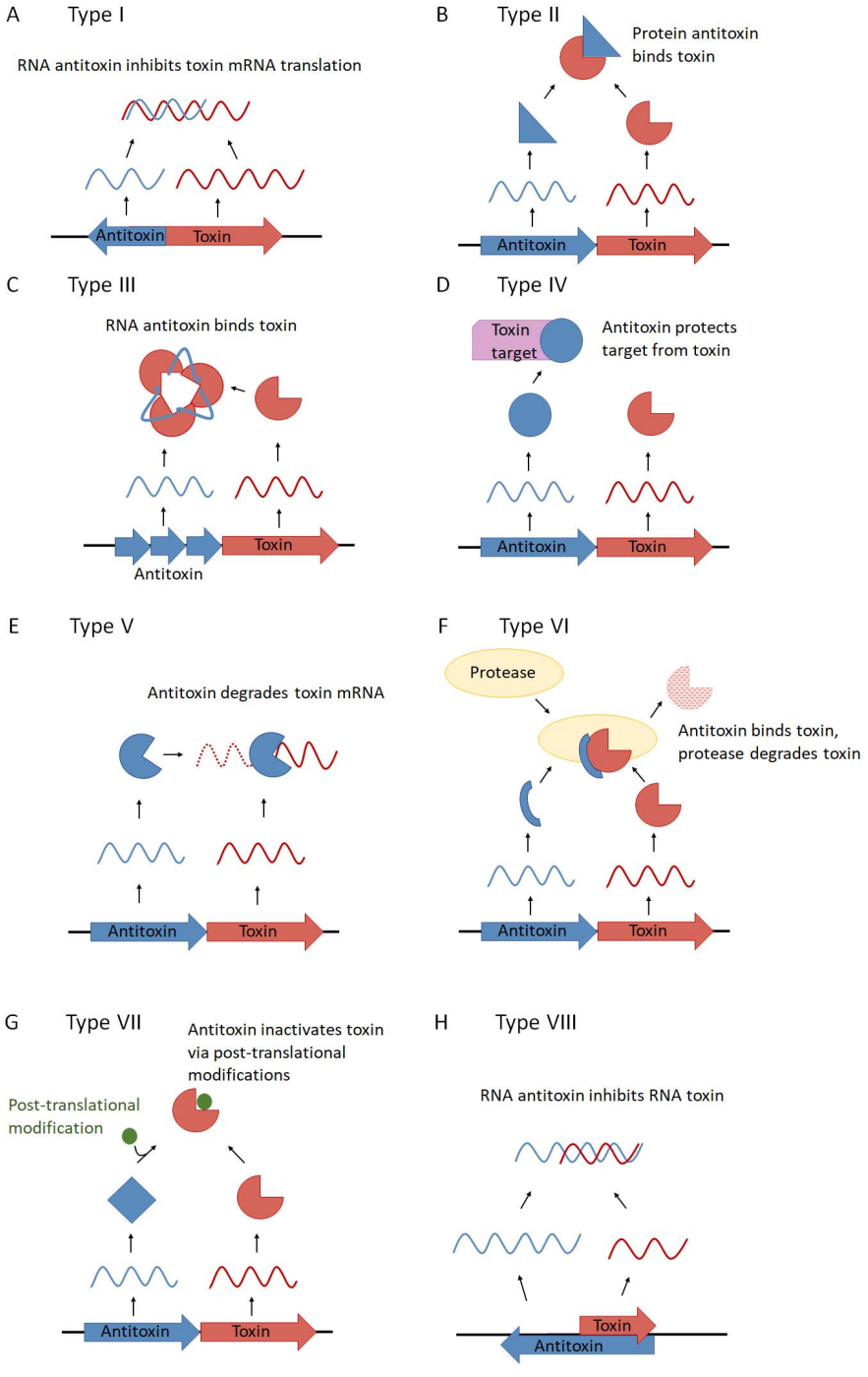

Figure 2. Types of TA systems characterized to date. Schematic illustration of how antitoxin (blue) neutralizes its cognate toxin (red).

Type II TA systems are the most prevalent TA systems in bacteria and thus also the most thoroughly characterized. They encode an antitoxin that is a protein and complex formation between antitoxin and toxin keeps the toxin inactive (Fig 2B). Examples of TA systems belonging to this group are the very first discovered TA system *ccdAB* (Ogura & Hiraga, 1983), but also other extensively studied TA systems such as *relBE*, *mazEF*, *mqsRA*, *vapBC*, *parDE*, *mbcTA, ataTR*, *doc-phd* and many more. Toxins of Type II TA systems are often ribonucleases and they can cleave mRNAs either ribosome-dependently (e.g. RelE of *E. coli*) (Pedersen *et al.*, 2003, Christensen & Gerdes, 2003) or independently (e.g. MazF and MqsR of *E. coli*) (Zhang *et al.*, 2003, Yamaguchi *et al.*, 2009). Some Type II RNase toxins can also target tRNAs (e.g. VapC of *Salmonella enterica*) (Winther & Gerdes, 2011) or rRNAs (e.g. MazF-mt6 of *M. tuberculosis*) (Schifano *et al.*, 2013). Moreover, they can also have other toxic effects to the cell, such as poisoning DNA gyrase (e.g. CcdA of mini-F plasmid and ParE of RK2 plasmid) (Bernard & Couturier, 1992, Jiang *et al.*, 2002), depleting cells of NAD+ (e.g. McbT of *M. tuberculosis*) (Freire *et al.*, 2019), or blocking translation either by modifying initiator $tRNA^{fmet}$ (e.g. AtaT of *E. coli*) (Jurenas *et al.*, 2017) or by phosphorylating translation elongation factor EF-Tu (e.g. Doc of *E. coli*) (Castro-Roa *et al.*, 2013).

In addition to Type I antitoxins, Type III antitoxins are also RNAs. But while Type I antitoxins bind the toxin mRNA and inhibit toxin production, Type III antitoxins bind the toxin protein and inhibit its activity (Fig 2C). To date, multiple Type III TA systems have been described, but the first and the most thoroughly studied Type III TA system is ToxIN and it was first discovered in *Pectobacterium atrosepticum* (Fineran *et al.*, 2009). The toxin ToxN is an endoribonuclease that is also responsible for processing its cognate antitoxin's RNA into active form which is a 36-nucleotide RNA pseudoknot ToxI (Blower *et al.*, 2011, Short *et al.*, 2013). To neutralize the toxic effect of the toxin ToxN, three ToxI RNAs must form a complex with three ToxN proteins (Blower *et al.*, 2011).

Antitoxins of Type IV TA systems are proteins. Yet, differently from Type II antitoxins, they do not directly interact with the toxins, but instead bind to and protect the toxin's target, or detoxify the cell (Fig 2D). The first discovered Type IV TA system is CbeA/CbtA of *E. coli* (Masuda et al., 2012). The toxin CbtA binds to and inhibits polymerization of the cytoskeleton proteins MreB and FtsZ which affects cell division and morphology (Tan *et al.*, 2011, Heller *et al.*, 2017). The antitoxin CbeA can also interact with MreB and FtsZ, promoting polymerization and protecting them from the toxin (Masuda *et al.*, 2012). In addition to CbeA/CbtA system, multiple different Type IV TA systems have been found. One interesting example is a recently described ToxSAS/antiToxSAS TA system of *Cellulomonas marina* that is comprised of a toxin and two antitoxins (Jimmy *et al.*, 2020). The toxin is a small alarmone synthetase (SAS) which can harm the cell by producing high amounts of ppGpp and its analogue ppApp, leading to depletion of GTP and ATP in the cell. One of the antitoxins is a Type II antitoxin which neutralizes only the cognate toxin by binding to it. However, the other antitoxin belongs to Type IV family and encodes a small alarmone hydrolase

(SAH) which can suppress the toxic effect of different SASs by degrading their synthesis product ppGpp (Jimmy *et al.*, 2020).

So far, the only discovered Type V TA system is *E. coli ghoST*. The antitoxin GhoS is an RNase that specifically cleaves the cognate toxin GhoT mRNA (Fig 2E) and thus prevents the accumulation of the toxin (Wang *et al.*, 2012). Similarly to Type I toxins TisB and Hok (Gerdes *et al.*, 1986a, Unoson & Wagner, 2008), GhoT can also affect cell viability by forming pores in the cell membrane. GhoT forms transient transmembrane pores which lead to loss of membrane integrity and formation of so-called ghost cells (dead or dying cells with damaged membranes) (Wang *et al.*, 2012, Kim *et al.*, 2018). Unexpectedly, another TA system from Type II TA family, *mqsRA*, is involved in *ghoST* regulation. When toxin MqsR is produced, it preferentially cleaves the antitoxin *ghoS* mRNA over the toxin *ghoT* mRNA, because the *ghoT* mRNA does not have primary MqsR cleavage sites, and this leads to toxin GhoT activation (Wang *et al.*, 2013).

SocA/SocB of *Caulobacter crescentus* is the only Type VI TA system described to date. The antitoxin SocA is a proteolytic adapter and is needed for the protease ClpXP to be able to degrade the toxin SocB (Fig 2F). The toxin SocB competes with various replication proteins for binding to the β-sliding clamp, thus accumulation of SocB in the cells blocks the replication elongation (Aakre *et al.*, 2013).

Antitoxins of Type VII TA systems are enzymes that neutralize the toxins by post-translational modifications (Fig 2G). The first discovered Type VII TA system is *E. coli hha/tomB*. The antitoxin TomB renders the toxin Hha harmless by promoting oxidization of a conserved cysteine residue of the toxin (Marimon *et al.*, 2016). As the oxidation happens only in the presence of oxygen, Hha/TomB is the first described TA system that is oxygen-dependent. Two other Type VII TA systems have been described. The antitoxin TakA of *tglT/takA* TA systems of *M. tuberculosis* is a serine protein kinase that phosphorylates a conserved serine residue of the toxin TglT to neutralize it (Yu *et al.*, 2020) and the antitoxin MntA of *hepT/mntA* TA system of *Shewanella oneidensis* is an adenylyltransferase that uses ATP as substrate to disarm the toxin by transferring three AMPs to its conserved tyrosine residue (Yao *et al.*, 2020).

The latest addition to the TA systems family is Type VIII TA systems. While all the other TA systems encode for a toxin that is a protein, Type VIII TA systems have a toxin that is an RNA. The antitoxin for Type VIII TA systems is also an RNA and inhibits the activity of its cognate toxin by antisense binding or by recruiting a Cascade protein to inhibit toxin RNA expression (Fig 2H). There are two Type VIII TA systems discovered so far – *sdsR/ryeA* of *E. coli* (Choi *et al.*, 2018) and *creTA* of *Haloarcula hispanica* (Li et al., 2021). The toxin *sdsR* is expressed only in stationary phase cells and is involved in regulation of multiple genes (Choi *et al.*, 2018, Parker & Gottesman, 2016, Gutierrez *et al.*, 2013). The toxic effect of *sdsR* most probably comes from repressing an inner membrane protein YhcB expression leading to problems in cell envelope development. Antisense binding of the antitoxin *ryeA* to the toxin *sdsR* abolishes the toxic effect (Choi *et al.*, 2018). The *creA* antitoxin works in a similar manner, but

to repress the expression of the toxin *creT*, the antitoxin *creA* has to recruit the Cascade protein of CRISPR-Cas system (Li *et al.*, 2021). When the toxic *creT* RNA is expressed, it sequesters rare arginine $tRNA^{UCU}$ which impedes translation leading to inhibition of cell growth and division (Li *et al.*, 2021)

2.1.2. Regulation of TA systems

Toxin and antitoxin genes are closely linked and generally encoded as one operon. In most cases, the antitoxin is the first gene of the operon, e.g. *E. coli mazEF* (Aizenman *et al.*, 1996) and *relBE* systems (Christensen & Gerdes, 2003). Having the antitoxin first in the operon helps to ensure that there is more antitoxin made than toxin, thus decreasing the risk of accidental self-poisoning. But there are also TA systems with reverse gene order, meaning that the toxin gene is the first gene of the operon, e.g. *E. coli mqsRA* (Fraikin *et al.*, 2019) and *hicAB* systems (Turnbull & Gerdes, 2017), and *Vibrio cholerae higBA* system (Christensen-Dalsgaard & Gerdes, 2006). To make sure that the antitoxin is produced more than the toxin, the antitoxin gene can have additional promoters. For example, the *mqsA* antitoxin has two additional promoters within the *mqsR* toxin gene which combined are stronger than the *mqsRA* operon promoter (Fraikin *et al.*, 2019). The *hicAB* operon has in total of two promoters and the transcription from the upstream promoter results in production of both the toxin and the antitoxin, but the expression from the downstream promoter leads to a transcript from which only antitoxin HicB can be produced (Turnbull & Gerdes, 2017). Interestingly, the transcription from the downstream promoter is induced in the excess of free HicA toxin, thus the antitoxin production is directly regulated by the amount of free toxin in the cells (Turnbull & Gerdes, 2017).

TA systems' expression must be tightly regulated to prevent unintentional self-harm. One way to ensure the appropriate toxin-antitoxin ratio in the cell is through transcriptional autoregulation, which is common for most Type II TA systems. Type II antitoxins generally have a toxin binding domain and a DNA binding domain (Loris & Garcia-Pino, 2014). Antitoxins can bind to the TA system operator to repress its expression and in many cases the DNA binding affinity of the antitoxin-toxin complex is higher than that of the antitoxin alone (Magnuson *et al.*, 1996, Gotfredsen & Gerdes, 1998, Afif *et al.*, 2001). However, when TA complexes get saturated with toxin, they can no longer bind to operators and TA operon repression is relieved, thus antitoxin can now be produced to neutralize the excess of toxin (Afif *et al.*, 2001, Overgaard *et al.*, 2008, Garcia-Pino *et al.*, 2010, Winther & Gerdes, 2012). This regulation is known as conditional cooperativity, because depending on the toxin:antitoxin ratio, toxin acts either as a corepressor (low toxin:antitoxin ratio) or derepressor (high toxin:antitoxin ratio) (Overgaard *et al.*, 2008, Garcia-Pino *et al.*, 2010, Winther & Gerdes, 2012). However, not all Type II TA systems are regulated through conditional cooperativity. It was shown that in some cases antitoxin binding to toxin reduces antitoxin's ability to bind to DNA. This seems to be more common for TA systems with reverse gene order, such as *mqsRA* (Brown *et al.*, 2013) and *hicAB* (Turnbull &

Gerdes, 2017) of *E. coli*, *higBA* of *Proteus vulgaris* (Schureck *et al.*, 2019) and *graTA* of *P. putida* (Talavera *et al.*, 2019).

2.1.3. TA systems' distribution

TA systems were initially discovered on plasmids, but when more and more microbial genomes were sequenced, it became evident that TA systems are also widespread in bacterial and archaeal chromosomes (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011, Xie *et al.*, 2018). Earlier studies attempting to identify TA systems in chromosomes found that most free-living bacteria encode TA systems and bacteria that tend to not have TA systems are mostly obligate intracellular parasites or symbionts (Pandey & Gerdes, 2005, Makarova *et al.*, 2009). For example, the facultative intracellular bacterium *M. tuberculosis* was predicted to have 38 chromosomal TA systems, while its close relative and obligate intracellular pathogen *Mycobacterium leprae* has none. Thus, it was proposed that TA systems are necessary for free-living bacteria as they often encounter different stresses and TA systems can help to manage stress whereas bacteria that live in a more stable environment simply do not require TA systems (Pandey & Gerdes, 2005). Additionally, it was also speculated that prokaryotes with smaller genome sizes have less TA systems, hence suggesting that TA systems might not be biologically relevant to bacteria (Makarova *et al.*, 2009). Both hypotheses were refuted when a subsequent study showed that intracellular pathogens also encode TA systems, e.g. some *Rickettsia* species can have over 30 predicted TA systems (Leplae *et al.*, 2011). These authors think that many intracellular pathogens have less or no TA systems compared to their free-living relatives, because intracellular bacterial genomes often go through massive reductive evolution and this can eliminate their TA systems. The study also showed that there is no direct correlation between the genome size and the number of TA systems (Leplae *et al.*, 2011). Additionally, another study focusing on *P. putida* TA systems' distribution noticed that clinical isolates and environmental strains tend to have different TA systems (Molina *et al.*, 2016). This could mean that certain TA systems are beneficial to the bacteria in specific growth conditions.

While the distribution of TA systems was under investigation, it was observed that virulent bacteria could have more TA systems. For example, this was the case for *S. enterica* different isolates (Di Cesare *et al.*, 2016). Also, a study focusing on pandemic bacteria concluded that pandemic bacteria compared to their non-pathogenic or less-pathogenic relatives have smaller genomes and they encode more TA systems. This could mean either that TA systems are relevant for bacterial virulence, or that even though pandemic bacteria have gone through genome reduction, they struggle to get rid of TA systems (Georgiades & Raoult, 2011).

Various studies found that the number of TA systems in different bacterial species can vary greatly (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011). Some bacteria might contain no TA systems, e.g. *Chlamydophila pneumoniae AR39* or *Buchnera aphidicola str. Sg* (Leplae et al., 2011), the common laboratory *E. coli* strain K-12 has at least 39 TA systems (Song & Wood,

2020a) and *M. tuberculosis* and *Microcystis aeruginosa NIES-843* can have as many as 88 and 97 predicted TA systems, respectively (Leplae *et al.*, 2011, Ramage *et al.*, 2009). It was also noted that even closely related bacteria do not necessarily encode the same TA systems and TA systems are often located within or in close proximity to different mobile elements in the genome, e.g. prophages or integrons. This indicates that TA systems are not part of the bacterial core genome and propagate through horizontal gene transfer (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011, Ramisetty & Santhosh, 2016). However, this might not be true in regards of Type I TA systems, as different studies have rarely linked them to mobile elements, and they have rather seemed to evolve through lineage-specific duplication (Fozo *et al.*, 2010, Coray *et al.*, 2017). But as Type I TA systems with RNA antitoxins are harder to detect than Type II TA systems with protein antitoxins, it is possible that when better tools are developed to mine for Type I TA systems, different data could come to light about their distribution.

