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LIST OF PUBLICATIONS

The current dissertation is based on the following original publications, which will be referred to by the corresponding Roman numerals in the text:

I. **Kasari V**, Kurg K, Margus T, Tenson T, Kaldalu N: The *Escherichia coli mqsR* and *ygiT* genes encode a new toxin-antitoxin pair. *Journal of Bacteriology* 2010, 192(11):2908–2919.

II. Kaldalu N, **Kasari V**, Atkinson G, Tenson T: Type II Toxin-Antitoxin Loci: The Unusual *mqsRA* Locus. In: *Prokaryotic Toxin-Antitoxins*. Edited by Gerdes K: Springer Berlin Heidelberg; 2013: 93–105.

III. **Kasari V**, Mets T, Tenson T, Kaldalu, N: Transcriptional cross-activation between toxin-antitoxin systems of *Escherichia coli*. *BMC Microbiology* 2013, 13:45.

Author's contribution

I. Performed all transcription and biofilm assays, participated in writing the manuscript
II. Participated in writing the chapter of the monograph
III. Performed all of the experiments, except growth resumption assay and western blot. Participated in writing the manuscript.

LIST OF ABBREVIATIONS

Abi – phage abortive infection
E. coli – *Escherichia coli*
EDF – extracellular death factor
IPTG – isopropylthio-β-D-galactoside
ORF – open reading frame
PCD – programmed cell death
PSK – post-segregational killing
RBS – ribosome binding site
ROS – reactive oxygen species
TA – toxin-antitoxin
TADB – toxin-antitoxin database

INTRODUCTION

Free-living bacteria are often exposed to variable environmental conditions that require appropriate adaptation strategies to survive in. Besides planktonic lifestyle, bacteria often attach to surfaces and form a biofilm, which provides better communication and protection against harmful factors such as antibiotics and host immune response. It has been proposed that most chronic infections are caused by bacteria growing in biofilms. This slime-enclosed bacterial community is enriched with specialized cells that have periods of low metabolic activity without genetic change and, thus, have both good tolerance against bactericidal antibiotics and ability to repopulate after treatment cycles. Researchers have long looked for a genetic basis for this type of dormancy. Gene expression profiles of these so-called persister-like cells are distinct from normally growing bacteria: among others, the genes encoding toxin-antitoxin systems are highly upregulated. A toxin-antitoxin (TA) system codes for an autotoxic protein that attacks an essential cellular process when its antidote is not available. TA systems are thus able to temporarily halt cell growth. Therefore, TA pairs have long been considered good candidates to play a key role in developing persister phenotypes and their role within bacterial cell physiology has been actively studied for over two decades. However, due to the redundancy of chromosomal TA loci, their function is often difficult to test and validate.

Elucidating the mechanism and physiological roles that TA systems play in persister cells may contribute to the development of a method to treat recalcitrant chronic infections. This dissertation has two main objectives. The first is to test whether the most highly upregulated genes in persister-like cells constitute a new TA system, and the second is to examine the cross-regulation between chromosomally encoded TA systems in *Escherichia coli*.

I. REVIEW OF LITERATURE

I.1. Discovery of toxin-antitoxin systems

The first toxin-antitoxin (TA) systems were discovered in plasmids (Ogura & Hiraga, 1983, Gerdes*, et al.*, 1985) and were shown to stabilize low copy plasmids during the segregation of daughter cells. The molecular mechanism for the plasmid maintenance system, termed post-segregational killing (PSK) (Gerdes*, et al.*, 1986), was solved nearly ten years later (Van Melderen*, et al.*, 1994). The daughter cells in a growing bacterial population that have not inherited a TA system containing plasmid are selectively killed. Because toxin is normally inactive and tightly bound to its antitoxin protein, the killing relies on the differential stability of toxin and antitoxin. Toxins are more stable to proteolysis than antitoxins. Without a copy of the PSK^+ plasmid, the unstable antitoxin is not produced and the more stable toxin becomes free from the T-A complex and poisons the cell (Fig. 1).

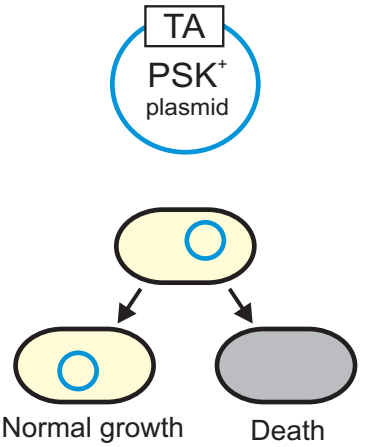

Figure 1. Toxin-antitoxin (TA) systems increase plasmid maintenance in growing bacterial population by post-segregational killing (PSK). Adapted from (Van Melderen & Saavedra De Bast, 2009).

Sequencing of prokaryotic genomes (Eubacteria and Archaea) led to the discovery of chromosomal TA loci (Masuda*, et al.*, 1993, Gotfredsen & Gerdes, 1998, Pandey & Gerdes, 2005). Protein sequences of toxins and antitoxins belonging to seven known TA families were used in a BLAST search (Pandey & Gerdes, 2005). The large-scale search on 126 sequenced prokaryotic genomes revealed a high abundance of TA loci in free-living but not in obligate host-associated prokaryotes with reduced genome size. Similar results were reported two years later, in which more than 500 annotated genomes were subjected to a comprehensive TA search tool named RASTA-Bacteria (Sevin & Barloy-Hubler, 2007). Some slow-growing bacteria, i.e. *Mycobacterium*

tuberculosis and *Nitrosomonas europaea*, contain more than 50 TA loci, while a maximum of 167 were found in *Microcystis aeruginosa* NIES-843. The vast majority of genomes contain less than 30 TA loci. In high TA numbered genomes, each TA family is often represented with multiple TA systems with the TA content often changing between isolates of the same species. These results support the belief that TA loci are frequently acquired by lateral gene transfer. Recent comprehensive comparative genomic analyses of TA systems provide a list of new putative TA pairs, including also new TA families (Makarova*, et al.*, 2009, Fozo*, et al.*, 2010, Leplae*, et al.*, 2011). The popular model organism *Escherichia coli* K12, wherein new TA families and types have been mostly characterized, has been found to host nearly 40 TA loci, including 12 validated Type II systems (see next paragraph for TA classification) (Yamaguchi*, et al.*, 2011) (Table 1).

Table 1. Type II toxin-antitoxin systems identified in the chromosome of *Escherichia coli* K12 strain.

TA system	Toxin	Antitoxin	TA family	Activity of toxin	Proteases degrading antitoxins	Reference to activity of toxin
chpB	ChpBK	ChpBI	*mazEF*	mRNA cleavage	Unknown	(Zhang*, et al.*, 2005)
dinJ-yafQ	YafQ	DinJ	*relBE*	mRNA cleavage[r]	Lon, ClpXP	(Prysak*, et al.*, 2009)
higBA	HigB	HigA	*relBE*	mRNA cleavage[r]	Lon	(Christensen-Dalsgaard*, et al.*, 2010)
hicBA	HicA	HicB	*hicBA*	mRNA, tmRNA cleavage	Lon	(Jorgensen*, et al.*, 2009)
hipBA	HipA	HipB	*hipBA*	phosphorylates EFTu	Lon	(Schumacher*, et al.*, 2009)
mazEF (chpA)	MazF	MazE	*mazEF*	mRNA,rRNA cleavage	Lon, ClpAP	(Zhang*, et al.*, 2003)
mqsRA	MqsR	MqsA	*relBE*	mRNA cleavage	Lon, ClpXP	(Yamaguchi*, et al.*, 2009)
prlF-yhaV	YhaV	PrlF	*relBE*	mRNA cleavage[r]	Unknown	Schmidt*, et al.* (2007)
relBE	RelE	RelB	*relBE*	mRNA cleavage[r]	Lon	(Pedersen*, et al.*, 2003)
rnlAB	RnlA	RnlB	*rnlAB*	mRNA cleavage	Lon, ClpXP	(Koga*, et al.*, 2011)
yafNO	YafO	YafN	*relBE*	mRNA cleavage[r]	Lon	(Christensen-Dalsgaard*, et al.*, 2010)
yefM-yoeB	YoeB	YefM	*relBE*	mRNA cleavage[r]	Lon	(Christensen*, et al.*, 2004)

[r] ribosome-dependent mRNA cleavage

1.2. Classification of bacterial TA systems

Toxin-antitoxin systems are classified into five types based on the nature and functionality of the antitoxins. Types I and II are well characterized, whereas types III–V have only recently been described. While antitoxins can be either RNAs or proteins, the toxins are universally proteins in all types and seem to be more constrained in their mode of action.