2.2. Functions of TA systems

After the initial discovery of TA systems on plasmids, it became evident that they help to maintain plasmids in the cells. However, when numerous different TA systems were found in chromosomes of various bacterial species, it sparked the interest of researchers to determine their importance in chromosomes. As TA systems are very widespread in bacterial chromosomes and one bacterium can harbor multiple TA systems, they were proposed to have an important physiological role in bacteria.

One proposed function for chromosomal TA systems has been stabilization of mobile genetic elements in the chromosome (Wozniak & Waldor, 2009, Yao *et al.*, 2015) – a similar function to TA systems in plasmids (Fig 1). In addition to that, over the years there have been various studies showing that TA systems could be part of the bacterial stress response through either regulation of growth rate (Pedersen *et al.*, 2002, Jørgensen *et al.*, 2009), programmed cell death (PCD) (Aizenman *et al.*, 1996), persister cell formation (Dörr *et al.*, 2010, Kim & Wood, 2010), biofilm regulation (Kim *et al.*, 2009, Wang *et al.*, 2011) or defense against phages (Hazan & Engelberg-Kulka, 2004, Guegler & Laub, 2021). However, various research groups have obtained conflicting results while trying to determine the functions of chromosomal TA systems, because often TA systems' functions are studied using artificial overexpression of the toxin that can lead to erroneous results. At the same time, little emphasis is put on the fact that the deletion of the whole TA system from the chromosome has no effect on the bacteria (Keren *et al.*, 2004, Lee *et al.*, 2014, Zhang *et al.*, 2018). Therefore, it is possible that TA systems are just selfish elements that generally give no benefit for their host bacterium. Thus, the importance of chromosomal TA systems for bacteria remains unclear and is further discussed in detail in this chapter.

2.2.1. DNA maintenance

One of the functions attributed to chromosomal TA systems is stabilization of mobile DNA. Since TA systems in plasmids are responsible for plasmid maintenance in the cells (Fig 1), it is not surprising that their chromosomal counterparts can also have a similar function. For example, it was shown that MosAT system encoded in the *V. cholerae* integrative and conjugative element SXT promotes the maintenance of the SXT element in the cells (Wozniak & Waldor, 2009). It is beneficial for the bacteria not to lose the SXT element, because it encodes multiple antibiotic resistance genes. Also, in *Streptococcus suis* the pathogenicity island SsPI-1 can only be deleted from the chromosome if the Type II TA system SezAT is disrupted, indicating that the TA system is there to maintain the pathogenicity island (Yao *et al.*, 2015).

TA systems can also be beneficial for prophages as after excision from the bacterial chromosome, TA systems can help to stabilize the extrachromosomal circular prophage in the cells until reintegration and thus prevent the prophage from getting eliminated. This has been shown for a Type II TA system $ParE_{SO}$/$CopA_{SO}$ encoded in *Shewanella oneidensis* prophage CP4So (Yao *et al.*, 2018) as well as for four Type I TA systems in *Clostridioides difficile* prophage phiCD630-1 (Peltier *et al.*, 2020).

Moreover, TA systems can also promote the integrity of other genetic elements important for bacteria. For example, it was recently shown that the Type VIII TA system CreTA of archaebacterium *Haloarcula hispanica* protects the *cascade* genes of CRISPR-*cas* locus from getting disrupted by transposable elements. If the *cascade* genes are disrupted, the antitoxin CreA can no longer silence the toxin gene as it requires the Cascade protein for it, thus the free CreT toxin can inhibit cell growth by sequestering the rare tRNA-Arg (Li *et al.*, 2021).

Thus, TA systems may be considered to be addiction modules, as they promote the maintenance of their host genetic element (whether it is a plasmid, prophage, pathogenicity island or something else) by making it difficult for the bacterium to eliminate this mobile DNA without enduring harm themselves. However, chromosomal TA systems can also act as anti-addiction modules to their plasmid counterparts. A study showed that the Type II CcdAB TA system encoded in the F-plasmid is responsible for killing bacteria that do not inherit the F-plasmid due to *ccdAB*-mediated PSK. However, bacteria with a chromosomal *ccdAB* TA system (e.g. *Erwinia chrysanthemi*) are protected from PSK upon plasmid loss (Saavedra De Bast *et al.*, 2008), meaning that chromosomal TA systems can prevent bacteria from getting addicted to plasmids with homologous TA systems.

2.2.2. Growth inhibition and programmed cell death

It is widely believed that TA systems can regulate growth rate when bacteria encounter stress and thus can help bacteria survive. This belief is mostly based on two things. Firstly, TA systems' expression is often increased under stress (Christensen *et al.*, 2001, Christensen & Gerdes, 2003, Jørgensen *et al.*, 2009, Christensen-Dalsgaard *et al.*, 2010, LeRoux *et al.*, 2020). And secondly, overexpression of the toxin reduces the growth of the bacteria, while simultaneous overexpression of the antitoxin restores the bacterial growth (Pedersen *et al.*, 2002, Budde *et al.*, 2007, Schmidt *et al.*, 2007, Jørgensen *et al.*, 2009, Kasari *et al.*, 2010). Some researchers have proposed the programmed cell death (PCD) model in which the activation of the toxin can lead to certain cells being directed to die if it benefits the rest of the population (Aizenman *et al.*, 1996).

E. coli Type II TA system *mazEF* was one of the first chromosomal TA systems to be studied thoroughly, and to be implemented in PCD. Initial studies on *mazEF* proposed that *mazEF* is the main reason behind PCD in *E. coli* and it is dependent on (p)ppGpp (Aizenman *et al.*, 1996). Limiting nutrients will lead to (p)ppGpp accumulation, which was proposed to inhibit transcription from *mazEF* locus, initially leading to degradation of antitoxin MazE due to its inherent instability, and resulting in free toxin MazF, and subsequently ending in cell death. It was claimed that when nutrients run low, the bacterial population will benefit from some of the members dying, as nutrients will be released from lysed cells, and this could ensure the preservation of the population (Aizenman *et al.*, 1996). Following studies by the same group showed that not only starvation, but also other stresses can induce *mazEF* dependent PCD, e.g. antibiotics inhibiting transcription (rifampicin) or translation (chloramphenicol, spectinomycin) (Sat *et al.*, 2001), DNA damage caused by either thymine starvation (Sat *et al.*, 2003), UV irradiation, nalidixic acid or mitomycin C, high temperatures, and oxidative stress by hydrogen peroxide (Hazan *et al.*, 2004). Moreover, *mazEF* was also shown to induce PCD in *E. coli* during P1 phage attack, thus halting the spread of the phage in the population (Hazan & Engelberg-Kulka, 2004).

However, another group challenged the *mazEF* dependent PCD model, as they showed that the overexpression of the MazF toxin does not lead to cell death but is only bacteriostatic and reversible when the antitoxin MazE is expressed (Pedersen *et al.*, 2002). Also, it was initially claimed that starvation induced (p)ppGpp accumulation inhibits *mazEF* transcription (Aizenman *et al.*, 1996), but in contrast, Kenn Gerdes's group showed that amino acid starvation instead induces *mazEF* expression and it is not dependent on (p)ppGpp (Christensen *et al.*, 2003). Nevertheless, Hanna Engelberg-Kulka's group showed in support of *mazEF* mediated PCD model that there is "a point of no return", i.e. a certain time of MazF toxin overproduction after which MazE antitoxin can no longer resuscitate the cells (Amitai *et al.*, 2004), and the previous study (Christensen *et al.*, 2003) was simply just not conducted long enough. Since the involvement of *mazEF* in PCD was unclear, Laurence Van Melderen's group set out to get some clarity. They used an *E. coli* strain deficient in 5 Type II TA systems, one of which was

mazEF. They did not see TA system mediated PCD under various tested stress conditions such as amino acid starvation, nutritional downshift, acidic-shift tolerance, heat shock, and transcription or translation inhibition by antibiotics (Tsilibaris *et al.*, 2007). Moreover, they discovered that the strains used in the initial studies by Hanna Engelberg-Kulka's group had RelA^{-} phenotype due to a mutation in the *relA* gene and deletion in the *mazG* gene (Tsilibaris *et al.*, 2007). As the initial *mazEF* dependent model was claimed to be dependent on (p)ppGpp (Aizenman *et al.*, 1996), and *mazG* was shown to affect (p)ppGpp levels and *mazEF* dependent PCD (Gross *et al.*, 2006), these findings discredited the *mazEF* mediated PCD model even more. Additionally, another study also pointed out that the *mazEF* deficient strain used by Hanna Engelberg-Kulka's group (Aizenman *et al.*, 1996) had lower fitness and stress tolerance than the wild-type strain, and they were unable to reproduce *mazEF* dependent PCD using the exact same strains (Ramisetty *et al.*, 2016b). Thus, problems with the strains and reproducibility discredit the *mazEF* dependent PCD model.

Even though ectopic production of toxins inhibits bacterial growth (Pedersen *et al.*, 2002, Budde *et al.*, 2007, Schmidt *et al.*, 2007, Jørgensen *et al.*, 2009, Kasari *et al.*, 2010) and stress often upregulates TA systems' expression (Christensen *et al.*, 2001, Christensen & Gerdes, 2003, Jørgensen *et al.*, 2009, Christensen-Dalsgaard *et al.*, 2010, LeRoux *et al.*, 2020), there is no direct evidence showing that abiotic stress can activate toxins. In fact, a recent study showed that while TA systems' expression is increased during different stresses, there is no liberation of the toxin from antitoxin's control (LeRoux *et al.*, 2020). Authors show that TA systems' expression is increased, because stress triggers the degradation of free antitoxins and thus relieves transcriptional autoregulation, but antitoxins bound to toxins are protected from proteolysis and hence the toxins remain inactive (LeRoux *et al.*, 2020). Thus, there is no convincing evidence showing that bacteria use TA systems to modulate their growth to cope with abiotic stress.

2.2.3. Persistence

Persisters are a dormant subpopulation of bacterial cells that are genetically identical to the rest of the population, but they are not susceptible to antibiotics (Balaban *et al.*, 2004). During antibiotic treatment, persisters remain unaffected and will survive, in contrast to the rest of the bacterial population, as they are either extremely slow-growing or non-growing and do not divide. After antibiotic exposure is over, persisters can start growing and dividing to reinstate the population. Persisters are one of the reasons behind relapsing of bacterial infections and hence trying to understand how persisters are formed and how to eradicate them is crucial to help people with chronic infections (Lewis, 2007, Fisher *et al.*, 2017).

Persister cells seem to appear stochastically in the population (Lewis, 2007), but it is believed that certain conditions, e.g. activation of SOS response (Dörr *et al.*, 2009) or TA systems (Wang & Wood, 2011), can promote persister cell formation. The first indication that TA systems might be important in persister

formation came when mutations in a gene named *hipA* led to higher number of persisters (Moyed & Bertrand, 1983). Further studies found that *hipA* is the toxin gene of *hipAB* TA system (Korch *et al.*, 2003). While mutations in the *hipA* toxin gene increased persistence (Moyed & Bertrand, 1983), the deletion of the whole TA system had no effect on *E. coli*'s ability to form persisters in growing cells (Keren *et al.*, 2004). Yet, stationary phase cells and biofilm encountered reduction in persister cell formation when *hipAB* was absent (Keren *et al.*, 2004). However, the same study showed that in the absence of another TA system, *relBE*, persister formation has not changed compared to the wild-type strain (Keren *et al.*, 2004) indicating that not all TA systems promote persistence.

Over the next few years, more evidence supporting TA systems' involvement in persistence arose. Firstly, TA systems' expression was upregulated in *E. coli* persister cells (Keren *et al.*, 2004, Shah *et al.*, 2006). Secondly, Type I TA system *tisB/istR* was shown to affect persister formation in *E. coli* treated with ciprofloxacin. The *tisB* toxin knockout strain had decreased and antitoxin knockout strain had increased persister formation compared to the wild-type strain (Dörr *et al.*, 2010). Thirdly, the deletion of either the toxin *mqsR* or the whole TA system *mqsRA* both decreased the amount of *E. coli* persisters (Kim & Wood, 2010). Also, overexpression of toxin in the cells frequently leads to increased persistence (Keren *et al.*, 2004, Dörr *et al.*, 2010, Kim & Wood, 2010, Cheverton *et al.*, 2016, Verstraeten *et al.*, 2015). Additionally, uropathogenic *E. coli* isolate lacking Type II TA system *pasTI* had decreased persistence after treatment with ampicillin or ciprofloxacin, although the deletion of the same TA system from *E. coli* MG1655 did not affects its persistence (Norton & Mulvey, 2012). Moreover, perhaps the most convincing evidence linking TA system to persistence was presented by Kenn Gerdes's group when they showed that Type II TA systems are responsible for *E. coli* persistence (Maisonneuve *et al.*, 2011). They deleted a total of 10 TA systems from *E. coli* genome and showed that the strain devoid of 10 TA systems had significantly reduced persistence (Maisonneuve *et al.*, 2011). Subsequent studies by the same group resulted in a widely accepted model for TA systems-dependent persister formation. According to this model, the toxin HipA activation inhibits glutamyl-tRNA synthetase, leading to accumulation of uncharged $tRNA^{Glu}$ and stalling ribosomes (Germain *et al.*, 2015). This activates RelA-dependent (p)ppGpp production and (p)ppGpp in turn inhibits exopolyphosphatase. This leads to accumulation of polyphosphate which triggers Lon protease to degrade antitoxins, thus activating toxins which promote the formation of persister cells (Germain *et al.*, 2015, Maisonneuve *et al.*, 2013). However, as the results of other groups did not fit the model proposed by Gerdes's group, they started to question whether the 10 TA systems had such a major part in *E. coli* persistence (Ramisetty *et al.*, 2016a, Shan *et al.*, 2017, Van Melderen & Wood, 2017). Finally, the authors of the original publication showing that TA systems are of major importance to *E. coli* persistence had to retract their papers (Maisonneuve *et al.*, 2011, Maisonneuve *et al.*, 2013, Germain *et al.*, 2015) as they discovered that the Δ10TA strain used in their studies had been contaminated with bacteriophage φ80 and this led to inaccurate results (Harms *et al.*, 2017).

New strains devoid of 10 TA systems were created independently by two different groups and both came to the same conclusion that the 10 TA systems have no significant importance to *E. coli* persistence (Harms *et al.*, 2017, Goormaghtigh *et al.*, 2018). This revelation bestowed serious doubt upon the connection between TA systems and persistence.

However, while the link between the 10 TA systems and persistence in *E. coli* was proposed, a similar story also unraveled with regards to *S. enterica* serovar Typhimurium. It was shown that after phagocytosis by murine macrophages, *S. enterica* encounters acidification and nutrient limitation in the vacuole which in turn activates TA systems' toxins to promote persistence. *S. enterica* encodes 14 Type II TA systems and most single TA-deletion mutants have decreased persistence (Helaine *et al.*, 2014), indicating TA systems' functional redundancy. But similarly to *E. coli*, the involvement of TA systems in *S. enterica* persistence was questioned by another independent study showing that the persistence of *S. enterica* strain devoid of 12 TA systems has not changed compared to the wild-type strain (Pontes & Groisman, 2019). Likewise, it was shown that the deletion of three Type II TA system does not affect *Staphylococcus aureus* persistence (Conlon *et al.*, 2016).

In conclusion, even after years of extensive research, the importance of TA systems in persister cell formation has remained unclear. Perhaps the only way to get clarity in this matter is to find out the specific conditions that trigger each toxin's activation. Nevertheless, it is already clear that most of the TA systems studied so far do not promote persistence (Conlon *et al.*, 2016, Harms *et al.*, 2017, Goormaghtigh *et al.*, 2018, Pontes & Groisman, 2019).

2.2.4. Biofilm formation

Biofilm is a microbial community that can form from bacteria attaching to a surface or to each other. It is a three-dimensional structure, and the main components of biofilm matrix are polysaccharides, proteins and extracellular DNA (Karatan & Watnick, 2009). Encountering stress can promote bacteria to start forming biofilm. The ability to form biofilm can also be an important virulence factor for some bacteria (Taylor *et al.*, 2014).

TA systems have been associated with biofilm regulation. The first indication that TA systems could affect biofilm production came from a study that identified genes that are upregulated in biofilms. The study found two toxin genes – *mqsR* from *mqsRA* system and *hha* from *hha/tomB* system – to be upregulated in *E. coli* biofilm (Ren *et al.*, 2004). Subsequent studies indicated that the toxin MqsR can promote biofilm formation as deletion of *mqsR* gene reduced biofilm production (González Barrios *et al.*, 2006). In coherence with that, the antitoxin MqsA was reported to be a global regulator that decreases biofilm formation. The overproduction of MqsA repressed the expression of the stress response regulator RpoS which led to reduced amounts of c-di-GMP in the cells, and an increase in motility (Wang *et al.*, 2011). In addition to *rpoS* repression, overproduction of MqsA was also shown to inhibit the expression of biofilm regulator CsgD, which

in turn negatively affects curli formation and thus biofilm production (Soo & Wood, 2013). However, the involvement of MqsRA in biofilm regulation was challenged by another group (Fraikin *et al.*, 2019). They showed that neither the deletion of the *mqsRA* locus nor mild overexpression of the antitoxin MqsA affects the biofilm production at all. Nor could they detect that MqsA is a global regulator that affects the expression of *rpoS* or *csgD* genes (Fraikin et al., 2019). As the initial studies (Wang *et al.*, 2011, Soo & Wood, 2013) were conducted using strong overexpression of MqsA, it is possible that it led to nonspecific binding of the antitoxin and thus inaccurate results. Hence, MqsRA seems not to be involved in biofilm regulation.

Nevertheless, there are indications that some TA systems can be important in biofilm formation. For example, *E. coli* cells devoid of 5 Type II TA systems (*mazEF*, *relBE*, *chpB*, *yoeB/yefM*, *yafQ/dinJ*) produce less biofilm after 8h and more biofilm after 24h than the wild-type *E. coli* (Kim *et al.*, 2009). The authors explain that the mutant's biofilm production increases after 24h because the dispersal of biofilm decreases. A different study focusing on the same TA systems tried to determine the impact of each of these systems in biofilm regulation. They saw that the deletion of *mazEF* or *dinJ/yafQ* had the biggest effect on biofilm production out of the 5 TA systems, reducing biofilm formation at both 8h and 24h (Kolodkin-Gal *et al.*, 2009). The authors claim that the mutants produce less biofilm because the TA system-mediated cell death pathway is defective, and since there is no extra nutrients being released from the dead cells, biofilm production is halted (Kolodkin-Gal *et al.*, 2009).

On some occasions, results have been presented about TA systems' role in biofilm production that fail to be wholly convincing. For example, *E. coli* produces less biofilm when Type V TA system *ghoST* toxin *ghoT* is deleted from the genome and more biofilm when the antitoxin *ghoS* is disrupted (Wang *et al.*, 2012). However, there is no data presented whether the absence of the whole *ghoST* system affects *E. coli* biofilm production. It is important to investigate the effect of the whole TA system's absence to the bacteria, as for example in *Xylella fastidosa* it has been shown that the deletion of either the toxin or antitoxin gene of *dinJ/relE* or *mqsR/ygiT* systems affects biofilm formation, but deletion of the whole TA loci have no effect on biofilm production (Lee *et al.*, 2014). This indicates that these TA systems are not integral for *X. fastidosa* biofilm regulation. Another example is the Type II TA system *higBA* of *Pseudomonas aeruginosa*. HigBA is claimed to be important in biofilm regulation, because when the antitoxin gene *higA* is disrupted and only the toxin HigB is produced, it leads to biofilm repression (Wood & Wood, 2016, Zhang *et al.*, 2018). Yet, the deletion of the whole *higBA* system has no effect on biofilm regulation (Zhang *et al.*, 2018). There is also no data presented about at what conditions the toxin would be released from the antitoxin's control and thus being able to affect biofilm production. Hence, the evidence about *higBA* involvement in *P. aeruginosa* biofilm regulation remain unconvincing.

All things considered, biofilm regulation does not seem to be a universal function for TA systems, but there is evidence that some TA systems can affect biofilm formation. It is important to look at TA systems as a whole when trying to determine their function. If TA systems are integral in biofilm regulation, then we should see effects on biofilm production when the whole TA system is deleted.

2.2.5. Protection from bacteriophages

Bacteria need to constantly protect themselves against bacteriophages and require different tools to withstand phage attacks. Restriction-modification systems and CRISPR-Cas systems are the most well-known mechanisms that can help individual bacteria to counteract phage infection (Hampton *et al.*, 2020). But in addition to that, different abortive infection (Abi) systems, that cause the death of phage-infected cells, can help the bacteria to halt phage spread in the population. This means that while the infected bacteria will die, there will be no new phages released and thus the population as whole is protected (Lopatina *et al.*, 2020).

There is accumulating evidence showing that TA systems can work as Abi systems to help the bacterial population during phage attack (Song & Wood, 2020a, LeRoux & Laub, 2022, Zhang *et al.*, 2022). The toxin is generally neutralized by the antitoxin, but during phage invasion the toxin is released from the antitoxin's control, which leads to the death of the infected bacteria and termination of phage propagation.

Multiple TA systems have been shown to protect bacteria from phage invasion. For example, Type II TA systems *mazEF* and *rnlAB* were shown to protect *E. coli* from phages P1 (Hazan & Engelberg-Kulka, 2004) and T4 (Koga *et al.*, 2011), respectively. Additionally, Type II TA system *darTG* of *E. coli* (LeRoux *et al.*, 2022) and Type III TA system *toxIN* of *P. atrosepticum* (Fineran *et al.*, 2009) were shown to provide resistance against multiple phages. Until recently, the mechanisms that trigger the toxin's activation upon phage infection had remained undescribed. Yet, a recent study focused on the chromosomal TA system *toxIN* from *E. coli* environmental isolate revealed the role of ToxIN upon phage infection in more detail (Guegler & Laub, 2021). Authors showed that when bacteria get infected and phage infection shuts off host transcription, it also includes the *toxIN* locus, meaning that bacteria cannot make more antitoxin. This leads to liberation of the toxin and as toxin primarily targets viral RNAs, the production of mature viral particles cannot be completed. To achieve the maximum protection provided by *toxIN*, the phage infection-mediated shutoff of host transcription has to be complete (e.g. in case of phage T4), as when it is only partial (e.g. in case of phage T7), the phage particles can still be produced, although in lesser amount (Guegler & Laub, 2021). ToxIN is not a canonical Abi system, as the overexpression of toxin ToxN does not directly kill the bacteria but only halts the bacterial growth and is reversible when the antitoxin ToxI is produced. Authors propose that the infecting phage manages to trigger the process that leads to the host cell death just before ToxN can shut off transcription and thus production of new phage particles (Guegler & Laub, 2021).

In addition to transcription shutoff, phage capsid protein recognition can also trigger toxin's activation. This is the case for Type II TA system CapRel encoded by *Salmonella* phage SJ46, which is a special kind of TA system as the toxin and the antitoxin are fused together (Zhang *et al.*, 2022). The C-terminal part of the fused protein is the antitoxin and in addition to neutralizing the toxin part of the protein, it also recognizes and binds to the newly synthesized major capsid protein during phage infection, leading to the activation of the N-terminal toxin. Activated toxin pyrophosphorylates tRNAs to inhibit translation and thus impedes phage production (Zhang *et al.*, 2022). Hence, new phage particles cannot be made, the phage does not spread in the population and the infection is restricted to the initially infected cells only, making this TA system a useful tool to stop phage invasion.

Moreover, a recent study revealed that *Bacillus subtilis* TA system *mazEF* is incorporated into the arbitrium signaling pathway of SPbeta and phi3T phages, which is responsible for the phage lysis-lysogeny decision during phage attack (Cui *et al.*, 2022). Upon infection, the non-coding RNA AimX of arbitrium system is expressed, leading to suppression of the lysogeny genes (Erez *et al.*, 2017). At the same time, the arbitrium peptide AimP is produced by the infected cells and transported into neighboring bacteria (Erez *et al.*, 2017). Accumulation of the arbitrium peptide in the cell induces the expression of phage phi3T operon which is downstream from the arbitrium locus (Cui *et al.*, 2022). One of the genes from the upregulated operon called *phi3T_93* was shown to suppress lytic propagation. Interestingly, to prevent the phage from completing the lytic cycle and instead lysogenize, *phi3T_93* requires the presence of the *mazEF* TA system (Cui *et al.*, 2022). phi3T_93 binds to the antitoxin MazE of the TA complex and can facilitate the mRNase activity of MazF *in vitro*. There are still some factors yet unknown that are responsible for the MazF toxin activation, but it was shown that the toxin activation during phage infection leads to bacterial growth arrest and halts the production of new phage particles (Cui *et al.*, 2022). Thus, the SPbeta phage relies on its host's TA system to switch from lytic to lysogenic cycle, and the presence of the *mazEF* TA systems protects *B. subtilis* from getting annihilated by the lytic phage.

Seeing as bacteria can use TA systems to protect themselves against phages, phages have come up with different ways to try to bypass TA systems. For example, the DarT toxin of *E. coli darTG* TA system ADP-ribosylates viral DNA which inhibits phage DNA and RNA synthesis and thus there is not enough viral DNA to pack into newly synthesized phage particles (LeRoux *et al.*, 2022). One way for the phage to overcome the DarTG defense is to modify its DNA polymerase so that it can bypass the DNA modification and maintain the ability to synthesize DNA regardless of the actions of DarT. Another strategy for the phage to overcome DarT effects is to modify an already existing anti-DarT factor (phage-encoded antitoxin) to neutralize the DarT toxin (LeRoux *et al.*, 2022).

Indeed, a very straightforward way for phages to bypass host TA systems is to encode their own antitoxin against the bacterial toxin. For example, phage T4 harbors a gene called *dmd* that encodes an antitoxin which can neutralize the

toxins of both *rnlAB* and *lsoAB* TA systems of *E. coli* (Otsuka & Yonesaki, 2012). In addition, the *P. atrosepticum* phage θTE was seen to encode a similar sequence to antitoxin *toxI* of *toxIN* TA system. Search for phage mutants that were no longer sensitive to *toxIN* system led to the discovery of mutants expressing additional copies of the *toxI* resembling gene (Blower *et al.*, 2012). ToxIN also protects *E. coli* from phage T4. While *toxI* is an RNA antitoxin, phage T4 encodes a protein TifA that can also neutralize the toxic effect of ToxN (Srikant *et al.*, 2022).

To conclude, TA systems are used as tools in the evolutionary arms race between bacteria and phages. As bacteria can use TA systems to stop the phages from spreading within the population through abortive infection, the phages must come up with different strategies to bypass TA systems to be able to successfully infect bacteria and form progeny. The most common way seems to be for the phage to encode its own antitoxins that can neutralize the toxins of TA systems.

2.2.6. Selfish elements

Even though most bacterial species have chromosomal TA systems, it is possible that the TA systems are not beneficial to their host. TA systems have many traits indicating that they are simply selfish elements and responsible only for their own maintenance in the genome no matter the cost for their host (Magnuson, 2007, Van Melderen & Saavedra De Bast, 2009). Firstly, TA systems' addictive nature makes it difficult for the bacteria to get rid of them. Similarly to plasmid addiction, losing the chromosomal TA locus leads to a situation where free toxin can harm the bacteria after antitoxin is degraded and there is no means to produce more antitoxin. Promoting their own maintenance could be one of the reasons why chromosomal TA systems are so widespread among bacteria (Van Melderen, 2010). Secondly, the sparse distribution of TA systems in closely related strains indicates that they are not part of the core genome and do not retain a substantial importance to the bacterial species (Makarova *et al.*, 2009, Leplae *et al.*, 2011, Fiedoruk *et al.*, 2015, Ramisetty & Santhosh, 2016). In support of that, there are multiple studies showing that the deletion of TA systems from the genome has no measurable benefit on bacterial fitness (Tsilibaris *et al.*, 2007, Unoson & Wagner, 2008, Tamman *et al.*, 2014, Li *et al.*, 2016, Harms *et al.*, 2017, Pontes & Groisman, 2019, LeRoux *et al.*, 2020, Goormaghtigh *et al.*, 2018). Also, selfish elements (mobile DNA like plasmids and transposons) easily spread horizontally between bacteria. The sporadic occurrence of TA systems and the notion that TA systems are often found close to or within mobile genetic elements implies that they propagate through horizontal gene transfer (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Mine *et al.*, 2009, Ramisetty & Santhosh, 2016). Even though TA systems are not able to move horizontally between bacteria on their own, they can hitch a ride with other mobile elements, thus stabilizing the mobile element they are part of in the host genome.

TA systems' degeneration in chromosomes also refers to their little importance to bacteria. *E. coli* chromosomal ccd_{O157} locus was shown to encode a defective toxin in about 1/3 of investigated loci, and the locus was shown to be under

neutral evolution, thus indicating the lack of biological importance to *E. coli* (Mine *et al.*, 2009). Another study looking into different *E. coli* TA systems found that truncated versions of most of the 10 TA systems under investigation could be found among various *E. coli*. Yet, two TA systems, *chpBS* and *mazEF*, were intact in all the strains, which could indicate their relevance to the bacteria (Ramisetty & Santhosh, 2016). But since the study could only identify truncated TA systems, it is highly likely that there were even more non-functional TA systems as toxins can be rendered harmless by smaller mutations. For instance, a study looking into how toxins are inactivated showed that the toxins are mostly inactivated due to mutations in the promoter area (Fernandez-Garcia *et al.*, 2019). Thus, functional studies are required to determine the integrity of TA systems.

2.3. Pseudomonas putida TA systems

Ubiquitous soil bacterium and laboratory model organism *P. putida* strain KT2440 (Nelson *et al.*, 2002) (isogenic to PaW85 (Bayley *et al.*, 1977)) is predicted to have 15 Type II TA operons in its chromosome (Xie *et al.*, 2018). Only four of them – *graTA* (Tamman *et al*., 2014, Tamman *et al*., 2016, Ainelo *et al*., 2016, Talavera *et al*., 2019), *mqsRA* (Sun *et al.*, 2017), *mazEF* (Miyamoto *et al.*, 2016) and *res-xre* (Skjerning *et al.*, 2019) – have been studied previously.

2.3.1. GraTA

The first and most thoroughly studied *P. putida* TA system belongs to the HigBA family and is named *graTA* for the growth rate affecting toxin (Tamman *et al.*, 2014). Similarly to other Type II TA systems, the antitoxin GraA has to form a complex with the toxin GraT to disarm it (Tamman *et al.*, 2014). Akin to many Type II toxins, the toxin GraT is an mRNase that cuts mRNA ribosome dependently (Talavera *et al.*, 2019). Still, GraTA is an unusual TA system in many ways. Firstly, while most TA systems encode highly poisonous toxins that are deadly to bacteria without antitoxin's presence (Shah *et al.*, 2006, Baba *et al.*, 2006, Budde *et al.*, 2007), GraT is mildly toxic and affects bacterial growth only somewhat when the antitoxin GraA is absent (Tamman *et al.*, 2014). However, GraT is temperature dependent, which means that its toxic effect is more prominent at lower temperatures. For instance, GraT-caused growth defect is minor at 30 °C, while at 20 °C bacteria are unable to form colonies on LB agar plates (Tamman *et al.*, 2014).

In addition to growth inhibition, GraT activity was shown to result in ribosome biogenesis defect (Ainelo *et al.*, 2016). Interestingly, chaperone protein DnaK, which is involved in ribosome biogenesis (Maki *et al.*, 2002, Al Refaii & Alix, 2009), was found to affect GraT toxicity and relieve the ribosome maturation defect (Ainelo *et al.*, 2016). DnaK has N-terminal nucleotide-binding domain (NBD), C-terminal substrate-binding domain (SBD) and C-terminal unstructured tail (Bertelsen *et al.*, 1999, Smock *et al.*, 2011). ATP binding and hydrolysis by

NBD is necessary for DnaK to bind to its substrate and to refold the target protein (Mayer & Bukau, 2005). The conserved C-terminal tail of DnaK is not essential for DnaK chaperone activity but can still affect its efficiency (Aponte *et al.*, 2010, Smock *et al.*, 2011). According to two different studies, the C-terminus either enhances (Smock *et al.*, 2011) or represses (Aponte *et al.*, 2010) the DnaK protein refolding activity. It was shown that GraT binds to DnaK and DnaK C-terminal tail disruption suppresses GraT toxicity (Ainelo *et al.*, 2016). Unfortunately, the consequence of the DnaK and GraT interaction in context of ribosome biogenesis defect remains unclear, but there are two possibilities. Firstly, GraT could inhibit ribosome biogenesis by binding to DnaK and blocking its activity (Ainelo *et al.*, 2016). In this case, if DnaK C-terminal tail decreases DnaK chaperone activity (Aponte *et al.*, 2010), then the disruption of the DnaK C-terminal tail could overcome the GraT-caused inhibiting effect and alleviate ribosome biogenesis defect (Ainelo *et al.*, 2016). The second possibility is that DnaK C-terminal tail enhances DnaK refolding ability by establishing additional interactions with misfolded proteins (Smock *et al.*, 2011) and since GraT has an intrinsically disordered N-terminus (Talavera *et al.*, 2019), DnaK could help GraT to obtain its active toxic form (Ainelo *et al.*, 2016). Disruption of the DnaK C-terminal tail could decrease DnaK's ability to fold GraT, thus there would not be enough active GraT to affect ribosome maturation (Ainelo *et al.*, 2016).

The antitoxin GraA is uncommonly stable for an antitoxin (Tamman *et al.*, 2016). For instance, *E. coli* RelB and *S. aureus* MazE antitoxins half-lives are less than 20 minutes (Christensen *et al.*, 2001, Donegan *et al.*, 2010), while GraA's is at least 4 hours (Tamman *et al.*, 2016). While most antitoxins have an intrinsically disordered region that obtains its proper folding when antitoxin forms a complex with the toxin (Brzozowska & Zielenkiewicz, 2013), GraA is fully folded on its own (Talavera *et al.*, 2019). The antitoxins' unfolded regions are thought to be responsible for their instability, as they can make the proteins attractive to cellular proteases (Brzozowska & Zielenkiewicz, 2013, Loris & Garcia-Pino, 2014). Many antitoxins are degraded by Lon and Clp proteases (Brzozowska & Zielenkiewicz, 2013), however that is not the case for GraA and the protease responsible for GraA degradation remains unidentified (Tamman *et al.*, 2016). Similarly to most Type II TA systems, GraA can bind the *graTA* operator and autoregulate the *graTA* locus expression (Tamman *et al.*, 2014). However, when many Type II TA operons are regulated by conditional cooperativity, involving the toxin-antitoxin complex binding to the operator (Overgaard *et al.*, 2008, Garcia-Pino *et al.*, 2010, Winther & Gerdes, 2012), then GraT prevents the GraTA complex from binding to the *graTA* operator and this is due to the toxin's disordered tail (Talavera *et al.*, 2019). This means that GraT is the derepressor of the *graTA* locus (Talavera *et al.*, 2019).