Type I. Antitoxin is an antisense RNA that controls translation of the toxin mRNA by RNA interference (Fig. 2). The toxin-antitoxin RNA duplex is degraded by an RNase, most likely by RNase III. Transcription of the toxin mRNA is controlled either by weak constitutive promoter (i.e. *hok* toxins) or by SOS responsible LexA repressor (i.e. *symE, tisB* toxins). Antitoxin is normally synthesized constitutively at a high rate, however, it is much less stable compared to toxin. Toxins are mostly small hydrophobic proteins with an integral membrane domain. Hence, Type I toxins localize in the inner membrane resulting in membrane depolarization and permeabilization (Gerdes & Wagner, 2007, Fozo*, et al.*, 2010).

Type II. Toxin and antitoxin are both proteins and encoded by genes of the same operon. Antitoxin inhibits toxin by direct protein-protein interaction forming a tight complex (Fig. 2). Antitoxin has a DNA binding motif, through which it autorepresses transcription of its operon. The toxin-antitoxin complex also binds to the promoter region and usually even more avidly than the antitoxin alone, causing strong transcriptional repression (see paragraph 1.5). Type II toxins most frequently destabilize mRNA by endoribonucleolytic cleavage, but some can also attack other targets (see paragraph 1.4).

Type III. Antitoxin is a small pseudoknot RNA that counteracts the proteic toxin by direct RNA–protein interaction (Fig. 2). Pseudoknot is an RNA structure that is composed of at least two helical segments connected by single-stranded regions or loops (Staple & Butcher, 2005). Toxin and antitoxin of Type III are transcribed from a single promoter, however, the relative levels of toxin and antitoxin synthesis are regulated by a transcriptional terminator between the genes (Fineran*, et al.*, 2009). Antitoxins are synthesized from short tandem repeats upstream of the toxin gene. Type III ToxN toxin is a sequence-specific RNAse (structural homolog of Type II MazF/Kid toxin family) that cleaves also its own antitoxin precursor mRNA to generate mature 36 nucleotide long pseudoknot (Blower*, et al.*, 2011, Short*, et al.*, 2013). The first Type III system was discovered as a phage protection system encoded by a plasmid within the plant pathogen *Erwinia carotovora* (Fineran*, et al.*, 2009). ToxIN TA system functions as an abortive infection (Abi) system which is based on “altruistic” suicide of infected bacterium (Chopin*, et al.*, 2005).

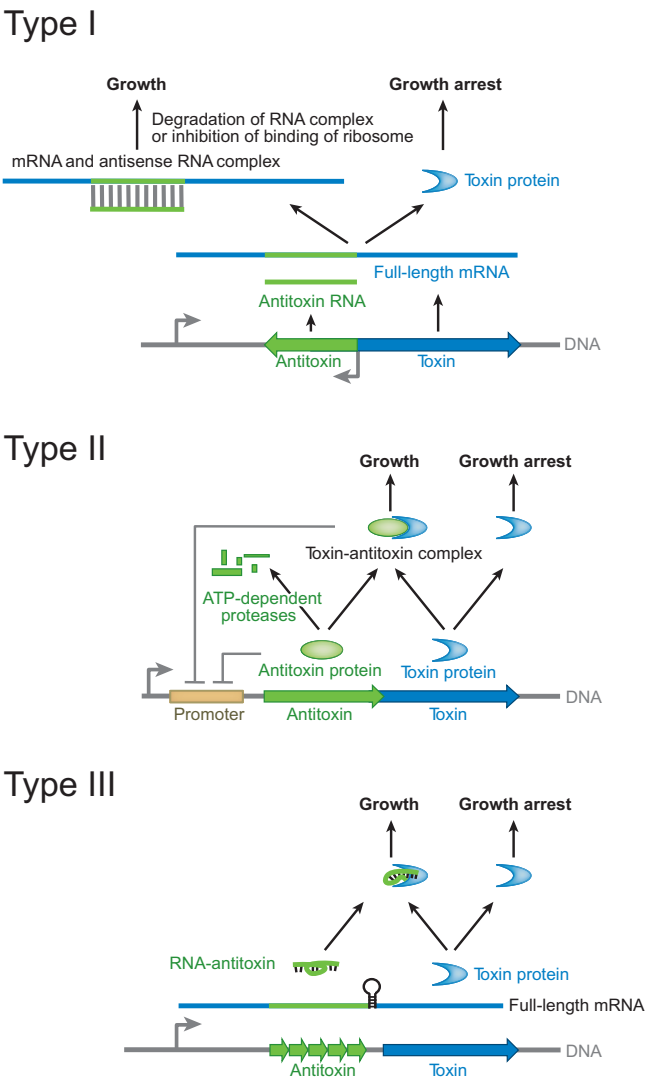

Figure 2. Regulation of TA systems: Type I–III. Adapted from (Yamaguchi*, et al.*, 2011).

Type IV. Antitoxin is a protein and protects the target of the toxin from getting poisoned (Masuda*, et al.*, 2012). It is a unique example of a putative TA system, in which YeeV (CbtA) toxin blocks polymerization of cytoskeletal proteins MreB and FtsZ, thereby inhibiting cell division in *Escherichia coli* (Tan, *et al*., 2011). The YeeU (CbeA) antitoxin presumably promotes bundling of linear cytoskeleton polymers by interacting directly with MreB and FtsZ. The molecular mechanism, of how exactly YeeU antagonizes the activity of YeeV toxin, is not known.

Type V. Proteic antitoxin neutralizes its toxin by cleaving its mRNA. In this unique TA system, the GhoT (previously YjdO in *E. coli* K12) toxin is a lytic membrane peptide. The formation of ghost cells is counteracted by GhoS (YjdK in *E. coli* K12) antitoxin through sequence-specific cleavage of *ghoT* mRNA. *ghoS* and *ghoT* are co-transcribed similarly to Type II systems. However, unlike Type II proteic antitoxins, GhoS does not regulate its promoter and is not labile (Wang*, et al.*, 2012b).

1.3. Main characteristics of Type II toxin-antitoxin systems

A polypeptide that is a part of a two-component protein complex may become toxic when being overexpressed or when its interaction partner is eliminated. However, this alone does not show that these two proteins constitute a TA system. Therefore, to define a new toxin-antitoxin system the candidates have to fulfill several criteria: (1) the system is formed by a pair of adjacent genes that encode a relatively stable autotoxic protein (toxin) and its short-lived inhibitor (antitoxin), (2) transcription of the TA operon is autorepressed by the antitoxin alone or by toxin-antitoxin complex, and (3) the main biological function of the toxin must be inhibition of cell growth.

Some Type II TA systems may have exceptional features. For instance, a well characterized Type II TA system, ω-ε-ζ, has a separate transcriptional regulator ω as a third component (Zielenkiewicz & Ceglowski, 2005). Analogous systems containing additional transcriptional repressor were recently found in the ParE family of toxins (Hallez*, et al.*, 2010). The third component can also function as a chaperon by preventing rapid degradation or aggregation of the antitoxin as was recently described for the *higBA* locus in *Mycobacterium tuberculosis* (Bordes*, et al.*, 2011).

1.4. Enzyme activities of Type II toxins

Most Type II TA toxins in *E. coli* K12 act as endoribonucleases, also termed mRNA interferases, that cleave mRNA (Table 1). Active mRNA interferases drastically increase the number of stalled ribosome-mRNA complexes which are rescued by tmRNA (Christensen & Gerdes, 2003, Christensen*, et al.*, 2003). Bacterial translation of an mRNA starts before the end of its transcription from DNA. If an mRNA is truncated and lacks a stop codon, the ribosome stalls at the 3' end. This happens because the release factors that should terminate translation only recognize stop codons (Brown & Tate, 1994). At this point, tmRNA enters into the trapped ribosome and mimics tRNA and a piece of mRNA that encodes a proteolytic tag (Keiler*, et al.*, 1996). tmRNA with an in frame stop codon helps to disassemble the stalled complex, and the faulty protein and mRNA are sent to degradation.