GraTA is certainly an intriguing TA system, as the toxin GraT can affect *P. putida* stress tolerance in the absence of the antitoxin GraA and depending on the stressor, the effect can be either positive or negative (Tamman *et al.*, 2014). For example, GraT makes *P. putida* cells more tolerant to translation inhibiting antibiotics kanamycin and streptomycin or DNA damaging chemicals mitomycin C and ciprofloxacin, but increases sensitivity to osmotic stress caused by NaCl

and oxidative stress caused by paraquat (Tamman *et al.*, 2014). However, it is important to note that GraT can affect *P. putida* stress tolerance only in the absence of the antitoxin GraA and when the whole *graTA* system is deleted from *P. putida* genome, there is no detectable change in the bacteria's stress tolerance (Tamman *et al.*, 2014). Thus, the importance of GraTA in *P. putida* stress tolerance remains questionable.

2.3.2. MqsRA

Similarly to the *graTA* system, the *mqsRA* locus also encodes a mild toxin, because while overexpression of the toxin MqsR is toxic to *P. putida*, the deletion of the antitoxin *mqsA* from the genome has no significant effect on bacterial growth (Sun *et al.*, 2017). The *mqsRA* operon regulation is analogous to *graTA* locus. The antitoxin MqsA represses the *mqsRA* operon expression, while the toxin MqsR inhibits the antitoxin MqsA from binding to the promoter region (Sun *et al.*, 2017). Also, this study tried to determine if *mqsRA* could affect *P. putida* persister cell and biofilm formation. It was seen that even though the toxin MqsR can increase *P. putida* persistence in the absence of the antitoxin *mqsA*, the deletion of the *mqsRA* locus has no effect on persister formation (Sun *et al.*, 2017). However, the MqsRA system seems to have importance for *P. putida* in biofilm regulation. It was shown that after 2 hours biofilm production is decreased in a similar manner when either the toxin, antitoxin or the whole TA system is absent from the genome (Sun *et al.*, 2017). Unfortunately, the mechanism behind it is not investigated and there is no additional information provided whether such inhibition in biofilm formation can also be seen at the later stages of biofilm development.

2.3.3. MazEF and RES-Xre

Both *mazEF* and *res-xre* have been shown to encode functional TA systems. The toxin MazF is an mRNase and was shown to preferably cleave the UAC sequence in *in vitro* experiments (Miyamoto *et al.*, 2016). RES is structurally similar to NADase toxins, and its expression depletes cells of NAD^+, leading to growth arrest (Skjerning *et al.*, 2019). So far, the biological importance of these two TA systems to *P. putida* has not yet been investigated.

3. THE AIM OF THE THESIS

TA systems are intriguing genetic elements, seeing as they encode for a toxin that when released from antitoxin's control, can poison their host. Once it was evident that TA systems are widely distributed on chromosomes (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011), it raised the question as to why bacteria would encode such systems. The significance of chromosomal TA systems to bacteria has been under thorough investigation ever since they were discovered and remains controversial. Some TA systems have been shown to stabilize mobile genetic elements (Wozniak & Waldor, 2009, Yao *et al.*, 2015), while others protect bacteria from phages (Hazan & Engelberg-Kulka, 2004, Guegler & Laub, 2021), and some have been claimed to be important in the stress regulation (Pedersen *et al.*, 2002, Kim *et al.*, 2009, Jørgensen *et al.*, 2009, Kim & Wood, 2010, Dörr *et al.*, 2010, Wang *et al.*, 2011). However, there are many TA systems that seem not to contribute to its host fitness (Tsilibaris *et al.*, 2007, Harms *et al.*, 2017, Pontes & Groisman, 2019). Therefore, they can be considered as selfish elements that use mobile DNA elements to invade bacterial genomes.

The common soil bacterium *P. putida* has predicted to encode as many as 15 TA systems in its genome (Xie *et al.*, 2018). The most thoroughly described *P. putida* TA system, *graTA*, is in many ways an unusual TA system. The antitoxin GraA is uncommonly stable and neutralizes the toxin GraT efficiently (Tamman *et al.*, 2014, Tamman *et al.*, 2016). GraT is a ribosome-dependent mRNase with an unusual N-terminal unstructured part that is involved in regulation of GraT toxicity (Talavera *et al.*, 2019). GraT activity results in growth inhibition and ribosome biogenesis defect, which are more prominent at lower temperatures (Tamman *et al.*, 2014, Ainelo *et al.*, 2016). Yet, previous studies did not reveal the changes that take place in *P. putida* on cellular level that lead to these effects. Interestingly, the major cellular chaperone DnaK was implicated in GraT-caused ribosome biogenesis defect (Ainelo *et al.*, 2016), however, the exact role of DnaK in GraT toxicity remained unclear in previous investigations.

It is fascinating that GraT can affect *P. putida* stress tolerance in the absence of the antitoxin GraA (Tamman *et al.*, 2014). However, the deletion of the whole *graTA* system had no effect on *P. putida* stress tolerance (Tamman *et al.*, 2014), raising the question about *graTA* biological significance to *P. putida* along with other *P. putida* TA systems. Thus, to get more insight into *graTA* and other TA systems' importance to *P. putida*, the aims of this thesis were to

- describe the cellular response of *P. putida* to GraT activity
- determine the role of chaperone protein DnaK in GraT toxicity
- determine the biological importance of chromosomal TA systems to *P. putida*

4. MATERIALS AND METHODS

Most of the methods used in this study have been described in detail in the Materials and Methods sections of publications I, II and III. Methods not described in publications I, II and III are described in detail in the following chapters. Additionally, some main principles of the main approaches are outlined here.

To study TA systems' toxins effects to *P. putida*, we chose to use antitoxin deletion strains rather than kill/rescue assay that is commonly used to study toxin effects. The kill/rescue assay uses high copy-number plasmids and artificial overexpression of the toxin. Yet, it is extremely unlikely that from a single genomic copy such a high amount of toxin is ever produced. Thus, we chose to study toxins' effects in antitoxin deletion strains, because we consider this to be more relevant to natural conditions when one genomic copy of toxin could escape the antitoxin's control (Ref I, III).

We used an efficient method to create markerless gene deletions (Martinez-Garcia & de Lorenzo, 2011), which allows us to study the effects of deletion of (a) gene(s) without additional changes to the bacterial genome (Ref I, II, III).

We used the competition assay to compare the fitness of different strains (Ref II, III). For that, we marked each of the strains used in competition assays with either kanamycin or streptomycin resistance genes to distinguish between the strains. We chose the combination of these two antibiotic resistance genes, because they do not affect the fitness of the bacteria (Ref II, Supplementary Fig S2).

4.1. Phage isolation

P. putida phages were isolated from various soil and water samples. Environmental sample size used in one isolation experiment was ~100 ml. In the case of soil samples, water was added until volume of 100 ml was reached. The enrichment method was used to isolate phages, meaning that nutrients and bacterial host culture were added to the environmental samples. *P. putida* PaW85 strain lacking 13 TA systems and 4 genomic prophages (Δ13TAΔ4φ) was used as the host bacterium in the isolation process. These genetic elements have been shown to have a role in phage resistance: TA systems have been shown to offer protection against phage invasion (Hazan & Engelberg-Kulka, 2004, Fineran *et al.*, 2009, LeRoux *et al.*, 2022, Zhang *et al.*, 2022), and prophages can encode various mechanisms to prevent phage superinfection (Bondy-Denomy *et al.*, 2016, Dedrick *et al.*, 2017).

To enrich the environmental samples with phages, 5 ml of 10x LB medium, 2 ml of exponential phase (OD_{580}~1) Δ13TAΔ4φ culture, $CaCl_2$ (final concentration 10 mM) and ciprofloxacin (final concentration 0.01 μg/ml) were added to the 100 ml of environmental samples. Ciprofloxacin was added to LB medium to induce DNA stress which can potentially trigger prophage activation in environmental bacteria and prevent temperate phages from entering the lysogenic cycle

in the host bacterium. $CaCl_2$ was added to enhance phage infection. Next, samples were incubated in flasks overnight at 70 rpm at 20 °C. The next day, to get rid of the bacteria, samples were centrifuged for 30 min at 5000 rpm (Hettich Universal 320R centrifuge) and the supernatant was filtrated (0.22 µm filter). To see if the phage isolation was successful, 1 ml of the filtrate was mixed with 200 µl of exponentially growing Δ13TAΔ4φ culture and 4 ml of 0.3% melted LB agar (42 °C) containing 10 mM of $CaCl_2$, and overlayed on LB plates supplemented with ciprofloxacin (0.03 µg/ml). Plates were incubated overnight at 20 °C. If plaques had formed, single plaques were isolated and purified. To do that, a single plaque was picked and suspended in 100 µl of SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin). 5 µl of chloroform was added to lyse the bacteria. Ten-fold dilutions of the phage suspension were made and 2 µl drops were spotted on bacterial lawn plates. Plates were incubated overnight at 20 °C. Next, a new single plaque was picked, and the purification step was repeated twice more. Purified phages were stored in phage storage buffer (SM buffer) at 4 °C.

4.2. Phage infection assays

4.2.1. Plaque assay

Bacteria were grown in LB medium overnight at 20 °C. Overnight bacterial cultures were diluted 15-fold into fresh LB medium and grown until OD_{580}~1 at 20 °C. Next, 200 µl of the bacterial culture was added to 5 ml of melted 0.3% LB agar medium (42 °C) containing 10 mM $CaCl_2$, and overlayed on 1.5% LB agar plates containing 0.03 µg/ml ciprofloxacin to create bacterial lawn. Ten-fold dilutions of phage stocks were made using SM buffer and 2 µl drops were spotted on the bacterial lawns. Plates were incubated overnight at 20 °C. The following day, the number of plaques formed was counted.

4.2.2. Infection with phages in liquid media

Bacteria were grown in LB medium overnight with shaking at 120 rpm at 20 °C. The overnight bacterial cultures were diluted about 50-fold to fresh LB medium or LB medium supplemented with 0.03 µg/ml ciprofloxacin and 10 mM $CaCl_2$, and the cultures were grown 2–3 hours on the microtiter plate without shaking at 20 °C. Next, dilutions of phage stocks were made into SM buffer and different amounts of phages were added to the bacterial cultures which is indicated by the multiplicity of infection (MOI). SM buffer was added to the control cultures that were not infected with phages. The microtiter plate was incubated at 20 °C without shaking and the optical densities (OD_{580}) of the culture were measured with Tecan Infinite 200 PRO plate reader every 10 minutes.

4.3. Kill/rescue assay for testing *hicAB-2* functionality

For the kill/rescue assay, the toxin and the antitoxin genes had to be cloned into separate expression vectors. The antitoxin *hicB-2* (PP_3899) gene encoding sequence was amplified from the *P. putida* PaW85 genome with primers 3900Kpn and 3899EcoRI (Table 1). The PCR product was digested with restriction enzymes EcoRI and Eco147I, and the pBRlacItac plasmid (Ojangu *et al.*, 2000) with EcoRI and SmaI. Next, the antitoxin gene was inserted into the pBRlacItac plasmid under the IPTG-inducible tac promoter. The toxin *hicA-2* (PP_3900) gene encoding sequence was amplified from the *P. putida* PaW85 genome with primers 3900Kpn and 3900Hindend (Table 1). Both the PCR product and pBAD33 plasmid (Guzman *et al.*, 1995) were digested with restriction enzymes KpnI and HindIII, and the toxin gene was inserted into the pBAD33 plasmid under the arabinose-inducible pBAD promoter. The toxin gene could only be cloned into the pBAD33 plasmid when the *E. coli* cells already harbored the antitoxin-encoding pBRlacItac plasmid. Plasmids were introduced into *E. coli* DH5α cells by electroporation. Successful construction of the plasmids was verified with sequencing.

To conduct the kill/rescue assay, single colonies of *E. coli* DH5α (Hanahan & Meselson, 1983) cells were picked that contained both the pBRlacItac-hicB-2 and pBAD33-hicA-2 plasmids. The cells were grown overnight in LB medium supplemented with 20 μg/ml chloramphenicol and 100 μg/ml ampicillin to select for the plasmids, 5 mM IPTG to induce the antitoxin expression and 0.2% glycose to repress the toxin expression. The optical densities of overnight bacterial cultures at 580 nm were measured and the bacteria were diluted into fresh medium for OD_{580} to be 0.1. The LB growth medium was always supplemented with 20 μg/ml chloramphenicol and 100 μg/ml ampicillin for plasmid selection but could additionally contain either 5 mM IPTG for antitoxin induction or 10 mM arabinose for toxin induction or both IPTG and arabinose for the induction of expression of both genes. The bacterial cultures were grown on a microtiter plate at 400 rpm at 30 °C and the optical densities of the cultures (OD_{580}) were measured with POLARstar Omega plate reader every 14 minutes.

4.4. Construction of the strains used in prophage P1 stability assays

4.4.1. Construction of the *ΔhicA-2* (ΔT) strain

To delete the *hicA-2* (PP_3900) toxin gene from *P. putida* genome, the pEMG-based plasmid was constructed according to the protocol described elsewhere (Martinez-Garcia & de Lorenzo, 2011). The downstream region of the toxin PP_3900 gene (899 bp), including the antitoxin PP_3899 gene, was amplified with primers 3899EcoRI and 3900del (Table 1), and the upstream region (588 bp) with primers 3900delpikk and 3901Bam (Table 1). Next, the two PCR fragments were joined into one fragment (1476 bp) by overlap extension PCR using primers

3899EcoRI and 3901Bam (Table 1). The generated PCR fragment and pEMG plasmid were digested with restriction enzymes EcoRI and BamHI, and then ligated together. The obtained plasmid pEMG-Δ3900 was delivered into *P. putida* PaW85 by electroporation, and after 3h of growth in LB medium, the bacteria were plated onto LB agar supplemented with kanamycin (50 μg/ml). Kanamycin-resistant cointegrates were selected and electrotransformed with the I-SceI expression plasmid pSW(I-SceI). To resolve the cointegrate, the plasmid-encoded I-SceI nuclease was induced with 1.5 mM 3-methylbenzoate overnight. Kanamycin-sensitive colonies were selected and the PP_3900 gene deletion was verified with PCR. The plasmid pSW(I-SceI) was eliminated from the deletion strain by growing the cells overnight in LB medium without antibiotics. Intactness of antitoxin *hicB-2* (PP_3899) gene in the Δ3900 (ΔT) strain was verified by sequencing.

4.4.2. Construction of the *hicAB-2::Sm* (TA::Sm) strain

The previously constructed pEMG-Δ3900 plasmid was used for the construction of the *hicAB-2::Sm* (TA::Sm) strain. The pEMG-Δ3900 was digested with restriction enzyme MunI, so that the antitoxin PP_3899 gene is disrupted. The streptomycin (Sm) resistance gene was cut out from the pUTmini-Tn5Sm/Sp plasmid (de Lorenzo *et al.*, 1990) with restriction enzyme VspI. Both the digested pEMG-Δ3900 plasmid and Sm resistance gene were blunt-ended with Klenow DNA polymerase. Next, the Sm resistance gene was inserted into the pEMG-Δ3900 plasmid and ligated together. The obtained plasmid pEMG-Δ3900/3899::Sm was delivered into *P. putida* PaW85 by electroporation, and after 3h of growth in LB medium, the bacteria were plated onto LB agar supplemented with streptomycin (200 μg/ml). Next, streptomycin resistant and kanamycin sensitive colonies were picked and Sm resistance gene insertion into the correct locus was verified by PCR and sequencing.

4.5. qPCR for testing prophage P1 stability

For the qPCR assay, bacteria were grown ~18 hours in LB medium or LB medium supplemented with 0.03 μg/ml ciprofloxacin to induce DNA stress and the genomic DNA was isolated with GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

To measure the excision frequency of P1 from *P. putida* genome, qPCR primers were designed that are flanking the P1 locus (Table 1) and the PCR product is detected only in the case of P1 excision. To determine whether the excised P1 can form an extrachromosomal circle, we created qPCR primers that cover the predicted junction of the extrachromosomal circle (Table 1). To measure the copy number of P1 in the cells, we designed primers for a DNA region within the prophage P1 (Table 1). We used *rpoD* as the reference gene (Table 1). The qPCR assay was performed on the Rotor-Gene Q system (QIAGEN) using the

SOLIScript® 1-step SolisGreen® Kit (Solis BioDyne) according to the manufacturer's protocol. For each reaction, 1 or 10 ng of genomic DNA was used. Raw data were analyzed with the Rotor-Gene Q software v. 2.02 (QIAGEN) and DNA amounts were calculated using the LinRegPCR software v. 2013.0 (Ruijter *et al.*, 2009). Data from at least five independent biological replicates were averaged and normalized against *rpoD* levels.

Table 1. Oligonucleotides.