Some endoribonucleases are sequence specific cutters (ChpBK, MazF, MqsR, YafQ), others do not exhibit any sequence specificity (HicA, HigB, RelE, YafO, YoeB). Besides, some mRNA interferases (most of the RelE family toxins) are ribosome-dependent and cleave only mRNA that is bound to the ribosome. The well-studied RelE is mRNA interferase, which cleaves mRNA between the second and third nucleotide located in the ribosomal acceptor site A (Christensen & Gerdes, 2003, Pedersen, *et al.*, 2003). The well-known sequence specific MazF and MqsR nucleases are ribosome-independent interferases. MazF toxin cleaves single-stranded mRNA at ACA motifs (Zhang, *et al.*, 2003) and it also cleaves an ACA sequence near the 3' end of 16S rRNA. By cutting off the anti-Shine-Dalgarno sequence, MazF promotes formation of specialized ribosomes that can translate leaderless mRNAs (Vesper, *et al.*, 2011) and causes significant changes in the protein expression profile (Amitai, *et al.*, 2009). VapC, a toxin of the largest TA family, is also a sequence-specific RNA interferase. VapC protein contains a metal-binding PIN (PilT N-terminal) domain, thus, depending on bacterial species, it is a Mg^{2+}– and/or Mn^{2+}–dependent endoribonuclease (Clissold & Ponting, 2000, Arcus, *et al.*, 2004, Arcus, *et al.*, 2005, Bunker, *et al.*, 2008). The sequence specificity of VapC varies between species. For example, in the human pathogen *Mycobacterium tuberculosis*, VapC cuts at GC-rich 4-mers of mRNA, whereas, GG^(U/G)G is the optimal target in *Pyrobaculum aerophilum* (McKenzie, *et al.*, 2012, Sharp, *et al.*, 2012). Enteric VapC proteins were shown to specifically cleave initiator $tRNA^{fMet}$ between the anticodon stem and loop *in vitro* and *in vivo* (Winther & Gerdes, 2011), but they do not cut model mRNAs, ribosomal RNAs or other tRNAs (Winther & Gerdes, 2009).

Another observed target of Type II toxins is replication, in which the toxin poisons DNA gyrase (topoisomerase II) activity on DNA. DNA gyrase is responsible for ATP-dependent negative supercoiling of DNA, which is essential for the initiation as well as elongation of DNA replication and general transcription (Gellert, *et al.*, 1976, Kreuzer & Cozzarelli, 1979, Nollmann, *et al.*, 2007). To maintain the negative supercoiling of DNA, gyrase introduces temporal double-strand brakes into the DNA and religates them afterwards. The $CcdB_F$ toxin encoded by F-plasmid attacks gyrase by forming a trapped ternary complex of DNA-Gyrase-$CcdB_F$, in which gyrase cannot religate double-strand breaks within the DNA (Bernard, *et al.*, 1993). Subsequent dissociation of the complexes by an unknown mechanism results in accumulation of fragmented DNA, which is lethal for the cell. The quinolone class of antibacterial drugs function by a similar mechanism (Drlica, *et al.*, 2009).

HipA toxin of the *hipBA* TA system in *E. coli* is a protein kinase that phosphorylates elongation factor EF-Tu (Schumacher, *et al.*, 2009). EF-Tu mediates aminoacyl-tRNA binding to the ribosome. Normally, GTP hydrolysis triggers conformational change in EF-Tu, which then dissociates from the ribosome for recycling. Phosphorylated Thr^{382} of the EF-Tu blocks formation of GTP-bound closed form of EF-Tu, which cannot bind aminoacyl-tRNA

(Lippmann, *et al.*, 1993, Alexander, *et al.*, 1995). Hence, the translation process halts due to inactivated EF-Tu. However, these results were obtained from *in vitro* study and should be validated *in vivo*.

Zeta toxins of the *epsilon/zeta* TA systems attack bacterial cell wall synthesis. Zeta toxin is a kinase that phosphorylates UDP-*N*-acetylglucosamine (UNAG) using ATP as phosphate donor (Mutschler, *et al.*, 2011). UNAG is an essential precursor metobolite required for biosynthesis of pepidoglycan. The 3' hydroxyl group of *N*-acetyl-glycoseamine, that is the target of zeta toxin, is needed for pentapeptide linkage (Barreteau, *et al.*, 2008). Phosphorylation of this 3'OH blocks synthesis of peptidoglycan which ultimately leads to cell autolysis. In addition, accumulation of UNAG-3P in the cytosol competitively inhibits MurA, the enzyme of the first step in synthesis of peptidoglycan (Mutschler, *et al.*, 2011).

1.5. Regulation of Type II TA Transcription

In addition to the interaction with its toxin, an antitoxin has a DNA binding domain. Through this domain, the antitoxin dimer binds to a palindrome in its promoter region and acts as a transcriptional repressor. The binding affinity of the antitoxin dimer to DNA is strongly affected (enhanced or inhibited) by the toxin. This regulatory property, termed conditional cooperativity, is described in different TA families, including, for example, the PhD/Doc (Magnuson & Yarmolinsky, 1998), CcdAB (Madl, *et al.*, 2006), and RelBE systems (Overgaard, *et al.*, 2008). Stressful conditions activate proteases that degrade unstable antitoxins faster than toxins. Hence, the relative amount of cellular toxin and antitoxin varies between growth conditions. Depending on the ratio between the toxin and the antitoxin, the TA complexes with different stoichiometry can be formed. In the RelBE system of *E. coli*, two complexes form: when the concentration of RelB exceeds that of RelE, a $RelB_2$-$RelE_1$ complex is observed; when the amount of RelE is higher, a $RelB_2$-$RelE_2$ complex is observed. The first complex (2:1) binds strongly to palindromes in the promoter region and represses the trancription, whereas the second (2:2) cannot bind to DNA. RelB alone binds to the operator DNA only *in vitro* with weak affinity, therefore, the toxin is required for efficient autorepression (Overgaard, *et al.*, 2008). The molecular mechanism of conditional cooperativity in the PhD/Doc system was recently solved (Garcia-Pino, *et al.*, 2010) (Fig. 3). The Doc toxin molecule has actually two binding sites for the PhD antitoxin: high (H) and low (L) affinity sites. The H site is conventional and the L is the key interaction site allowing Doc to bridge together two PhD dimers bound to two successive palindromes. The L site binding only appears in a complex wherein at least one of the H affinity sites is unoccupied. However, if the amount of Doc toxin exceeds that of PhD, the rigid Doc-PhD_2-Doc complex, involving only H site of toxin, is formed. As such complex cannot bind to two

palindromic operator sites, this results in promoter derepression. Conditional cooperativity of RelBE functions in much the same way. Transcription of *vapBC* in *Salmonalla enterica* is also regulated by conditional cooperativity, albeit, with a slighly different mechanism. The bridging of DNA-bound antitoxin dimers is mediated by dimerization of two VapC toxins. Excess of VapC, however, disrupts the cooperative DNA binding of the $[(VapBC)_2]_2$ complex by breaking the VapC dimers (Winther & Gerdes, 2012). It was proposed that all Type II TA systems are regulated by conditional cooperativity. However, a recent study states that the transcription of the MqsRA system in *E. coli* is not enhanced by cooperative binding of the MqsR-MqsA complex to its operator DNA (Brown*, et al.*, 2013). The DNA and the MqsR toxin binding sites on MqsA antitoxin are mutually exclusive as they partially overlap. Affinities of the binding sites are also similarly strong. Unlike most antitoxins, MqsA is completely structured in a free state while being stabilized by a zinc ion (Brown*, et al.*, 2009). Therefore, MqsA alone is an effective repressor. The excess of MqsR results destabilization of the MqsA-DNA complex and, as a consequence, in derepression of TA operon transcription.