Name	**Sequence (5'-3')***	**Use**
3900Kpn	AATGGTACCTAACTGGAATGATTGACC	Construction of pBRlacItac-hicB-2 and pBAD33-hicA-2
3899EcoRI	CGGAATTCTACATAGCCGCCCTGG	Construction of pBRlacItac-hicB-2 and pEMG-Δ3900
3900Hindend	CTGAAGCTTGCCGAATAGGCAATT	Construction of pBAD33-hicA-2
3900del	AAGCAGGAGGATTGATCTGA	Construction of pEMG-Δ3900
3900delpikk	TCAGATCAATCCTCCTGCTTCTTGCTTAGACATTTGGGC	Construction of pEMG-Δ3900
3901Bam	GTGGATCCGGTGATGGAGCGAGT	Construction of pEMG-Δ3900
delP1qFw	AGTTTCGGAACTCTTGCCTTT	To measure the excision frequency of P1
delP1qRev	GCCGAGTAGCAAAGTGGTTAT	To measure the excision frequency of P1
P1circ-R	CAAGCCTTCCAAATGATGAAGTT	To determine if P1 is an extrachromosomal circle
P1circ-L	ATCGACCCTTGTAGAATGCG	To determine if P1 is an extrachromosomal circle
PP_3886qFw	TGACGAATTGGCGAGTGTT	To measure the copy number of P1
PP_3886qRev	CCCACGCTGACTGTTACC	To measure the copy number of P1
rpoDqFw	GCAACAGCAGTCTCGTATCA	To amplify the *rpoD* gene
rpoDqRev	ATGATGTCTTCCACCTGTTCC	To amplify the *rpoD* gene

*The sites of restriction enzymes used in cloning are underlined.

5. RESULTS AND DISCUSSION

5.1. Cellular response to GraT toxicity (Ref I)

GraT is a peculiar toxin. Firstly, it has an intrinsically disordered N-terminus that is uncommon for toxins as toxins are usually fully structured (Talavera *et al.*, 2019). Also, GraT activity leads to growth and ribosome biogenesis defects, which are more severe at lower temperatures (Tamman *et al.*, 2014, Ainelo *et al.*, 2016). Although some toxins' activity has been shown to be affected by temperature (Jahn *et al.*, 2012, Janssen *et al.*, 2015, Rocker & Meinhart, 2015, Zhao *et al.*, 2016), GraT caused temperature-dependent growth inhibition along with ribosome maturation defect is a unique trait for a toxin. It is interesting why a ribosome-dependent mRNase toxin would lead to ribosome biogenesis defect. To shed some light into what could cause the ribosome maturation defect and how *P. putida* copes with GraT toxicity, we performed a whole-cell proteome comparison of the *P. putida* wild-type and the antitoxin deletion strain Δ*graA*. We chose to use the antitoxin deletion strain rather than overexpressing the toxin, because it is a closer approximation of natural conditions when the one genomic copy of toxin could escape the antitoxin's control. Also, we can observe the toxin's effects on growing cells, as GraT only inhibits growth rate of the Δ*graA* strain about 2-fold at 25 °C and even less at 30 °C (Tamman *et al.*, 2014), as opposed to toxin overexpression that often leads to cell growth arrest.

A previous study showed that the ribosomal subunits accumulate in the Δ*graA* strain grown at 25 °C, indicating a ribosome maturation defect (Ainelo *et al.*, 2016). Thus, we wanted to see whether this defect is the result of lack of some ribosomal biogenesis factors that could be the target of GraT. However, much to our surprise, the whole-cell proteome analysis did not detect any significant decrease in the abundance of known proteins involved in ribosome maturation in the Δ*graA* strain at neither 30 °C nor 25 °C (Ref I, Fig 3A,B). On the contrary, we observed that some known ribosome biogenesis factors' expression has been significantly upregulated in the Δ*graA* strain, including RNA helicase DeaD/CsdA, which participates in ribosome assembly in *E. coli* (Charollais *et al.*, 2004, Peil *et al.*, 2008) and rRNA processing enzyme RNase III (Ref I, Table 1, Fig 3A,B). Further examination revealed the general trend of upregulation of ribosome biogenesis factors at both 30 °C and 25 °C in the absence of the antitoxin GraA, although the changes were not always statistically significant (Ref I, Fig 3A,B). Out of analyzed 39 ribosome biogenesis factors, only chaperones DnaK/J and GroEL/ES were observed to have slightly decreased expression, however these changes were insignificant (Ref I, Table S4). Thus, the upregulation of most ribosome biogenesis factors indicates that *P. putida* is trying to alleviate the GraT caused ribosome maturation defect, but unfortunately no specific reason was uncovered why ribosome biogenesis is deficient. Since GraT is a ribosome-dependent mRNase with low sequence specificity (Talavera *et al.*, 2019), we propose that the ribosome biogenesis defect in the Δ*graA* strain is the cumulative

effect of degradation of various GraT targets. Interestingly, a recent study revealed that the induction of different *E. coli* toxins also leads to ribosome biogenesis defect (Culviner *et al.*, 2021). The authors explained that ribosome independent mRNase toxins, such as MazF and MqsR, cleave both rRNA precursors and mRNAs of ribosomal proteins and this inhibits ribosome maturation (Culviner & Laub, 2018, Culviner *et al.*, 2021). But they also saw that the expression of ribosome-dependent mRNase toxins, such as RelE and HigB, leads to ribosome biogenesis defect and proposed that this is mainly due to cleavage of ribosomal protein transcripts (Culviner *et al.*, 2021). However, this does not seem to be the reason for GraT caused ribosome biogenesis defect in *P. putida*, as we did not detect any significant change in the abundance or stoichiometry of ribosomal proteins (Ref I, Fig S2). Also, it has been shown previously that GraT does not target rRNAs as they are intact in the Δ*graA* strain (Ainelo *et al.*, 2016).

The proteome analysis also revealed that *P. putida* central metabolism is altered due to GraT toxicity. This is indicated by significant downregulation of tricarboxylic acid (TCA) cycle enzymes isocitrate dehydrogenase Idh, α-ketoglutarate dehydrogenase subunit SucA and succinate-CoA ligase subunit SucD at 25 °C (Ref I, Fig 3D). Also, other TCA cycle enzymes tend to be downregulated at 25 °C and a similar trend is observed at 30 °C, however these changes are statistically insignificant (Ref I, Fig 3C,D, Table S4). It is possible that the changes in the TCA cycle enzyme levels are due to GraT targeting the mRNAs of these metabolic enzymes. However, a more likely explanation is that the downregulation of central carbon metabolism is the consequence of GraT inhibiting translation and ribosome biogenesis, and adjusting metabolism helps *P. putida* cope with GraT-induced stress.

To conclude, the proteome comparison of wild-type and Δ*graA* strain revealed that *P. putida* seems to increase the expression of ribosome biogenesis factors to alleviate the GraT-inflicted harm and altering the central carbon metabolism might help *P. putida* to adapt to the GraT-caused growth and ribosome biogenesis defects.

5.2. Regulation of GraT toxicity by chaperone DnaK (Ref II)

5.2.1. C-terminus of DnaK enhances GraT toxicity (Ref II)

Previous studies showed that the chaperone protein DnaK is implicated in the GraT activity, as the disruption of the C-terminal tail of DnaK alleviated the GraT-caused cold-sensitive growth and ribosome biogenesis defects (Ainelo *et al.*, 2016). So far, it has remained unclear whether ribosome biogenesis and growth defects in the Δ*graA* strain is the result of DnaK being the target of the toxin GraT and thus being unable to facilitate ribosome maturation (Fig 3A), or the result of DnaK aiding GraT to attain its proper toxic fold which would lead to self-poisoning (Fig 3B).

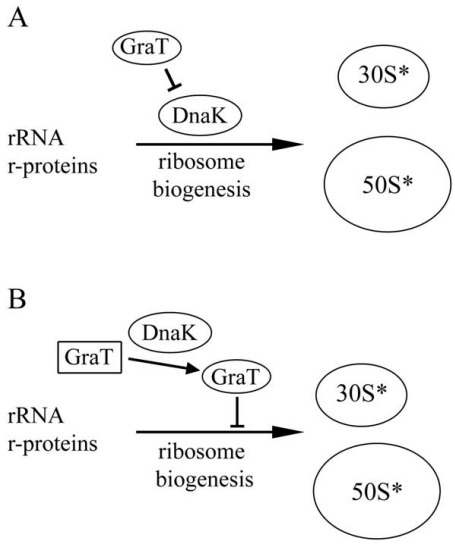

Figure 3. Hypothetical mechanisms how GraT activity leads to ribosome biogenesis defect (Ainelo *et al.*, 2016). 50S* and 30S* represent the accumulation of immature 50S and 30S subunits, respectively. A: DnaK is the target of GraT and thus unable to facilitate ribosome biogenesis. B: GraT affects unknown factor(s) that leads to ribosome biogenesis defect. DnaK assists inactive GraT (rectangle) to attain its active toxic fold (oval).

It seems plausible that GraT could target DnaK, since GraT is a ribosome-dependent mRNase (Talavera *et al.*, 2019) and the whole-cell proteome comparison of the Δ*graA* and the wild-type strains revealed that there is less DnaK in the antitoxin deficient strain, although the difference is not statistically significant (Ref I, Table S4). To determine whether DnaK mRNA is the target of GraT, the DnaK mRNA levels were measured in the wild-type and the *graA* deficient strain. The cultures were grown at 25 °C, because GraT hinders growth significantly at 25 °C in the absence of the antitoxin GraA. We detected very similar DnaK mRNA levels in the two strains (Ref II, Fig 1), which indicates that GraT does not specifically target DnaK mRNA. This means that GraT-caused growth and ribosome biogenesis defects are not the result of depletion of DnaK in the cells.

The other possibility is that since DnaK is a chaperone that helps misfolded proteins to obtain their proper folding (Mayer *et al.*, 2000), and GraT has a 22 amino acid long N-terminal unfolded region (Talavera *et al.*, 2019), then DnaK could facilitate GraT N-terminus folding and thus enhance its toxicity. To establish DnaK importance to GraT, we tried to delete the *dnaK* gene from *P. putida* wild-type and Δ*graA* strains. Unfortunately, we could not obtain strains lacking the *dnaK* gene, which indicates that DnaK is essential for *P. putida*. Next, we decided to use a different approach and wanted to check if overexpressing DnaK could further enhance GraT toxicity. To test this, we introduced a copy of *dnaK* under the inducible tac-promoter into the chromosome of Δ*graA*, as well as into the wild-type strain as a control, and checked the growth of the bacteria at different temperatures. Growth comparison on LB plates at both 30 °C and 25 °C

showed that *dnaK* overexpression in the Δ*graA* strain slightly enhanced the GraT-caused growth retardation (Ref II, Fig 2A). At the same time, overexpression of *dnaK* in the wild-type strain had no effect on bacterial growth (Ref II, Fig 2A). To additionally validate the positive effect of DnaK to the GraT toxicity, we overexpressed DnaK in the Δ*graA* $dnaK_{\Delta 41}$ strain, which encodes DnaK that lacks 41 amino acids from the C-terminus. This truncated DnaK has been previously shown to extensively alleviate the GraT-caused growth inhibition (Ainelo *et al.*, 2016). Overexpressing the full sized DnaK in the Δ*graA* $dnaK_{\Delta 41}$ strain revokes the relieving effect of the $dnaK_{\Delta 41}$ allele and restores the GraT-specific growth defect (Ref II, Fig 2A), thus confirming that DnaK is facilitating the GraT activity. Even more so, it implies that the DnaK C-terminal tail, which is not essential for DnaK ability to refold proteins and is proposed to only fine-tune the DnaK chaperone activity (Aponte *et al.*, 2010, Smock *et al.*, 2011), is particularly important in GraT toxicity.

There is a highly conserved negatively charged region in the extreme end of DnaK C-terminus (Ref II, 2B) and we wanted to find out whether this specific motif is responsible for enhancing GraT activity. To test this, we replaced four negatively charged amino acids (D629, E631, E633 and E634) with their respective polar residues (Asn or Gln) in the DnaK conserved C-terminus (Ref II, Fig 2B) in the wild-type strain and Δ*graA* genomes and named this substitution $dnaK_{mut4}$. Growth comparison at 25 °C shows that the mutated DnaK C-terminus significantly alleviates the GraT-caused growth inhibition in the Δ*graA* strain, effect that is also inherent when DnaK C-terminus is truncated (Ref II, Fig 2A). Furthermore, since GraT has been shown to affect *P. putida* stress tolerance in the absence of the antitoxin GraA either positively or negatively depending on the particular stressor (Tamman *et al.*, 2014), we tested whether mutations in the DnaK conserved C-terminus can reverse those effects. To determine that, we compared the stress tolerance of the wild-type and Δ*graA* strains with $dnaK_{mut4}$ locus against their parental strains. As anticipated, the C-terminally mutated DnaK increased the Δ*graA* tolerance to NaCl and tetracycline (Ref II, Fig 2C). This further confirms that the conserved charged motif in the DnaK C-terminus is responsible for aiding GraT toxicity. But unexpectedly, the mutated DnaK C-terminus did not affect the Δ*graA* streptomycin tolerance (Ref II, Fig 2C). However, the $dnaK_{mut4}$ strain exhibited increased tolerance to streptomycin compared to the wild-type strain (Ref II, Fig 2C). This indicates that while DnaK conserved C-terminus can affect *P. putida* streptomycin tolerance, it does not do it through affecting GraT.

5.2.2. DnaK likely assists GraT folding (Ref II)

It is interesting that GraT requires the major cellular chaperone DnaK to obtain its full toxic potential. But perhaps it is not that surprising considering that GraT N-terminus is not fully folded. TA system's chaperone addiction has been described before, e.g. for *M. tuberculosis* toxin-antitoxin-chaperone (TAC) system. However, differently from the *graTA* system, the antitoxin of *M. tuberculosis*

TAC system is chaperone dependent, as it requires the SecB-like chaperone to attain its proper folding and thus to be able to neutralize the cognate toxin (Bordes *et al.*, 2011).

Previous studies showed that DnaK binds GraT and even though the C-terminal tail of DnaK is somehow important for GraT toxicity, it is not essential for DnaK to be able to bind GraT (Ainelo *et al.*, 2016). However, *in vivo* data indicated that DnaK truncated variants ($DnaK_{\Delta 41}$ and $DnaK_{\Delta 66}$) are less efficient chaperones for GraT (Ainelo *et al.*, 2016), hence indicating that merely binding to DnaK SBD is not enough to fully activate GraT. Since DnaK C-terminus has been proposed to support DnaK binding with unstructured proteins (Smock *et al.*, 2011), we wanted to test whether DnaK C-terminus can give additional interaction with GraT to strengthen the binding. To do this, we performed a pull-down assay with His_6-tagged 57-amino acids long C-terminal peptide of DnaK and tagless GraT. Since full length GraT is toxic, we used nontoxic $GraT_{\Delta 1C}$ that lacks the C-terminal conserved histidine. We could only detect purification of the C-terminal peptide of DnaK, but not GraT from the cell lysate with nickel affinity columns (Ref II, Fig 3A). While disappointing, this is yet in coherence with a previous study from *E. coli* that showed that the DnaK C-terminus fusion with the glutathione S-transferase could not pull out any proteins from *E. coli* lysates (Smock *et al.*, 2011). Additionally, we performed a BACTH assay (Karimova *et al.*, 1998) to determine whether we can detect any interactions between DnaK C-terminus and GraT *in vivo*. For this, the DnaK C-terminus was fused with T18 fragment of CyaA and GraT was fused with T25 fragment of CyaA. In the case of interaction, we would detect *lacZ* reporter gene activity upon induction of the two plasmids. However, no reporter activity beyond the negative control value was observed (Ref II, Fig 3B). Thus, we could not detect that DnaK C-terminus could interact stably with GraT in either of the assays. Nevertheless, we cannot rule out the occurrence of transient interactions between GraT and DnaK C-terminal domain.

GraT has an unfolded N-terminal region and since DnaK interacts with unfolded proteins, we wondered whether the intrinsically disordered N-terminal part of GraT is required to bind to DnaK. To test this, we carried out a pull-down assay with His_6-tagged DnaK and GraT variant that lacks the 22 N-terminal amino acids. As we observed the co-purification of His_6-DnaK and Δ22GraT (Ref II, Fig 4), this means that the N-terminus of GraT is not essential for binding to DnaK. However, despite of not being crucial in DnaK binding, the intrinsically disordered N-terminus has major importance to GraT. Firstly, the GraT N-terminus participates in the *graTA* operon regulation, as it prevents the GraTA complex from binding to the promoter region which leads to the derepression of the operon (Talavera *et al.*, 2019). This is extremely important, as when the toxin:antitoxin ratio is high, it allows to relieve the antitoxin-mediated operon repression and proceed with de novo antitoxin synthesis to keep the toxin neutralized. Even more importantly, the GraT N-terminal tail is necessary for GraT activity (Talavera *et al.*, 2019). Just like its closest homolog HigB of *P. vulgaris*, GraT is a ribosome-dependent mRNase (Hurley & Woychik, 2009, Talavera *et al.*, 2019). The structures of *P. vulgaris* HigB and GraT are very similar, except for the N-terminal

part, as HigB has its N-terminus fully folded while GraT has it unstructured (Schureck *et al.*, 2014, Talavera *et al.*, 2019). *P. vulgaris* HigB N-terminus is important for the protein's ability to bind to ribosomes and its activity (Schureck *et al.*, 2016). Mutations in the HigB N-terminal region decrease HigB toxicity and this is reckoned to be the consequence of the mutant HigB reduced ability to bind to the ribosomes (Schureck *et al.*, 2016). We presume that similarly to HigB of *P. vulgaris*, GraT N-terminus is crucial for the toxin to be able to bind to the ribosomes, and this would explain why GraT lacking the N-terminus is nontoxic (Talavera *et al.*, 2019). However, as the N-terminus of GraT is unfolded, GraT seems to be unable to attain its proper fold without DnaK assistance and thus needs aid from DnaK to be able to bind to ribosomes, and especially important is the conserved motif in the C-terminus of DnaK to achieve the full toxic potential of GraT. To conclude, our data is in agreement with the hypothesis proposed by Ainelo and colleagues (Ainelo *et al.*, 2016) that DnaK helps inactive GraT to achieve its active toxic fold (Fig 3B).

5.2.3. Possible explanations for cold-sensitivity of GraT toxicity

The involvement of DnaK in GraT toxicity might explain why GraT toxic effects are more prominent at lower temperatures. While GraT has no effect on *P. putida* growth at 37 °C and only slightly inhibits growth at 30 °C, tremendous growth suppression can be observed at 20 °C (Tamman *et al.*, 2014). DnaK along with other chaperones refolds and disaggregates proteins and the number of impaired proteins in need of DnaK assistance increases at higher temperatures (Sherman & Goldberg, 1993, Mayer *et al.*, 2000). This could mean that while GraT requires aid from DnaK to attain its active form, at higher temperatures, DnaK is pre-occupied with other damaged proteins and cannot assist GraT, thus GraT remains nontoxic. But at lower temperatures, there is more available DnaK that can help GraT achieve its toxic form and we can detect GraT-caused growth inhibition. However, another but not mutually exclusive possibility is that GraT spontaneous folding is more accurate at lower temperatures, and this could be the reason why GraT is more toxic at 20 °C than 30 °C.

5.3. The conserved motif of DnaK C-terminus enhances *P. putida* competitiveness and affects stress tolerance (Ref II)

There is little known about the biological importance of DnaK C-terminus to bacteria. Thus far, we have observed that the negatively charged conserved motif in DnaK C-terminus enhances GraT toxicity (Ref II, Fig 2A) and can also affect *P. putida* streptomycin tolerance (Ref II, Fig 2C). Therefore, we wondered if there are other traits that the DnaK C-terminus contributes to. First, we tested whether the mutations in the DnaK conserved C-terminus can affect the growth

of *P. putida* in liquid media. We saw no significant difference between the wild-type and the $dnaK_{mut4}$ strain, not even under heat stress conditions (Ref II, Fig 5A). Next, we picked different stressors ($FeSO_4$, $CuCl_2$ and NaCl) that induce the accumulation of misfolded and aggregated proteins in the cells, and tested their effect to the two strains grown on solid media at different temperatures. Once again, we did not detect clear differences between the wild-type and the $dnaK_{mut4}$ strain at any tested conditions. However, the lack of difference between the strains might not be that surprising considering that other cellular chaperones can compensate for DnaK deficiency (Ullers *et al.*, 2004, Smock *et al.*, 2011). Thus, we wanted to see whether DnaK C-terminus mutations would change the stress tolerance of *P. putida* in the chaperone *secB* deficient background. We saw that the $dnaK_{mut4}$ *secB* double mutant has substantially decreased tolerance to NaCl at both 30 °C and 37 °C compared to the *secB* strain (Ref II, Fig 5B). However, an opposite effect was seen when the medium contained excess of iron or copper. At 37 °C, the double mutant had somewhat increased tolerance to these metals (Ref II, Fig 5B). These results demonstrate that the negatively charged conserved C-terminus affects DnaK functionality which in turn affects *P. putida* stress tolerance, but whether the effect is positive or negative depends on the specific stressor.

Additionally, we wanted to see if the conserved motif of DnaK C-terminus could affect the competitive fitness of the bacteria. To investigate that, we used a competition assay, meaning that we mixed the wild-type and the $dnaK_{mut4}$ strain together in equal amounts and co-cultivated them for 24 days to see if one strain could out-compete the other. To distinguish between the two strains, they were previously marked with either kanamycin or streptomycin resistance gene. We observed already at 30 °C that the conserved DnaK C-terminus contributes to the competitive fitness of *P. putida*, as there was significantly less $dnaK_{mut4}$ strain left in the mixture (Ref II, Fig 6A,B). Furthermore, the effect was even more prominent at 34 °C, as in most of the replicates the wild-type strain had out-competed the mutant strain (Ref II, Fig 6C,D). This demonstrates that the negatively charged motif in the C-terminus of DnaK is extremely important for *P. putida* when faced with long-term heat stress. Interestingly, this is the first time when mutations in DnaK C-terminus have been shown to result in measurable fitness defects in bacteria with otherwise wild-type genotype. The deletion of DnaK C-terminus in *E. coli* was shown to inhibit growth when *E. coli* encounters heat shock as well as in the absence of chaperone protein *secB* (Smock et al., 2011). However, the *E. coli* strain used in the study had decreased expression of *dnaJ* and a mutation in σ^{32} that reduces the heat shock response, in addition to *dnaK* C-terminal truncation.

To conclude, previous studies had shown that the C-terminal tail of DnaK either enhances (Smock *et al.*, 2011) or reduces (Aponte *et al.*, 2010) DnaK chaperone activity. Our results rather coincide with the model that the C-terminus enhances DnaK protein refolding ability. We see that the conserved motif of the C-terminal tail has an important role in enhancing GraT toxicity (Ref II, Fig 2) and increasing the long-term thermotolerance and competitive fitness of *P. putida* (Ref II, Fig 6).

However, we can see that in the *secB* deficient background, the mutated C-terminus of DnaK can entail opposite fitness consequences to *P. putida* depending on the applied stress (Ref II, Fig 5B). Nevertheless, most of our obtained results imply that the C-terminal part enhances DnaK chaperone activity.

5.4. Chromosomal TA systems' biological significance to *P. putida* (Ref III)

Despite thorough investigations, chromosomal TA systems' biological importance has remained unclear. There are some studies showing that TA systems within mobile DNA can help to maintain the genetic element in the bacterial genome (Wozniak & Waldor, 2009, Yao *et al.*, 2015). Also, more and more TA systems have been found to protect their host from phage attacks (Hazan & Engelberg-Kulka, 2004, Fineran *et al.*, 2009, Koga *et al.*, 2011, Cui *et al.*, 2022, LeRoux *et al.*, 2022, Zhang *et al.*, 2022). However, TA systems' involvement in stress regulation is the most controversial as some studies claim TA systems have major importance to bacterial stress tolerance (Christensen *et al.*, 2001, Keren *et al.*, 2004, Verstraeten *et al.*, 2015, Muthuramalingam *et al.*, 2019), while others detect no effects (Tsilibaris *et al.*, 2007, Harms *et al.*, 2017, Pontes & Groisman, 2019, LeRoux *et al.*, 2020) and rather propose that TA systems are selfish genetic elements (Magnuson, 2007, Van Melderen & Saavedra De Bast, 2009). To get some clarity on the matter, we wanted to determine the significance of chromosomal TA systems to the common soil bacterium *P. putida*. *P. putida* PaW85 has been predicted to encode up to 15 TA systems in its genome (Xie *et al.*, 2018), and thus far it has only been shown that the lack of the *graTA* system does not affect *P. putida* stress tolerance (Tamman *et al.*, 2014) and the *mqsRA* system may affect *P. putida* biofilm formation (Sun *et al.*, 2017). To investigate TA systems' importance to *P. putida*, we deleted 13 TA loci from *P. putida* genome in the following order: *graTA* (PP_1586-1585), *res-xre* (PP_2433-2434), *higBA* (PP_1199-1198), *hicAB-1* (PP_1480-1479), *relE*$_2$*-higA*$_2$ (PP_5435-PP_0274), *mqsRA* (PP_4205-4204), *mazEF* (PP_0770-0771), PP_1716-1717, *brnTA* (PP_4530-4529), PP_4151-4152, *relBE* (PP_1268-1267), *yefM-yoeB* (PP_2940-2939) and *relB*$_2$*-parE* (PP_2499-2500) (Ref III, Table 1); and named the strain Δ13TA. According to TADB 2.0 prediction, there are two additional TA systems in *P. putida* genome. The *hicAB-2* (PP_3899-3900) is encoded in prophage P1 and PP_3032-3033 in prophage P2 genomes (Martinez-Garcia *et al.*, 2015). Attempting to delete the *hicAB-2* system resulted in the loss of prophage P1 (see chapter 5.4.4). Since prophage loss has been shown to alter *P. putida* fitness (Martinez-Garcia *et al.*, 2015), we did not want it to interfere with interpretation of phenotypes inherent to deletion of other *P. putida* TA systems. Thus, we left the prophage-encoded TA systems intact in *P. putida* Δ13TA genome.

To be certain that the subsequent TA system deletions have not resulted in any unwanted alterations in the Δ13TA genome, we sequenced the genomes of the

wild-type PaW85 and the Δ13TA strains. The whole-genome sequencing confirmed the deletion of the 13 TA loci and no other major changes were found in the genome. The Δ13TA differed from the wild-type strain PaW85 only by 23 point mutations, most of which were found in intergenic regions, pseudogenes or transposase genes (Ref III, Table S1). The whole-cell proteome comparison of the wild-type and the Δ13TA strains grown in LB medium at 30 °C to mid-exponential phase confirmed that the multiple TA deletions have not essentially altered the protein expression levels of the Δ13TA strain. We observed no significantly differently expressed proteins between the strains among 1867 proteins detected in all three replicates of both strains (Ref III, Fig 1, Table S2).

5.4.1. TA systems do not affect growth, stress tolerance, persistence and biofilm formation of *P. putida* (Ref III)

We compared the growth of the wild-type and the Δ13TA strains in LB, 2YT and glucose minimal media at 30 °C and saw no differences between the two strains (Ref III, Fig 2A), meaning that the lack of 13 TA systems does not affect the growth rate of *P. putida*. Since inhibition of growth helps bacteria endure stress (Tuomanen *et al.*, 1986, Eng *et al.*, 1991) and TA systems have been proposed to be activated by stress resulting in toxin-mediated growth suppression (Gerdes *et al.*, 2005, Wang & Wood, 2011), we wanted to see whether TA systems can affect *P. putida* stress tolerance, antibiotic persistence and biofilm formation. Growth comparison of the wild-type and the Δ13TA strains on LB solid media plates supplemented with different chemicals that inhibit either replication (ciprofloxacin), transcription (rifampicin), translation (kanamycin, streptomycin, tetracycline), or cell-wall synthesis (benzylpenicillin), or induce either oxidative (4-nitroquinoline 1-oxide, paraquat) or osmotic stress (NaCl) showed no difference between the two strains at any tested conditions (Ref III, Fig 2B). This indicates that the lack of TA systems does not affect *P. putida* stress tolerance.

To determine whether the absence of 13 TA systems affects *P. putida* persistence, we measured the amount of persister cells left after treatment with lethal dose of streptomycin and benzylpenicillin. The killing curves of the wild-type and the Δ13TA strains were highly similar (Ref III, Fig 2C, D), indicating that TA systems have no role in *P. putida* antibiotic persistence. This is in agreement with recent studies conducted with *E. coli* (Harms *et al.*, 2017, Goormaghtigh *et al.*, 2018), *S. enterica* (Pontes & Groisman, 2019) and *S. aureus* (Conlon *et al.*, 2016) that showed that the deletion of multiple TA systems does also not affect the persistence of these bacteria.

We also compared the ability of the wild-type and the Δ13TA strains to form biofilm. The amount of biofilm produced after 24h was similar for the two strains (Ref III, Fig 2E), implying that TA systems do not regulate biofilm production in *P. putida*. As one of the deleted TA systems in the Δ13TA strain is *mqsRA*, then these results are in contrary to a previous study that claimed that the absence of the *mqsRA* system affects *P. putida* biofilm formation (Sun *et al.*, 2017). In this

study, Sun and colleagues measured biofilm after 2h and the *mqsRA* deficient strains used had gentamycin resistance gene (Sun *et al.*, 2017), but in our study, biofilm was measured after 24h and the Δ13TA strain had no additional antibiotic resistance genes. These differences could be the reason behind contradictory results.

To conclude, as we cannot detect any difference in *P. putida* growth, stress tolerance, persistence or biofilm formation in the absence of 13 TA systems, this indicates that TA systems are not part of *P. putida* stress regulation. However, we cannot rule out that TA systems benefit *P. putida* in some specific stress conditions that we have not discovered yet.

5.4.2. TA systems can decrease the competitive fitness of *P. putida* (Ref III)

The abundance of chromosomal TA systems in bacterial genomes suggests that they are of importance to bacteria. So far, however, we have not detected that TA systems are beneficial to *P. putida* (Ref III, Fig 2). To further investigate it, we conducted a competition assay. We mixed together the wild-type and the Δ13TA strains in equal amounts and cultivated the mixed culture for 20 days. The mixture was diluted to fresh media every two days and the CFU of both strains was determined every four days. The strains were marked with either streptomycin or kanamycin resistance gene to differentiate them from one another. We hypothesized that if the TA systems carry benefits to *P. putida*, then the wild-type strain would out-compete the Δ13TA strain. Curiously, after 20 days of co-cultivation in LB medium at 30 °C we could not detect greater abundance of the wild-type strain compared to the Δ13TA strain in the mixed culture (Ref III, Fig 3). On the contrary, in most experiments we saw that there is 10–100-fold more of the Δ13TA strain in the mixed culture by day 20, or in some cases we could not detect that either of the strains had obtained the majority in the mixed culture (Ref III, Fig 3, Fig S1). Thus, rather than contributing to the host fitness, TA systems can decrease the fitness of *P. putida*. However, since the Δ13TA strain could not out-compete the wild-type strain entirely, the cost of carrying TA systems seems quite minor.

Since TA systems are proposed to help bacteria cope with stress (Gerdes *et al.*, 2005, Wang & Wood, 2011), we wanted to see if adding various stressors to the competition assay might reveal TA systems importance to *P. putida*. We conducted the experiments at suboptimal growth temperature (20 °C), in different growth media (M9 minimal medium with glucose), or in the presence of sub-inhibitory concentrations of various chemicals (paraquat, ciprofloxacin, nitroquinoline, benzylpenicillin). Surprisingly, the addition of stress to the competition assay did not generally affect the ratio of wild-type and Δ13TA in the growth media (Ref III, Fig 4). However, when either nitroquinoline or benzylpenicillin was added to the growth media, then the Δ13TA strain achieved a slight competitive advantage over the wild-type strain (Ref III, Fig 4G,H). Remarkably,

we could never observe that the wild-type strain has a competitive advantage over the Δ13TA strain at any tested conditions.

As nutritional stress has been proposed to activate TA systems (Christensen *et al.*, 2001, Gerdes *et al.*, 2005), we also measured the competitiveness of the two strains in long-term starvation conditions. At the start of the experiment, we mixed together in equal amounts the stationary phase cells of the wild-type and the Δ13TA and cultivated them in M9 minimal media for 50 days without addition of fresh media. During prolonged nutrition deprivation we did not detect that either of the strains has any competitive advantage over the other (Ref III, Fig 4I,J).

Taken all together, we did not detect that carrying TA systems is in any way beneficial to the host bacterium (Ref III Fig 2, Fig 3, Fig 4). On the contrary, in competition assays at optimal growth conditions (rich LB medium, 30 °C), we can observe the competitive advantage of the strain lacking the 13 TA systems (Ref III Fig 3). This indicates that TA systems can be a burden to fast-growing bacteria. In coherence with that, the *shpAB* TA system was also observed to be costly to *Salmonella*, as it decreased the competitiveness of the wild-type strain compared to the TA system deletion strain in the absence of the antibiotic (Helaine *et al.*, 2014). However, when *P. putida* encounters stress, which in its natural habitats in soil and water is a common occurrence, the decrease in the competitiveness due to TA systems is small or even non-existent (Ref III, Fig 4). It is possible that in the non-ideal growth environment, the minor fitness cost conferred by the TA systems does not matter much, and this could explain why *P. putida* encodes multiple TA systems despite the possible fitness cost that comes with having them. Yet, we cannot say that TA systems are always a burden to their host because it was shown that the deletion of 5 TA systems does not affect *E. coli* competitiveness at all in a 7-day competition assay (Tsilibaris *et al.*, 2007). However, it is possible that the cost of TA systems to *E. coli* can come out at different growth conditions.

5.4.3. TA systems can be costly to *P. putida* under phage attack

Some TA systems have been shown to help bacteria endure phage invasion (Hazan & Engelberg-Kulka, 2004, Fineran *et al.*, 2009, Cui *et al.*, 2022, LeRoux *et al.*, 2022, Zhang *et al.*, 2022). Therefore, we wanted to see if TA systems could also help *P. putida* withstand phage attack. To test that, we required phages that can infect *P. putida*, but to our knowledge there has not yet been composed a *P. putida* phage library. Thus, we started out by isolating phages that can infect *P. putida* to create our own *P. putida* phage collection. The phage isolation protocol is described in detail in chapter 4.1. So far, we have managed to isolate about 80 different phages and sequenced most of them. Based on the current sequencing data, these phages belong to 24 new species and 9 genera (bioinformatic analysis was done by Age Brauer). Two phages were considered to belong to the same genus if they were at least 70% identical and to be of the same species if they

were at least 95% identical. We are still working on expanding the phage collection and will continue to sequence the isolated phages.

To see whether TA systems offer protection to *P. putida* against phages, we compared the phages' ability to infect *P. putida* wild-type and Δ13TA strains. For this, we made bacterial lawn plates of the two strains and spotted ten-fold phage dilutions on them (see chapter 4.2.1). The plates were incubated overnight at 20 °C and we compared the number of plaques formed on the wild-type and the Δ13TA plates on the following day. Mostly, we could observe a similar number of plaques for both strains and a representative picture with 8 phages from our collection is shown in Figure 4. This indicates that TA systems have no role in protection against these specific phages.