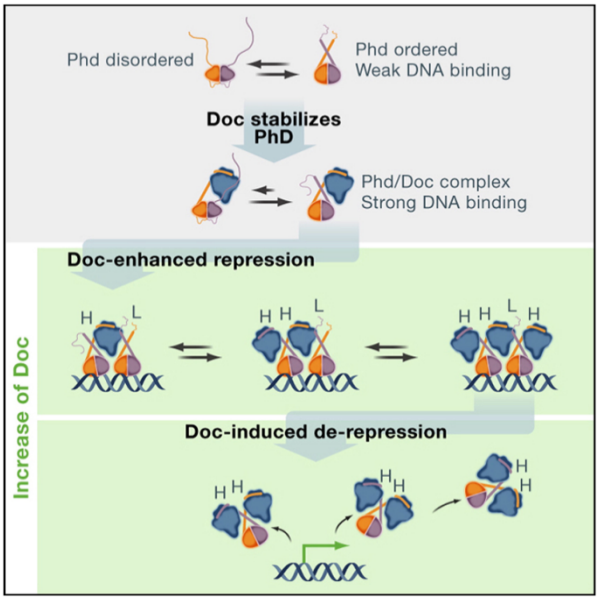

Figure 3. Molecular mechanism of conditional cooperativity in transcriptional regulation of *phd/doc* operon (Garcia-Pino*, et al.*, 2010).

1.6. Physiological roles proposed for chromosomal Type II TA systems

Since the discovery of chromosomally encoded TA loci, the role of these poisonous entities play in the cell continues to be debated. Their function in plasmids as providers of maintenance is credible for the microbiology community (See paragraph 1.1). However, stabilization of plasmid (or any mobile genetic element) could be a side effect that results from the properties of TA systems: a decay of less stable antitoxin releases active toxin, which in turn leads to cell stasis or killing in plasmid-free cells. This view is also supported by the fact that plasmids already have much more effective active segregation (partition) systems than the proposed TA loci (Vecchiarelli*, et al.*, 2012). In addition, some researchers have suggested an existence of excellent symbiosis between PSK and partition systems to provide cost-effective maintenance of plasmids (Brendler*, et al.*, 2004). Obviously, this role is questionable among highly widespread chromosomal TA loci, which suggests a different primary function. Therefore, many studies have been performed by disrupting single or multiple TA open reading frames to see differences in cell physiology, or by examining up- and downregulations of expression of TA systems under various stress conditions.

To date, at least eight different models have been proposed, wherein chromosomal Type II TA systems may have a regulatory function. The models, described below, are programmed cell death, growth adjustment in stress response, development, persistence, phage protection, genome stabilization, anti-addiction, and selfish entities.

Programmed cell death (PCD). This model is based on activation of the MazEF TA system in *E. coli* observed under various stresses uniquely by Engelberg-Kulka group (Aizenman*, et al.*, 1996, Sat*, et al.*, 2003, Hazan*, et al.*, 2004). Bacteriostatic antibiotics were shown to activate MazF toxin in *E. coli* MC4100 (Sat*, et al.*, 2001). Activated MazF was demonstrated to increase the level of reactive oxygen species (ROS), probably due to truncated detoxifying proteins, which lead to cell death in aerobic but not in anaerobic conditions (Kolodkin-Gal*, et al.*, 2008). Deletion of *mazEF* abolishes rifampicin-induced PCD in dense cultures but not at low cell densities (Kolodkin-Gal*, et al.*, 2007). That led to discovery of another MazF mediated but ROS-independent death pathway, which depends on a cell produced quorum-sensing-like linear pentapeptide Asn-Asn-Trp-Asn-Asn, termed as EDF (extracellular death factor) (Kolodkin-Gal*, et al.*, 2008). EDF was isolated from the supernatant of an exponential phase culture of *E. coli* MC4100 (Kolodkin-Gal*, et al.*, 2007). There is no protein in *E. coli* that contains this sequence. Therefore, it is not exactly known how EDF is generated, however, the authors demonstrate that EDF-mediated MazF-dependent killing does not occur in *clpP* (protease) and *zwf* deficient strains. The gene *zwf* encodes glucose-6-phosphate-1-dehydrogenase and contains a sequence (Asn-Asn-Trp-Asp-Asn) that differs by one

amino acid from the EDF sequence. This led the authors to hypothesize that a precursor of EDF may be generated by ClpPX protease during the degradation of Zwf, which may be further amidated to the EDF sequence (Kolodkin-Gal*, et al.*, 2007, Kolodkin-Gal & Engelberg-Kulka, 2008). Recently, it was reported that EDF outcompetes MazE antitoxin from the MazE-MazF complex *in vitro* by directly binding to the MazF. This in turn leads to enhanced RNA cleavage (Belitsky*, et al.*, 2011). EDF is supposed to lead to MazF-mediated killing after being in a growth arrested state longer than 'the point of no return' (Amitai*, et al.*, 2004). Several groups have tried, but could not reproduce the MazF-dependent killing (Pedersen*, et al.*, 2002, Tsilibaris*, et al.*, 2007, Fu*, et al.*, 2009, Christensen-Dalsgaard*, et al.*, 2010). These groups suggest that MazF operates like other endoribonucleases in *E. coli* inducing a state of reversible bacteriostasis. Probably, the MazF-dependent killing is a strain- and cell density-specific phenomenon, because, for instance, a widely used *E. coli* MG1655 strain does not produce EDF (Kolodkin-Gal & Engelberg-Kulka, 2008). Recently, novel EDF-like quorum-sensing peptides, which are able to induce the MazF-dependent cell death, were isolated from *Pseudomonas aeruginosa* and *Bacillus subtilis* (Kumar*, et al.*, 2013). Bacterial PCD cannot be a reasonable solution for an individual bacterial cell to resist the stress. However, a bacterial population that acts like a multi-cellular organism may possibly benfit from stress-induced PCD, which provides nutrients and/or signals for the suvivors, or protection against phages.

Growth adjustment in stress response. This model originates from studies of the RelBE TA system in *E. coli* and contrasts to the PCD (Christensen*, et al.*, 2001). Under normal growth conditions RelB and RelE proteins are in complex at a 2:2 ratio (Overgaard*, et al.*, 2008). However, under amino acid starvation, stress-induced Lon protease degrades RelB antitoxin, which leads to release of RelE toxin and subsequently to growth inhibition. Bacteriostatic properties were confirmed on RelE-arrested cells that were able to form colonies after the over-expression of the antitoxin RelB. The notion, that ectopic production of endoribonucleases is bacteriostatic rather than bactericidal, is consistent with studies performed by many research groups (see the PCD model section). Amino acid starvation induces most of the Type II TA loci in *E. coli*, which rapidly inhibit translation and reset the mRNA pool (Christensen*, et al.*, 2001, Christensen*, et al.*, 2003, Jorgensen*, et al.*, 2009, Christensen-Dalsgaard*, et al.*, 2010). Downregulating the global translation rate is probably beneficial for a cell to adapt to the limited supply and to maintain translation accuracy.

Development. A MazF homolog was reported to cause PCD during fruiting-body formation in *Myxococcus xanthus* (Nariya & Inouye, 2008). *M. xanthus* will develop myxospores under nutrient limitation, wherein 80% of the cell population undergoes cell lysis (Wireman & Dworkin, 1977). The *mazF-mx* gene was reported to form an unusual TA system with the key developmental regulator *mrpC*, which bears no resemblance to the *mazE* antitoxin. The relevance of MazF-mx in the PCD was discredited by Lee and colleagues (Lee*,*

et al., 2012). The deletion of *mazF-mx* in two different wild-type *M. xanthus* strains revealed only a marginal decrease of developmental PCD, in contrast to the reduction obtained previously from a mutant background strain. Recently, a third group demontrated that MrpC actually activates transcription of *mazF-mx* by binding to its promoter in developing cells (Boynton*, et al.*, 2013). Therefore, MazF-mx probably functions without an antitoxin and is not expressed in vegetative cells. The strain, in which MazF-mx-dependent PCD was described, has a mutant *pilQ* (encodes secretin homolog), which increases permeability of the outer membrane and, thus, was shown to be highly vulnerable to vancomycin drug (Wall*, et al.*, 1999). Introducing the *pilQ1* mutation together with deletion of the *mazF-mx* to a wildtype *M. xanthus* strain generated the same phenotype reported by Nariya and Inouye (Boynton*, et al.*, 2013). Hence, the MazF-mx involvement in PCD is observed by using *pilQ* mutant strain.