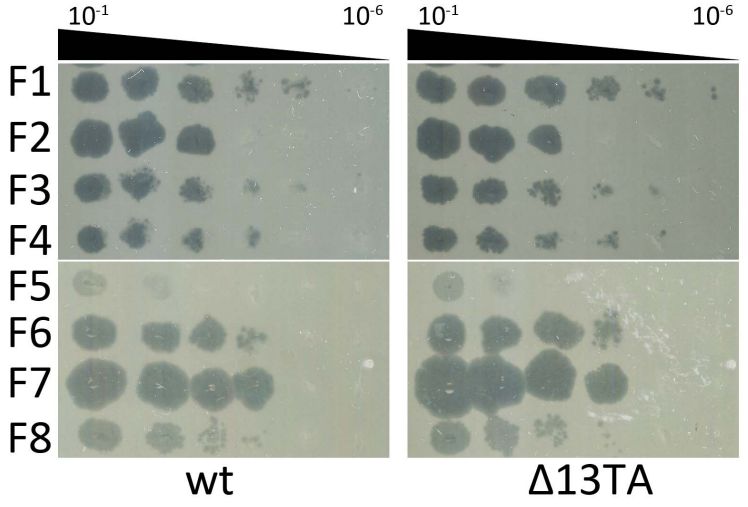

Figure 4. TA systems do not protect *P. putida* from phages. 10-fold serial dilutions of phage lysates (F1–F8) were spotted onto lawns of *P. putida* wild-type (wt) and Δ13TA strains. Bottom agar layer was supplemented with 0.03 μg/ml ciprofloxacin. Top layer (0.3% agar) contained 10 mM $CaCl_2$. Plates were incubated overnight at 20 °C.

Additionally, to assess the infection dynamics in more detail, we compared the growth curves of the wild-type and the Δ13TA strains following infection with our isolated phages at 20 °C (see chapter 4.2.2). Different amounts of phages were added to the bacterial cultures which is indicated by the multiplicity of infection (MOI). We saw that most phages infect the wild-type and the Δ13TA strains similarly also in liquid media. An example is shown in Figure 5A, when the two strains are infected with phage F8. However, we can observe that the sensitivity of the Δ13TA strain to phage infection has changed on some occasions. For example, the growth comparison of the wild-type and the Δ13TA strains after infection with phage F2 revealed that the TA systems can be costly to *P. putida* (Fig 5B). We can see that at MOI 10^{-3} the growth of the wild-type strain is significantly more affected by the phage F2 than that of the Δ13TA strain,

and at MOI 10^{-4}, F2 inhibits the growth of the wild-type strain but not at all the Δ13TA strain (Fig 5B). However, since we cannot detect any differences between the two strains when infected with F2 on plaque assays (Fig 4), the cost that TA systems bear to *P. putida* during phage invasion seems to be rather small.

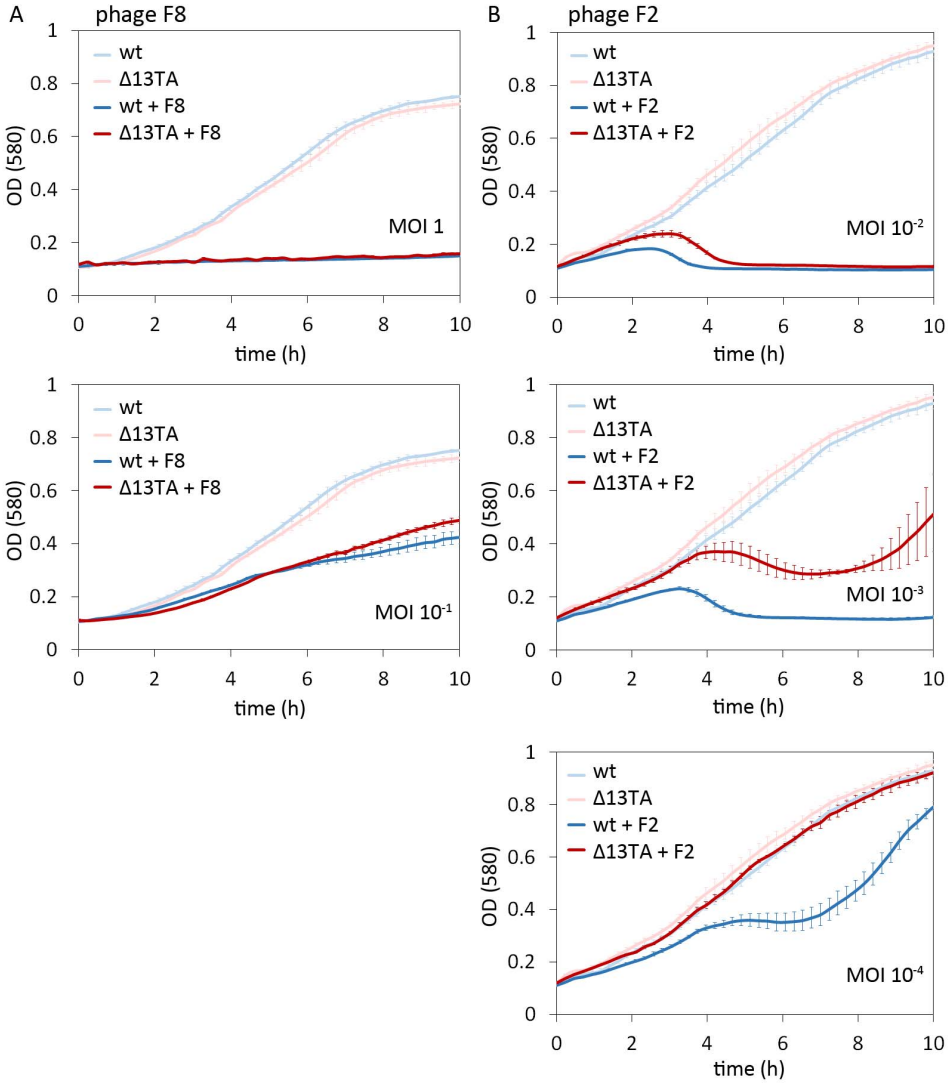

Figure 5. TA systems can be costly to *P. putida* during phage invasion. Growth curves of *P. putida* wild-type (wt) and Δ13TA strains without phages and after infection with phages F8 (A) and F2 (B) at varying MOI at 20 °C. Overnight bacterial cultures were diluted approximately 50-fold to fresh LB medium (phage F2) or LB medium supplemented with 0.03 µg/ml ciprofloxacin and 10 mM $CaCl_2$ (phage F8), and grown for 2–3 h on microtiter plates before infection with phages. Phages were added at t=0. Average of three technical replicates with standard deviation is presented.

The fact that we cannot detect that TA systems offer any kind of protection to *P. putida* against the phages we have isolated can mean that a specific TA system offers protection only to a specific phage or a small range of phages, and perhaps we have not managed to isolate the corresponding phages yet. It is also possible that since most *P. putida* TA systems are either nontoxic or mildly toxic (Ref III, Table 1), then perhaps these TA systems have lost their ability to protect *P. putida* from phages altogether. Another and perhaps more likely possibility is that TA systems are generally selfish elements, which is indicated by the cost they can have to *P. putida* (see chapter 5.4.2, Fig 5B), but since bacteria are in constant arms race with phages, they could evolve to use some TA systems to protect themselves from incoming threats.

5.4.4. Prophage P1 encoded TA system *hicAB-2* does not affect the stability of the prophage P1

In addition to the 13 TA systems that were removed from *P. putida* genome in the Δ13TA strain, *P. putida* has been predicted to have two additional TA systems – *hicAB-2* (PP_3899-3900) and PP_3032-3033 (Xie *et al.*, 2018). Both of these TA systems reside within prophage genomes, *hicAB-2* in prophage P1 and PP_3032-3033 in prophage P2 (Martinez-Garcia *et al.*, 2015). When we tried to delete the *hicAB-2* TA system from *P. putida* genome, we saw that it results in the loss of prophage P1 which is otherwise stably maintained in *P. putida* genome. Prophage loss was also observed when we attempted to delete the antitoxin *hicB-2* gene, however we were able to attain a mutant strain lacking the *hicA-2* toxin gene (see chapter 4.4.1). Therefore, we hypothesized that the *hicAB-2* locus encodes a functional TA system, and the *hicAB-2* system helps to stabilize prophage P1 in *P. putida* genome.

Since we were unable to delete the antitoxin gene without triggering prophage loss, we decided to test whether the *hicAB-2* locus constitutes a functional TA system with ectopic overexpression of the TA genes in *E. coli*. For that, the antitoxin *hicB-2* gene was cloned into the pBRlacItac plasmid under the IPTG-inducible tac promoter, and the toxin *hicA-2* gene was introduced into the pBAD33 plasmid under the arabinose-inducible pBAD promoter (see chapter 4.3). It is important to note that we could only clone the toxin gene into the pBAD33 plasmid when the *E. coli* cells already harbored the antitoxin-encoding pBRlacItac plasmid, indicating that the HicA-2 toxin is poisonous. The overexpression assay confirmed that the arabinose induced toxin production is indeed toxic to *E. coli*, as it inhibits the growth of the bacterium (Fig 6). Concurrent induction of the HicA-2 toxin and the HicB-2 antitoxin relieved the suppressing effect of the toxin on *E. coli* growth, while the induction of the antitoxin alone had no effect on *E. coli* (Fig 6). Thus, these results confirm that the P1 prophage-encoded *hicAB-2* is a *bona fide* TA system.

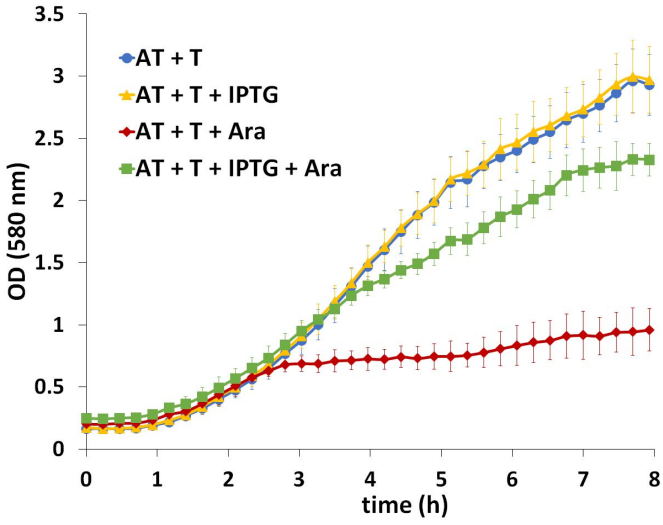

Figure 6. Toxin HicA-2 inhibits *E. coli* growth. Growth curves of *E. coli* DH5α cells harboring the pBRlacItac-hicB-2(AT) and pBAD33-hicA-2(T) plasmids for antitoxin HicB-2 and toxin HicA-2 overexpression, respectively. Antitoxin production was induced with 5 mM IPTG (+IPTG) and toxin production with 10 mM arabinose (+Ara). Average of six biological replicates with standard deviation is presented.

To determine whether HicAB-2 system helps to maintain prophage P1 in *P. putida* genome, we measured the excision frequency of P1 from *P. putida* genome (see chapter 4.5). For that we created qPCR primers that are flanking the P1 locus and the PCR product is detected only in the case of P1 excision. Since many prophage P1 genes have been shown to activate during DNA damage (Abella *et al.*, 2007), we examined the excision frequency of P1 in non-stressed bacteria and during ciprofloxacin induced stress. While we could not attain the *hicAB-2* deletion strain, we managed to disrupt the *hicB-2* antitoxin gene in *ΔhicA-2* background with streptomycin (Sm) resistance gene, creating a *hicAB-2* deficient strain called TA::Sm (see chapter 4.4.2). Thus, we measured the excision frequency of prophage P1 in the wild-type strain, toxin *hicA-2* deletion strain (ΔT) and *hicAB-2* deficient strain (TA::Sm).

We saw that the spontaneous excision frequency of prophage P1 from the wild-type strain is about 10^{-3} and DNA stress increases it by 100-fold (Fig 7A). To our surprise, we did not detect any significant change in either spontaneous or DNA damage-induced P1 excision frequency for the ΔT nor TA::Sm strains (Fig 7A). This indicates that *hicAB-2* is not important in prophage P1 stabilization in *P. putida* chromosome.

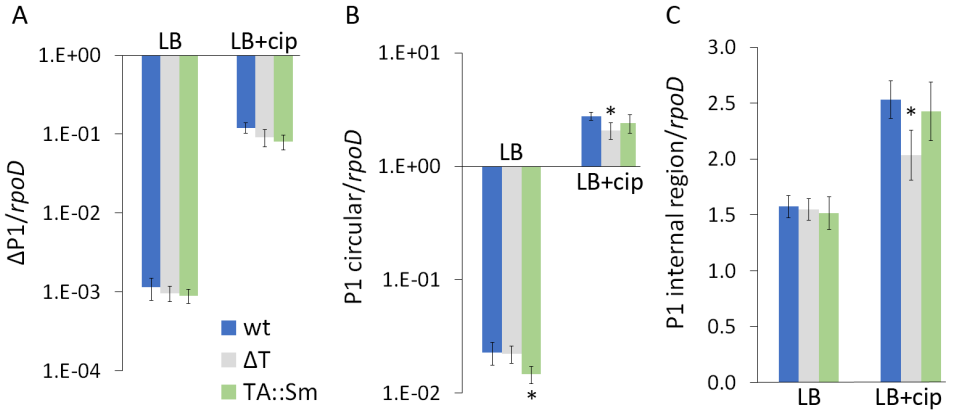

Figure 7. Prophage P1-encoded *hicAB-2* is not important for either spontaneous or DNA damage-induced excision of prophage P1. The excision frequency of P1 (A), relative abundance of the extrachromosomal P1 circle (B) and the P1 genome (C) in *P. putida* wild-type (wt), Δ*hicA-2* (ΔT) and *hicAB-2::Sm* (TA::Sm) strains was determined with qPCR. *rpoD* was used as reference gene. Bacteria were grown in LB or LB supplemented with 0.03 µg/ml ciprofloxacin (cip) for ~18 hours, after which genomic DNA was extracted for qPCR assay. The results are an average of at least 5 biological replicates with 95% confidence interval is presented. Statistically significant changes from wild-type are indicated (*P < 0.01, Student's T-test).

We also wanted to determine whether the excised P1 can form an extrachromosomal circle or is lost from the cells. For this, we created qPCR primers that cover the predicted junction of the extrachromosomal circle, as well as primers for a DNA region within the prophage P1 to measure the copy number of P1 in the cells (see chapter 4.5). The qPCR analysis revealed that P1 can exist as an extrachromosomal circle in *P. putida* cells after excision, and DNA stress increases the amount of circular P1 in the cells by two orders of magnitude (Fig 7B). We also noticed that the copy number of P1 is higher than that of the reference gene *rpoD*, suggesting that some cells can harbor at the same time P1 as a chromosomal and extrachromosomal copy (Fig 7C). Interestingly, it seems that during DNA stress in the absence of the *hicA-2* toxin gene, some of the circular P1 is lost from the cells. This is indicated by the approximately 1.3-fold decrease in the amounts of circular P1 and copy number of P1 in the cells (Fig 7B,C). However, we cannot detect significant changes between the wild-type and the TA::Sm strains in the amount of circular P1 or P1 copy number during ciprofloxacin induced stress (Fig 7B,C). Thus, since the decrease in the number of extrachromosomal P1 is rather small in the toxin deficient strain and the amount of extrachromosomal P1 is similar in the wild-type and TA::Sm strains, it suggests that the *hicAB-2* system does not have a role in the stabilization of the extrachromosomal P1 in *P. putida* cells.

Therefore, we conclude that *hicAB-2* is not important for P1 maintenance in *P. putida* cells. However, we have yet to test whether PP_3032-3033 has any role in prophage P2 stabilization in *P. putida* genome.

5.4.5. TA systems propagate horizontally and lose their toxicity over time (Ref III)

Since we cannot detect that TA systems are beneficial to *P. putida* (see chapters 5.4.1, 5.4.2 and 5.4.3) and the fitness costs inflicted by TA systems are rather minor (see chapters 5.4.2 and 5.4.3), we started to wonder whether the toxins of the 13 TA systems are toxic to *P. putida*. We decided to try to create antitoxin deletion strains for all the 13 TA systems to study the toxins' effects in natural genomic context, rather than using the kill/rescue assay that involves artificial toxin overexpression from a high copy-number plasmid. Despite multiple attempts, we were unable to delete the antitoxin of the *higBA*, *relE*$_2$*-higA2*, PP_4151-4152, *yefM-yoeB* and *relB*$_2$*-parE* systems, indicating that these five TA systems encode highly poisonous toxins (Ref III, Table 1). Interestingly, we were able to successfully create the antitoxin deletion strains for the other eight TA systems. A previous study had shown (Tamman *et al.*, 2014) and our experiments confirmed that *graTA* locus encodes a mild toxin that inhibits *P. putida* growth in the absence of the antitoxin GraA slightly at 30 °C and more severely at 25 °C (Ref III, Fig 5A,B). Additionally, we found two more TA systems, *res-xre* and *mqsRA*, to encode mild toxins, as both Res and MqsR mildly inhibited *P. putida* growth in the absence of their cognate antitoxins (Ref III, Fig 5A,B). Similarly to GraT, Res toxicity is more severe at lower temperature, while MqsR activity does not seem to be affected by temperature (Ref III, Fig 5A,B). While we know that GraT activity is dependent on the chaperone protein DnaK (Ref II) and leads to ribosome biogenesis defect that is more severe at lower temperatures (Ainelo *et al.*, 2016), we do not yet know whether the cold-sensitive effects of Res also indicate inhibition of ribosome maturation or suggest that Res expression or properties are temperature-sensitive.