Persistence. This model by nature is similar to that of the growth adjustment model. Cells that survive bactericidal antibiotics because of dormancy, which does not involve genetic resistance mechanisms, are termed persisters (Lewis, 2007). After antibiotic treatment, persister cells can start growing again. Biofilms are enriched for persisters, which might be the major cause of chronic infections (Costerton*, et al.*, 1999, del Pozo & Patel, 2007, Mulcahy*, et al.*, 2010). In planktonic cultures, persister cells form a small fraction that starts to increase in late-exponential and stationary phase (Spoering & Lewis, 2001, Keren*, et al.*, 2004a). In addition, persister frequency depends on different growth conditions, age of the inoculum, and the growth media (Jõers*, et al.*, 2010, Luidalepp*, et al.*, 2011). Different bactericidal antibiotics also leave a different persister fraction (Dorr*, et al.*, 2009, Ankomah*, et al.*, 2013, Kwan*, et al.*, 2013). The mechanism behind the formation of these rare cells has remained elusive for a long time. However, a recent publication appeared that presents compelling evidence for one plausible mechanism for persister generation (Maisonneuve*, et al.*, 2013). This study states that the dormancy of some cells in exponentially growing culture is caused by stochastic fluctuations in the level of (p)ppGpp. High (p)ppGpp concentration trigers polyphosphate mediated activation of Lon protease and consequent degradation of unstable antitoxins. A mutation that confers a high persistence phenotype (from persister frequency of 10^{-6} to 10^{-2}) was identified in the gene of the HipA toxin (*hipA7*) of *Escherichia coli* (Moyed & Bertrand, 1983). Bactericidal antibiotics and fluorescence-activated cell sorting (FACS) have been used to isolate cells that have a persister-like phenotype. The gene expression profile of non- lysed planktonic cells from ampicillin-treated culture as well as non-growing cells of FACS analysis revealed up-regulation of several TA systems (Keren*, et al.*, 2004b, Shah*, et al.*, 2006). The induced transcription is also an indirect sign of activated toxins since the levels of antitoxins, which autorepress the TA operons, must be dropped. Active TA toxins cause growth stasis and therefore form a strong basis for a persistence model. Single deletion of *relE*, *mazF*, *yafQ, mqsR* or *yoeB* toxin in *E. coli* K12 treated with ofloxacin did not reduce the

persister count, and only stationary phase cells of Δ*hipA* had an effect in rich medium (Keren*, et al.*, 2004b, Shah*, et al.*, 2006). Later, in another *E. coli* K12 strain (BW25113) deletion of *mqsR* decreased the persister level in response to ampicillin treatment (Kim & Wood, 2010) but only when the inoculum was taken from an aged culture (Luidalepp*, et al.*, 2011). In addition, deletion of the *yafQ* toxin produced a normal biofilm that was about 2400 times more susceptible to killing by certain bactericidal antibiotics compared to wild-type (Harrison*, et al.*, 2009). Thus, the importance of TA loci in the persistence phenotype may become evident under different assay conditions. Recently, it was demonstrated that successive deletion of the 10 endoribonucleolytic TA loci of *E. coli* gradually reduced the level of persisters (Maisonneuve*, et al.*, 2011). Hence, it is necessary to consider redundancy and possible cross-talk when we study TA-related phenotypes, because most bacterial genomes contain multiple TA loci. Persistence and growth adjustment models are not mutually exclusive. However, every toxin-arrested cell may not become a persister, because the vast majority of individual nondividing cells never restart growth (Roostalu*, et al.*, 2008, Jõers*, et al.*, 2010).

Phage protection. Phage abortive behaviour of TA systems was first described in Type I (RNA antitoxin) system (Pecota & Wood, 1996, Fineran*, et al.*, 2009), but was later found also in Type II MazEF (Hazan & Engelberg-Kulka, 2004) and RlnAB families (Koga*, et al.*, 2011, Otsuka & Yonesaki, 2012), and in Type III ToxIN system (Fineran*, et al.*, 2009). Phage abortive infection (Abi) is based on altruistic cell suicide of infected bacteria, which halts phage propagation within a bacterial population (Chopin*, et al.*, 2005). Many different Abi systems are described and one of them involves activation of TA toxins. Phage infection probably leads to degradation of labile antitoxins due to arrested gene expression of the host, thereby releasing toxins that poison the cell. Phages have developed their own antitoxins that mimics and replaces the cognate labile antitoxin to circumvent the phage abortion (Otsuka & Yonesaki, 2012).

Genome stabilization, *anti-addiction* or *selfish entities*. These three models are based on the addictive properties of TA loci. The stabilization model argues that the main purpose of chromosomal TA systems is to stabilize non-essential genome regions; the anti-addiction model theorizes on the possibility that a chromosomal TA system prevents PSK induced by a homologous TA module of the lost plasmid; and the selfish model suggests no function for TA loci, rather they just persist as selfish modules (Gerdes*, et al.*, 2005, Van Melderen & Saavedra De Bast, 2009).

1.7. Families of Type II TA systems

It is reasonable to expect that toxin-antitoxin operons have spread and evolved as modular units. Intriguingly, TAs are not conserved as pairs. On the basis of primary sequences, toxins and antitoxins unite into families and superfamilies of their own. General classification of Type II TA loci is based on the sequence similarity of toxin proteins (Gerdes*, et al.*, 2005, Van Melderen & Saavedra De Bast, 2009). Toxin-antitoxin database (TADB) was created to organize data about predicted and experimentally validated prokaryotic and archeal Type II TA loci (Shao*, et al.*, 2011). According to the TADB web-based database (http://bioinfo-mml.sjtu.edu.cn/TADB/) there are eleven two-component and three three-component TA families classified on the basis of the sequence similarity and tertiary structure of toxin proteins. The two-component families are *ccd*, *hicBA*, *hipBA*, *mazEF*, *pem*, *parDE*, *phd-doc*, *relBE*, *vapBC*, *mosAT*, and *yeeUV*, in which *pem* (*parD*) should be placed together with *mazEF* due to homology (Masuda*, et al.*, 1993) and *yeeUV* should be assigned as Type IV now (Masuda*, et al.*, 2012). The tree-component families are *ω-ε-ζ*, *pasABC*, and *paaRA-parE*.

Resolved structures reveal the similarity between several toxins with quite dissimilar primary sequences; e.g. RelE and YoeB of *E. coli* share only 13% sequence identity, but are structurally similar to each other (Kamada & Hanaoka, 2005, Takagi*, et al.*, 2005). RelE also shares a similar β-sheet core structure with MqsR, which was placed into a separate toxin family at first (Brown*, et al.*, 2009). Based on the tertiary structure, the RelBE superfamily currently contains five subfamilies: RelBE, YefM/YoeB, HigBA, MqsRA and PrlF-YhaV (http://bioinfo-mml.sjtu.edu.cn/TADB/).

Toxins of a particular toxin family can be paired with antitoxins representing different antitoxin families. That has led to building an alternative, TA domain pair-based classification (Makarova*, et al.*, 2009). Authors reported more than 6000 TA loci in 750 genomes that are organized into 44 TA domain pair groupings in TADB v1.1 database (http://bioinfo-mml.sjtu.edu.cn/TADB/browse_domain.php). Most surprisingly, toxins of the RelE-YoeB-like group are paired up with cognate antitoxins that belong to at least 4 different families: RHH (ribbon-helix-helix; e.g. RelB (Takagi*, et al.*, 2005)), Xre (e.g. HigA (Arbing*, et al.*, 2010)), PHD (e.g. YefM (Kamada & Hanaoka, 2005)) and AbrB (e.g. PrlF (Coles*, et al.*, 2005)). Folds and primary sequences of these proteins are completely different. Despite their general structural diversity, the structural homology of the toxin-binding regions of several antitoxins (e.g. RelB and MazE of *E. coli*) has been revealed (Takagi*, et al.*, 2005).

I.8. Interplay between Type II TA systems

The TA domain pair-based classification (Makarova, *et al.*, 2009) raises a hypothesis of gene shuffling between a few major classes of toxins and antitoxins (Anantharaman & Aravind, 2003). Here, the protein partners are stuck with the requirement of a functional and physical interaction in a newly assembled TA system. If a protein swaps partners, it will have a propensity to interact with several partners (Gophna & Ofran, 2011). The antitoxin must be able to control its newly acquired toxin partner from the very moment of shuffling; thus, these two proteins must have cross-reacted already before swapping. This hypothesis contradicts the key assumption in the field, which states that interaction between cognate toxin and antitoxin is exclusively specific: "one toxin for one antitoxin" (Gerdes, *et al.*, 2005). The latter notion is based on the observation that deletion mutants of the antitoxin genes usually cannot be obtained (Fiebig, *et al.*, 2010). Although solitary toxin and antitoxin genes have been found in many genomes (Makarova, *et al.*, 2009), expression and functionality of these orphan genes has been rarely demonstrated or tested (Nariya & Inouye, 2008).

Possible cross-reactivity between coexisting TA systems, mostly those that share relatively high sequence similarity, has been occasionally tested. Some authors report positive results (Santos-Sierra, *et al.*, 1997, Santos Sierra, *et al.*, 1998, Grady & Hayes, 2003, Wilbaux, *et al.*, 2007), while others did not see any cross-reaction (Ramage, *et al.*, 2009, Fiebig, *et al.*, 2010). A recent work states that at least limited non-cognate TA interactions occur in *Mycobacterium tuberculosis*, because several antitoxins of this organism could interact both functionally and physically with non-cognate toxins (Zhu, *et al.*, 2010). However, if toxins are expressed from the arabinose-inducible P_{BAD} promoter and antitoxins from an IPTG-inducible promoter, as was performed in the study of mycobacterial TA systems, it is important to consider that IPTG inhibits P_{BAD} directly (Lee, *et al.*, 2007). Therefore, these functional interactions between non-cognate TA partners, reported by Zhu and colleagues, may require retesting.

Another type of cross-talk between TA systems could derive from the activity of mRNA interferases. There is a possibility that a toxin could specifically degrade an mRNA of another TA system. Indeed, it was recently reported that MqsR controls the GhoST TA system, which is an unusual TA pair (Type V) where the antitoxin neutralizes toxin by cleaving its mRNA (Wang, *et al.*, 2012b). MqsR cleaves the 5' end of *ghoST* mRNA, specifically degrading the antitoxin-encoding part of mRNA (Wang, *et al.*, 2012a). This portion of the mRNA contains three GCU sites recognized by active MqsR. The 3' portion, which encodes for the GhoT toxin, does not have any GCU sequence. Hence, stress-induced MqsR leads to elevated level of *ghoT* mRNA and subsequent production of an active toxin.

2. AIMS OF THE STUDY

Numerous chromosomal toxin-antitoxin loci are activated in dormant subpopulation of bacteria that can tolerate bactericidal antibiotics. These persister cells are enriched in biofilms that often cause recurrent and chronic infections. The broader aim of this study is to elucidate the mechanism for simultaneous activation of TA systems, and to test whether they are linked to multidrug tolerance and growth heterogeneity due to their inhibitory effect on cell growth.

Specific objectives of this dissertation are:

- to test if the gene *mqsR*, that is upregulated in *E. coli* cells refractory to antibiotic treatment, encodes a toxin and is part of a novel TA system (Publication I, II)
- to characterize toxicity, transcription regulation, phyletic distribution and involvement in biofilm formation of the novel *mqsRA* TA system (Publication I, II)
- to examine potential transcriptional cross-talk between different TA systems (Publication III)

3. RESULTS AND DISCUSSION

3.1. MqsRA shares properties common to canonical TA systems (I, II)

3.1.1. MqsR toxicity

Transcription profiles of persister-like dormant cells revealed that, among other genes, TA loci were highly induced (Keren*, et al.*, 2004b, Shah*, et al.*, 2006). The most upregulated gene on these profiles was B3022 (*ygiU E. coli* K12, later renamed as *mqsR* (see paragraph 2.3) (Gonzalez Barrios*, et al.*, 2006)). *mqsR* is located next to B3021 (*ygiT*, renamed *mqsA*), that was similarly induced. This gene pair was predicted to be a TA system due to several intrinsic characteristics: a short intergenic region (1 bp between the two genes) suggests cotranscription and translational coupling, small size (98 amino acids MqsR and 131 MqsA), DNA binding fold in MqsA for putative repressor function, and the growth-arresting effect of MqsR (Shah*, et al.*, 2006).

We overexpressed MqsR in *E. coli* to test its toxicity. It inhibited bacterial growth, which was suppressed by production of the MqsA (YgiT) antitoxin (I, Fig. 1). MqsR does not kill bacteria, because colony formation ability did not decrease after the prolonged expression of the MqsR toxin. Similar resuscitation was observed by others, confirming that MqsR causes a temporal growth arrest that is reversible by its cognate antitoxin (Christensen-Dalsgaard*, et al.*, 2010). Fluorescence microscopy revealed that MqsR-arrested cells were elongated and fatter but not filamented (I, Fig. 2). The mechanism behind this phenotype is to be elucidated in the future.

Because the primary sequence of MqsR did not reveal its molecular target, we performed pulse-labeling experiments to measure MqsR toxicity on macromolecular synthesis *in vivo*. Induction of MqsR caused rapid inhibition of translation, whereas transcription was less affected, and the effect on replication was not clearly pronounced (I, Fig. 4). Further testing in an *in vitro* coupled transcription-translation system revealed no inhibition of translation by MqsR (I, Fig. 5). Similar contradictory results appeared for the HipA toxin (Korch & Hill, 2006), whereas RelE and MazF inhibited translation both *in vivo* and *in vitro* (Gotfredsen & Gerdes, 1998, Zhang*, et al.*, 2003). Shortly after submission of Publication I, two research groups reported, that MqsR toxicity is based on ribosome-independent GCU site-specific endoribonucleolytic activity of the protein (Yamaguchi*, et al.*, 2009, Christensen-Dalsgaard*, et al.*, 2010). Based on the observation that MqsR cleaves mRNA irrespective of translation, it should block translation *in vitro*. However, we ought to interpret our negative results with caution because the S30 extract and protein expression system is an artificial setup. Either substrate specificity or inactivity of MqsR may play a role here. In addition, a fourth group published crystal structures of MqsRA, which linked MqsR to the RelE superfamily of toxins and revealed unique

properties of MqsA antitoxin (Brown, *et al.*, 2009). Therefore, activation of MqsR toxin causes changes in mRNA pool, blocks translation, and leads to production of many truncated misfolded proteins. Bacteria have to overcome these obstacles to be able to start growing again.

3.1.2. Transcription of *mqsRA* operon

According to bioinformatic data, the consecutive *mqsR* and *mqsA* genes are expected to constitute an operon, which is regulated by binding of the MqsA to the promoter region. To confirm this, we performed RNA analysis using the northern blot method. We detected two *mqsRA*-specific transcripts (I, Fig. 3). Further mapping of detected mRNAs with DNA oligoprobes complementary to different regions nearby the locus validated cotranscription of *mqsR* and *mqsA*. Both detected transcripts covered the full operon, one extended at the 3' end into the intergenic region. Additionally, we mapped the exact transcription start site to 18 bp upstream of the start codon of *mqsR* using the 5' RACE method (I, Fig. 3). This is the same site reported by dr Gerdes lab, but different from that reported by dr Inouye lab, who mapped mRNA 5' end to 109 bp upstream; both groups used the primer extension technique (Yamaguchi, *et al.*, 2009, Christensen-Dalsgaard, *et al.*, 2010). To validate autoregulation by MqsA antitoxin, we constructed a P_{mqsRA} promoter region fusion to the *lacZ* reporter. β-galactosidase assay confirmed strong repression of the promoter when MqsA was induced (I, Fig. 3).

Summing up, the caracteristics of MqsR and MqsA proteins and the operon regulation allow us to conclude that *MqsRA* is a TA system.