The rest of the five TA systems seem to encode non-toxic proteins, as their toxins do not affect the growth of *P. putida* in LB at either 30 °C or 25 °C when the cognate antitoxin is deleted (Ref III, Fig 5A,B). However, toxins BrnT of *brnTA* system and RelE of *relBE* system can affect the sensitivity of *P. putida* to some chemicals (Ref III, Fig 5C), indicating that they have retained some activity. In contrast, the deletion of the HicAB-1, MazEF and PP_1716-1717 systems' antitoxin genes results in no growth defects in any tested conditions (Ref III, Fig 5), indicating that the toxins of these TA systems are not active.

Thus, we can see that the toxins of *P. putida* TA systems have various levels of toxicity (Ref III, Table 1). This implies that the TA loci evolve to be less toxic in time. To see whether we can also detect disrupted TA loci in other *Pseudomonads*, we searched for putative homologs of 13 *P. putida* PaW85 TA systems among 334 fully sequenced *Pseudomonas* strains. Firstly, our data suggests that one of the putative 13 TA systems, PP_1716-1717, is not actually a TA system. Since TA systems are thought to propagate horizontally and often even closely related bacterial strains do not encode the same TA systems (Pandey & Gerdes, 2005, Leplae *et al.*, 2011, Ramisetty & Santhosh, 2016), the operon PP_1716-1717 was not fitting the profile of a TA system. PP_1716-1717 locus is present

in almost all the 334 *Pseudomonas* strains analyzed and the homologous loci are in the exact same genomic context in all *Pseudomonads* (Ref III, Fig S2), indicating that the PP_1716-1717 locus is rather part of the core genome of *Pseudomonads*. Since we also could not detect any toxic effect of the putative toxin PP_1717 in the absence of the putative antitoxin PP_1716 (Ref III, Fig 5), it further confirms our suspicions that PP_1716-1717 is not a TA system after all. Thus, PP_1716-1717 is not involved in the subsequent discussion.

The bioinformatic analysis revealed that the number of homologous TA systems in *Pseudomonads* varies greatly for different TA systems. For example, antitoxins GraA of *graTA* and PP_4151 of PP_4151-4152 were found to have the most homologs in *Pseudomonads*, 180 and 72, respectively, while RelB of *relBE* and $HigA_2$ of $relE_2$-$higA_2$ had the least – less than 10 each (Ref III, Table 1, Fig S2). The sporadic occurrence of TA systems suggests that they have been acquired by different *Pseudomonas* strains independently, most likely through horizontal gene transfer. This is also supported by the finding that most TA homologs that are in the same genomic location as *P. putida* PaW85 belong to *P. putida* clade (Ref III, Fig S2). An exception to this is the $relB_2$-*parE* system, which is in the same genomic context in distantly related *Pseudomonas* strains (Ref III, Fig S2). However, since *the* $relB_2$-*parE* system is just upstream from an integrase gene and the other genes around the TA system are completely identical in various *Pseudomonas*, it seems that the DNA region containing $relB_2$-*parE* system is mobile and gained horizontally. We also found other TA systems' homologs to be in close proximity to integrase or transposase genes. Furthermore, finding several copies of a TA homolog in one strain can also suggest horizontal transfer (Ramisetty & Santhosh, 2016). For example, *P. koreensis* P19E3 has four *xre* homologs, two of which are located in the chromosome and the others on two different plasmids (Ref III, Fig S2). Interestingly, one of the chromosomal *xre* homologs is identical to the *xre* homolog in plasmid 3, implying gene transfer between replicons. Thus, the phylogenetic analysis of *P. putida* TA systems supports the concept that TA systems propagate horizontally.

Additionally, we saw that most *P. putida* antitoxin homologs have a toxin gene homolog next to them. However, we also found that several homologous TA loci had either a truncated toxin gene or no toxin gene at all next to antitoxin gene (Ref III, Table 1). For example, 52% and 27% of the toxin genes were disrupted among the homologous *res-xre* and *higBA* systems, respectively (Ref III, Table 1). In coherence with that, studies in *E. coli* have shown that defective TA systems' homologs can be found for many TA loci (Mine *et al.*, 2009, Ramisetty & Santhosh, 2016). But there are probably even more defective toxins among the *P. putida* PaW85 homologous TA systems than we found. A previous study showed that the major reason for toxin inactivation is mutations in their promoter region (Fernandez-Garcia *et al.*, 2019) and as we cannot detect the effects of point mutations on the toxins' expression or activity with this bioinformatic analysis, we can only assume that the number of defective TA systems' homologs is higher. All this indicates that TA systems undergo degradation during evolution, and suggests their lack of function to bacteria.

It is reasonable to presume that TA systems are selfish elements. We cannot see that chromosomal TA systems are in any way beneficial to *P. putida* (see chapters 5.4.1, 5.4.2 and 5.4.3). On the contrary, we can observe that TA systems can be costly to *P. putida* (see chapters 5.4.2 and 5.4.3). Yet, the burden of having TA systems seems to be rather small, as we can detect the decrease in competitiveness of the wild-type strain mostly only in ideal growth conditions (Ref III, Fig 3), and the wild-type strain is more susceptible only to some phages than the Δ13TA strain (Fig 5). The fact that bacteria encode multiple highly poisonous TA systems (*P. putida* has five) is another indication that they do not entail an immense burden, otherwise we would presume that the noxious toxins would be rendered harmless rather quickly by mutations. Since the antitoxin has a tight control over the toxin (by directly binding to it and controlling the TA operon expression), the selection against TA loci is probably relatively weak.

However, even though TA systems fit the criteria of being selfish elements, we cannot overlook the fact that more and more TA systems have been connected to protection against bacteriophages (Hazan & Engelberg-Kulka, 2004, Fineran *et al.*, 2009, Koga *et al.*, 2011, Cui *et al.*, 2022, LeRoux *et al.*, 2022, Zhang *et al.*, 2022). It is possible that some *P. putida* TA loci are also anti-phage systems, however thus far we have not been able to observe that. TA systems could be part of the bacterial pan-immunity (Bernheim & Sorek, 2020). According to the pan-immune system model, the anti-phage elements encoded by the different bacterial strains living in one population make up the immunity reservoir, which is accessible to the population members via horizontal gene transfer (Bernheim & Sorek, 2020). This is important, because one bacterium cannot encode all the phage defense elements due to the high fitness cost they entail. The fitness cost is the reason why defense systems do not accumulate and can frequently get lost from bacterial genomes even though they can give selective advantage during phage attacks (Bernheim & Sorek, 2020). It is possible that *P. putida* has acquired so many TA systems because they could protect the bacterium during phage attacks. However, since TA systems do come with fitness costs to *P. putida* (see chapters 5.4.2 and 5.4.3), they get selected against over time, and this can explain why we see TA systems with various toxicity levels in *P. putida* genome (Ref III, Table 1). If TA systems are part of the pan-immunity reservoir, then perhaps *P. putida* TA systems can protect other bacteria from phages when acquired by them via horizontal gene transfer.

6. CONCLUSIONS

TA systems can be found in most bacterial genomes (Makarova *et al.*, 2009, Leplae *et al.*, 2011). Despite extensive studies over the years, the biological significance of chromosomal TA systems to bacteria has remained unclear. The first and most thoroughly studied *P. putida* TA system GraTA (Tamman *et al.*, 2014) is in many ways a remarkable TA system. The purpose of this thesis was to further expand our knowledge about how GraTA system affects *P. putida* and to find out its importance to *P. putida* along with other TA systems.

The main results of this work can be summarized as follows:

- *P. putida* responds to the GraT-inflicted harm by
  - upregulation of the ribosome biogenesis factors, which probably alleviates the ribosome maturation defect,
  - altering the central carbon metabolism, which could help the bacterium to adapt with toxin-caused growth defect.
- The chaperone protein DnaK and particularly its C-terminus is important in enhancing GraT toxicity, likely by assisting GraT to attain its proper toxic fold.
- The conserved motif of DnaK C-terminus enhances DnaK chaperone activity and is needed for the competitive fitness of *P. putida*.
- TA systems are not beneficial to *P. putida*, as they do not affect *P. putida* growth, stress tolerance, persistence, biofilm formation, or offer protection from bacteriophages tested so far. Instead, TA systems can be costly to *P. putida*, as they can decrease the competitiveness of *P. putida* and its resistance to phage attacks.
- Prophage P1 encoded TA system *hicAB-2* is not important for P1 maintenance in *P. putida* cells.
- TA systems propagate horizontally and lose their toxicity over time.

These results show that despite the general belief that TA systems are beneficial to bacteria, this work did not detect that encoding TA systems comes with any advantages to *P. putida*. On the contrary, it was observed that having TA systems can entail a cost on *P. putida* fitness. Yet, the cost is small and this can explain the abundance of TA systems in bacterial genomes. In addition, less than half of the predicted *P. putida* TA systems have retained their lethality, indicating their degeneration over time and lack of function. Since TA systems propagate horizontally and they can be costly to *P. putida*, it is tempting to consider TA systems as selfish elements that are not meant to benefit their host. However, even though this work showed that TA systems do not protect *P. putida* from phages, it cannot be ruled out as the phage collection used in this study needs still expanding and

perhaps a corresponding phage is yet to be isolated. It is also possible that *P. putida* TA systems are part of the bacterial pan-immune system and contribute to the population's phage resistance potential. Or perhaps TA systems are generally selfish elements that can occasionally evolve into phage immunity elements beneficial for the host bacterium or larger microbial consortium.

SUMMARY IN ESTONIAN

Kromosomaalsete toksiin-antitoksiin süsteemide mõju *Pseudomonas putida* kohasusele

Bakterites leiduvad toksiin-antitoksiin (TA) süsteemid on geneetilised elemendid, mis koosnevad enamasti kahest osapoolest – kahjulikust toksiinist, mis võib häirida raku elutegevust ja antitoksiinist, mis toksiini tõhusalt neutraliseerib. TA süsteemid avastati umbes 40 aastat tagasi plasmiididest (Ogura & Hiraga, 1983), kus nende funktsiooniks on plasmiidi stabiliseerimine rakkudes (Gerdes *et al.*, 1986a). Suuremahuline bakterigenoomide sekveneerimine paljastas, et TA süsteemid on laialt levinud ka bakterite kromosoomides ning ühel bakteril võib olla genoomis kümneid TA süsteeme (Pandey & Gerdes, 2005, Makarova *et al.*, 2009, Leplae *et al.*, 2011). On äärmiselt intrigeeriv, miks nii paljudel bakteritel on sellised geneetilised elemendid, mis võivad neid kahjustada. Potentsiaalselt toksiliste TA süsteemide omamine oleks põhjendatav vaid siis, kui nad oleksid oma peremehele kasulikud. Pikalt on arvatud, et kromosomaalsed TA süsteemid osalevad bakterite stressivastuses ning et just stress indutseerib toksiini vabanemise antitoksiini kontrolli alt (Gerdes *et al.*, 2005). Kuna on näidatud, et toksiin pidurdab bakterite kasvu (Pedersen *et al.*, 2002, Budde *et al.*, 2007), siis võib oletada, et toksiini aktiveerumine ja seeläbi bakteri kasvukiiruse mõjutamine võiks bakteritel aidata stressiga toime tulla. Lisaks sellele stabiliseerivad mõned TA süsteemid genoomset mobiilset DNA-d (Wozniak & Waldor, 2009, Yao *et al.*, 2015) ning mõned võivad kaitsta baktereid bakteriofaagide rünnaku korral (Fineran *et al.*, 2009, LeRoux *et al.*, 2022). Samas leidub aga ka mitmeid viiteid, et TA süsteemid pole bakterile olulised ja on seega vaid isekad DNA elemendid (Tsilibaris *et al.*, 2007, Goormaghtigh *et al.*, 2018). Kuna erinevate uurimisgruppide tulemused on vastuolulised, siis on kromosomaalsete TA süsteemide tähtsus bakteritele jäänud ebaselgeks ning vajab täpsemat uurimist.

Ennustuste kohaselt on mullabakteril *Pseudomonas putida* 15 kromosomaalset toksiin-antitoksiin süsteemi (Xie *et al.*, 2018), kuid ainult nelja neist, GraTA (Tamman *et al.*, 2014), MqsRA (Sun *et al.*, 2017), Xre-RES (Skjerning *et al.*, 2019) ja MazEF (Miyamoto *et al.*, 2016), on varasemalt uuritud. Kõige põhjalikumalt on neist uuritud GraTA süsteemi, mis eristub teistest TA süsteemidest nii oma ebatavaliselt stabiilse ning täielikult struktureeritud antitoksiini GraA poolest (Tamman *et al.*, 2016, Talavera *et al.*, 2019) kui ka vaid mõõdukalt toksilise toksiini GraT tõttu (Tamman *et al.*, 2014), mille N-terminaalne osa on struktureerimata ning on ühtlasi äärmiselt oluline GraT toksilisuses (Talavera *et al.*, 2019). Nagu ka paljud teised toksiinid, on GraT ribosoom-sõltuv mRNaas (Talavera *et al.*, 2019), kuid erinevalt teistest põhjustab GraT temperatuurist sõltuvat kasvu- ja ribosoomi biogeneesi defekti (Tamman *et al.*, 2014, Ainelo *et al.*, 2016). Samuti on väga ebatavaline, et GraT toksilisust mõjutab šaperonvalk DnaK (Ainelo *et al.*, 2016), kuigi selle interaktsiooni mehhanism on ebaselge. Seega olid käes-

oleva töö esimesteks eesmärkideks välja selgitada DnaK roll GraT poolt põhjustatud kasvu- ja ribosoomi biogeneesi defektis ning kirjeldada, millised muutused leiavad aset *P. putida* rakkudes GraA antitoksiini puudumisel.

Antitoksiini GraA puudumisel võib toksiin GraT, sõltuvalt kemikaalist, nii suurendada kui vähendada *P. putida* stressitaluvust, kuid kogu *graTA* süsteemi deletsioon *P. putida* stressitaluvust ei mõjuta (Tamman *et al.*, 2014). See tõstatab küsimuse, et milline on *graTA* lookuse ning ühtlasi ka kõigi teiste TA süsteemide tähtsus *P. putida*'le. Seega oli antud töö eesmärgiks ka välja selgitada kromosomaalsete TA süsteemide olulisus *P. putida*'le.

Käesoleva töö peamised tulemused on järgmised:

- Proteoomi andmed viitavad, et vastusena GraT põhjustatud stressile suureneb *P. putida*'s ribosoomi biogeneesi valkude ekspressioonitase, mis ilmselt leevendab ribosoomi biogeneesi defekti. Samuti toimub GraT-mõjutatud bakteris tsentraalse süsinikumetabolismi ümber korraldamine, mis võib aidata *P. putida*'l kohaneda GraT-põhjustatud kasvudefektiga.
- GraT toksilisust suurendab šaperonvalk DnaK ja eriti oluline selles on DnaK mittestruktureeritud C-terminaalne osa. Tõenäoliselt abistab DnaK GraT'd voltumises.
- Konserveerunud motiiv DnaK C-terminaalses osas võimendab DnaK šaperoni aktiivsust ja on eriti oluline *P. putida*'le konkurentsitingimustes.
- TA süsteemid ei ole *P. putida*'le kasulikud, sest nende puudumine ei mõjuta *P. putida* kasvu, stressitolerantsust, persistorrakkude tekkimist, biofilmi moodustumist ega kaitse *P. putida*'t faagirünnaku korral. TA süsteemid võivad *P. putida*'le hoopiski kulukad olla, sest nad võivad vähendada *P. putida* konkurentsivõimet ja vastupidavust faagirünnakutele.
- Profaagis P1 leiduv *hicAB-2* TA süsteem ei mõjuta profaagi P1 stabiilsust *P. putida* rakkudes.
- TA süsteemid levivad horisontaalselt ning võivad kaotada aja jooksul järkjärgult oma toksilisuse.

Kuigi TA süsteeme peetakse bakteritele kasulikeks, siis minu tulemused näitavad, et kromosomaalsed TA süsteemid ei ole *P. putida*'le mingitki moodi kasulikud. Vastupidi, tulemused viitavad, et TA süsteemid võivad olla *P. putida*'le koormaks. Siiski on TA süsteemide kulukus väike ning see võib ühtlasi seletada, miks TA lookusi bakterite genoomides nii arvukalt leidub. Lisaks tuvastasin, et vähem kui pooled *P. putida* TA süsteemid kodeerivad letaalset toksiini, mis viitab sellele, et TA süsteemid degenereeruvad aja jooksul. Kuna TA süsteemid levivad horisontaalselt ning käesolev töö näitab, et nad võivad *P. putida*'le kulukaks osutuda, siis võib TA süsteeme pidada isekateks DNA elementideks. Sellegipoolest ei saa välistada võimalust, et TA süsteemid võivad bakterit kaitsta faagide

eest, kuigi antud töö ei näidanud TA süsteemide olulisust *P. putida* faagiresistentsuse suurendamisel. Selles töös kasutatud faagikollektsioon on aga veel väike ja vajab täiendamist ning on võimalik, et faag mille eest TA süsteemid *P. putida*'t kaitseksid, ei ole veel eraldatud. Samuti on võimalik, et *P. putida* TA süsteemid on osa bakterite pan-immuunsuse süsteemist ning panustavad populatsiooni faagiresistentsuse potentsiaali. Aga on ka võimalik, et TA süsteemid on üldiselt isekad DNA elemendid, kuid võivad evolutsioonilises võidurelvastuses leida mingil hetkel rakendust bakteri faagivastases kaitses.

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