3.2. Phylogenetic distribution of *mqsRA* (I, II)

To study the phylogenetic distribution of *mqsRA* we searched for genes homologous to MqsR in 914 fully sequenced bacterial genomes. A total of 42 homologs in 40 genomes were found, most of them in Gamma- and Betaproteobacteria (I, Fig. 6). Putative MqsR toxins were also found in Alpha- and Deltaproteobacteria, in Chlorobi, and in Acidobacteria, whereas we did not find any in phyla Firmicutes and Actinobacteria according to our search criteria. Independently, a more comprehensive comparative genomic study of Type II TA systems was conducted (Makarova, *et al.*, 2009). In this study, another more divergent, phylogenetically distinct group (Group 2) of the MqsR family emerged. Group 2 is present mostly in Firmicutes and Chlorobi, but also in some phages and prophages. However, only one of the toxin active site residues (residue Y81 of *E. coli* MqsR), and none of the antitoxin-interacting residues, is conserved in Group 2. This raises the possibility that Group 2 is functionally distinct from Group 1 (II, Fig. 6.2). Hence, two splitting groups become visible on the phylogenetic tree of MqsR homologs (II, Fig. 6.1).

Most MqsR-encoding genomes contain a single *mqsR* gene, with few exceptions where two or three *mqsR* homologs were found (I, Fig. 6, II) (Makarova*, et al.*, 2009). As a general rule, the presence of *mqsR* genes differs among closely related bacteria. For example, only about one-half of the sequenced *E. coli* genomes contain *mqsR* homolog gene suggesting that this gene was acquired recently through lateral gene transfer or lost due to selective pressure. As it is typical with TA loci, all the identified genes that code for the MqsR toxin homologs were followed by a putative antitoxin gene. Also, as usual in TA operons the genes were either overlapping or separated by a couple of nucleotides (I, Table S1). Notably, two distinct helix-turn-helix (HTH) subfamilies of antitoxins are linked to the MqsR toxins (Makarova*, et al.*, 2009). In Group I, the HTH motif of MqsA antitoxin contains an extra N-terminal zinc-finger domain. In the second subfamily, the HTH domain is fused to a domain of GepA (genetic element protein A, an uncharacterized phage-associated protein) family. Thus, MqsRA as well as other Type II toxins and antitoxins have probably assembled several times during their evolution (Anantharaman & Aravind, 2003, Leplae*, et al.*, 2011).

3.3. Effect of MqsRA on biofilm formation (I, II)

mqsR (motility quorum sensing regulator) was named by a phenotype in which disruption of the *mqsR* reduced autoinducer-2-mediated biofilm growth in *E. coli* (Gonzalez Barrios*, et al.*, 2006). Later, deletion of five chromosomal TA loci was reported to decrease biofilm formation, while individual disruptions had no effect (Kim*, et al.*, 2009). Therefore, we conducted biofilm measurements with deletion mutants of the *mqsRA*, *relBE*, and *mazEF* operons in three different *E. coli* K-12 strains. Our results indicate the complexity of the biofilm regulation, because no common pattern appeared in different TA deletion strains (I, Fig. 7). Deletion of *mqsR* alone in BW25113 had no effect on biofilm formation in our crystal violet staining assay. However, biofilm formation decreased in both HM21Δ*mqsRA* and BW25113Δ*mqsRA* (*mqsA* ≡ *ygiT*). A similar phenotype appeared after deletion of *relBE* and *mazEF* loci, whereas individual deletion of *relE* even enhanced biofilm production. The *E. coli* MG1655 strain, for which the biofilm-related phenotype of *mqsR* was originally reported, was a poor biofilm producer in our assay system, and Δ*mqsRA* biofilm formation resembled to that of the wild-type. Partially, these results can be explained by different strain backgrounds. MqsR is less toxic in MG1655 and BW25113 strains compared to HM21 (I, Fig. 1, S2). Also, we were not able to transform HM21Δ*mqsRA* with the MqsR expression plasmid, while it was possible in MG1655 background (Kim*, et al.*, 2010).

After submission of Publication I, reports about the ability of MqsA antitoxin to regulate other promoters than the *mqsRA* promoter were published (Brown*, et al.*, 2009, Kim*, et al.*, 2010). This was the first proof of auxiliary

transcriptional regulation conducted by an antitoxin. Whole-transcriptome analysis of cells with overexpressed MqsR (Kim, *et al.*, 2010) led to the discovery of the mechanism by which MqsRA loci may mediate environmental stress response in bacteria through RNA polymerase sigma factor σ^S (Wang, *et al.*, 2011). σ^S is a key regulator of stress-related genes. It signals a cell to modify, among other things, motility, flagella, curli, biofilm production, and stationary growth (Battesti, *et al.*, 2011) (II, Fig. 6.4). MqsA was shown to specifically bind to palindromic sequences in the promoter region of *rpoS*, and repress its transcription (Wang, *et al.*, 2011). Under stress, activated Lon protease degrades MqsA antitoxin, which leads to derepression of σ^S, ensuing increased biofilm production.

Taking together the results of recent studies: transcription of *mqsRA* is not regulated by conditional cooperativity (Brown, *et al.*, 2013); the transcription of *rpoS* and consequent production of biofilm is repressed by MqsA; translation of many genes can be affected by MqsR toxin. These findings suggest a multilevel regulation of biofilm production by the MqsRA TA system (II, Fig 6.4). However, we still cannot explain why deletion of the *mqsRA* operon leads to a reduction in biofilm formation, while deletion of *mqsR* toxin alone has no effect or even increases production (Kim, *et al.*, 2010).

3.4. Uninhibited toxins cross-activate TA systems (I, III)

Previous array-based gene expression analysis revealed simultaneous up-regulation of TA loci in an ampicillin tolerant fraction of *E. coli* cells (Keren, *et al.*, 2004b). These results support the hypothesis that a common regulatory mechanism exists for the simultaneous induction of different TA systems. Therefore we designed experiments to test possible cross-talk between different TA loci in *E coli*. We hypothesized that an activated (uninhibited) toxin can induce other toxins. The assumption was supported by the first relevant test, wherein we observed that the abundance of *mqsR* mRNA increased in response to activation of HipA toxin (I, Fig. 3). In further experiments, we overexpressed several toxins, one at a time, and monitored changes in various TA mRNAs using northern hybridization. As a result, we confirmed strong cross-induction of TA mRNAs in response to several toxins. Expression of MazF, MqsR, HicA, and HipA clearly induced the transcription of the *relBEF* operon. Expression of YafQ did not, at first glance, induce the *relBEF* mRNA (III, Fig. 1). However, considering that the smaller *relBEF*-specific fragments can be detected, the mRNA might be overexpressed, but subsequently degraded after cleavage by YafQ. In the reverse experiment, expression of RelE toxin strongly induced transcription of the *mqsRA*, *mazEF*, *dinJ-yafQ*, *hicAB*, *yefM-yoeB*, and *prlF-yhaV* TA systems (III, Fig. 2). Mostly, hybridization signals from the antitoxin portion of the transcripts were weaker regardless of whether the antitoxin was the first or the second gene in the operon. These results may reflect better cleavage of the antitoxin part and a higher stability of the toxin-encoding region.

Others have also noted some examples of transcriptional cross-induction between TA systems as well as cross-activation between toxins. Overproduction of MqsR induced transcription of *relBE* and *relF* (hokD) (Kim, *et al.*, 2010). Ectopic expression of VapC toxins originating from *Salmonella* and *Shigella* activated RNA cleavage by YoeB toxin (Winther & Gerdes, 2009) and production of the Doc toxin activated RelE in *E. coli* (Garcia-Pino, *et al.*, 2008). Transcriptional induction of a TA system appears only when the concentration of the toxin exceeds that of its antitoxin. Decreased antitoxin levels probably lead to activation of toxin. Hence, by detecting transcriptional induction one may conclude activation of the toxin.

To further analyze whether the transcriptional cross-activation is not a mere result of artificial overproduction of toxins, we conducted experiments under more physiologically relevant conditions. We induced amino acid starvation by addition of mupirocin and monitored *mqsR* and *mazF* mRNAs in both wild-type and *relBEF* deficient strains. Amino acid starvation has been reported to strongly induce *relBE* (Christensen, *et al.*, 2001) and *mazEF* (Christensen, *et al.*, 2003). We detected RelBE dependent induction of *mazEF* in response to amino acid shortage, whereas *mqsRA* mRNA (also strongly induced in amino acid starvation) was not affected by the deletion of *relBE* (III, Fig. 3). Hence, cross-activation between TA loci indeed occurs at least in amino acid starvation.

3.5. Cross-activation is not affected by deletion of proteases known to degrade the antitoxins (III)

Bacteria are frequently faced with nutrient limitation, which is the most common stress in nature. Stress response of bacteria involves induction of proteases that degrade ribosomal and truncated proteins to supply resources to synthesize stress-related genes (Kuroda, *et al.*, 2001, Choy, *et al.*, 2007, Durfee, *et al.*, 2008). Previous studies have revealed degradation of unstable antitoxins by the stress-induced ATP-dependent proteases Lon and ClpP (Van Melderen, *et al.*, 1994, Lehnherr & Yarmolinsky, 1995, Aizenman, *et al.*, 1996, Christensen, *et al.*, 2004, Overgaard, *et al.*, 2009, Christensen-Dalsgaard, *et al.*, 2010, Hansen, *et al.*, 2012). Given that antitoxins are transcriptional autorepressors, their degradation results in transcriptional burst of TA operons. Considering this, we hypothesized that overexpression of a toxin could lead to activation of stress-related proteases. Thus, we tested whether proteases, known to degrade antitoxins, are the key regulators for transcriptional cross-activation of TA operons. However, in deletion mutant strain lacking three major proteases (*lon*, *clpPX* and *hslVU*) we still detected strong induction of *relBEF* mRNA in response to overproduced MazF and MqsR toxins (III, Fig. 4). This indicates that either other proteases are involved or a protease-independent mechanism is responsible for TA operon cross-activation.

3.6. Selective mRNA cleavage by toxins as a potential mechanism for gene regulation (III)

In addition to cross-induction of TA transcription we observed extensive cleavage of TA mRNAs upon expression of mRNA interferases (III, Fig. 1, 2). Surprisingly, the mRNA cleavage pattern revealed the selective stability of *in vivo* cleaved TA mRNAs on northern blots: the toxin portion of the mRNAs accumulated, whereas the antitoxin portion, as well as control mRNAs, almost fully degraded (III, Fig. 1, 2, S2). To further characterize the *in vivo* cleavage of *relBEF* mRNA, we mapped the 5' ends of the cleavage products generated by different toxins using primer extension analysis (III, Fig. 5, S4, Table S3). We detected MqsR and HicA cutting sites all over the mRNA and did not see selective cleavage on the RelB encoding portion. MazF cutting was more clustered, probably because some ACA sites were masked by the mRNA secondary structures. Also RelE cleaved its own mRNA. Different primers were required to map the full mRNA. Therefore, cleavage efficiency of different regions can neither be estimated nor compared.

We detected mostly cleavage by the overexpressed toxins, because mRNA was cut at the sites that are unique for particular toxins. We detected few sites in the *relBEF* mRNA that were cut in response to production of several toxins. We wanted to determine whether these sites are a result of cutting by over-expressed toxin itself or by cross-activated toxins. We tested three of the sequence-specific cutting sites (ACA for MazF, GCU for MqsR) to detect possible cross-activation in response to expression of HicA. These stops were still detected in *mazF* and *mqsR* deficient strains suggesting that the cleavage was conducted by the HicA itself or by another activated RNase. MazF has a homolog ChpB in *E. coli* that may be involved in the ACA cutting (Zhang*, et al.*, 2005).

Our MazF over-expression resulted in truncated *relBEF* mRNAs that lack ribosome recruitment signals (RBS), but leave the open reading frames (ORF) intact. Leaderless mRNAs generated by MazF have been demonstrated before (Vesper*, et al.*, 2011). Some mRNAs have ACA sequences in front of AUG start codons that can be cleaved by MazF. Furthermore, the authors showed that MazF cuts off the anti-Shine-Dalgarno sequence at 3' end of the 16S rRNA. The cleaved 16S rRNA can assemble into specialized ribosome that can selectively translate the set of leaderless mRNAs. Hence, activation of MazF affects significantly composition of the proteome (Amitai*, et al.*, 2009). To test whether RelE protein can be translated from our MazF-cleaved leaderless *relBEF* fragments, we constructed two T7 expression plasmids. One transcript started next to the RelB start codon and contained intact RelB-RelE ORFs. The other transcript started in the middle of RelB ORF. For RelE detection on western blot, the RelE encoding gene was C-terminally His6-tagged and mutated to reduce its toxicity. We expressed both of the leaderless *relBEF* mRNAs and demonstrated translation of RelE toxin from the transcripts *in vivo*

(III, Fig. 6). However, the truncated leaderless mRNAs were translated less efficiently compared to the full-length mRNA with intact RBS. Thus, activation of MazF may lead to translation of leaderless *relBEF* transcripts resulting in unequal production of RelB and RelE proteins, biased towards RelE.

Increase of the T/A ratio may possibly trigger a positive feedback loop consisting of transcriptional activation of the TA operon, successive cleavage of the TA transcript, buildup of the toxin-encoding mRNA fragments, and translation of them, shifting the T/A balance in favour of toxin (III, Fig. 7). Thus, it can be related to TA-linked growth heterogeneity in bacterial populations and persister formation that is not dependent on activation of the Lon protease (III, Fig. S6) (Keren*, et al.*, 2004b, Shah*, et al.*, 2006, Rotem*, et al.*, 2010, Maisonneuve*, et al.*, 2013).

CONCLUSIONS

- *mqsR* and *mqsA* genes of *Escherichia coli* encode a toxin-antitoxin system, in which MqsR arrests cell growth by blocking translation and MqsA counteracts its toxicity.
- *mqsRA* constitute an autoregulatory operon, in which trancription is repressed by MqsA antitoxin.
- Although *mqsRA* homologs are common in gamma- and betaproteobacteria, not all closely related bacteria contain this TA operon.
- MqsRA play a role in the multi-level regulation of biofilm production.
- Uninhibited toxins cross-activate transcription of TA systems in *Escherichia coli* by an unknown mechanism.
- Cross-activation is not dependent on Lon and ClpXP proteases known to degrade antitoxins.
- Specific cross-activation between RelBE and MazEF TA systems is detected in nutrient limited stress.
- Toxins with mRNase activity cleave TA mRNA-s *in vivo.*
- Accumulation of cleaved transcripts that retain toxin-encoding part may serve as an additional source for toxin production.
- Selective mRNA cleavage by toxins could function as part of a potential gene regulatory mechanism.

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SUMMARY IN ESTONIAN

Bakterite toksiin-antitoksiin süsteemid: transkriptsiooniline rist-aktivatsioon ja uue *mqsRA* süsteemi iseloomustamine

Looduslikus keskkonnas peavad bakterid populatsiooni säilimiseks kohanema muutuvate keskkonnatingimustega. Seejuures on mitmed bakteriliigid, ka patogeensed, võimelised kinnituma pindadele ning moodustama biokilet. See limane, polüsahhariidse maatriksiga ümbritsetud bakterite kogukond on paremini vastupidav halbadele mõjuritele, nagu näiteks antibiootikumid või peremehe immuunsüsteem. Biokiled arvatakse põhjustavat suuremat osa inimese kroonilistest bakterinakkustest. Biokiledes elavate bakterite füsioloogia uurimisel on tuvastatud erineva metaboolse aktiivsusega bakterirakke. Võrreldes vabalt elavate bakteritega on biokiledes suurenenud ajutiselt mittejagunevate bakterite hulk, kelle genoom on identne kasvavate rakkude omaga, kuid kes oma uinunud oleku tõttu on surmavatele antibiootikumidele tundetumad. Selliseid rakke on hakatud kutsuma persistoriteks ning nad on võimelised antibiootikumiravi järgselt taastama populatsiooni arvukuse. Persistorrakkude geenide avaldumine on märgatavalt teistsugune võrreldes kasvavate rakkudega: muu hulgas on neil tugevalt indutseeritud toksiin-antioksiin (TA) süsteeme kodeerivad geenid. TA süsteemis on toksiiniks stabiilne autotoksiline valk, mille märklauaks on mõni oluline rakuline protsess. Enamus *Escherichia coli* II tüüpi TA toksiine lõikavad mRNA-d, seega häirivad tugevalt tranlatsiooni ning põhjustavad pöörduvat kasvuinhibitsiooni. Normaalselt jagunevates rakkudes neutraliseerib ebastabiilne antitoksiin toksiini mõju. II tüüpi TA süsteemides on antitoksiiniks valk, mis seondub otse toksiiniga. Kuna *E. coli* genoomis on mitmeid TA süsteeme kodeerivaid geene, siis võiks toksiinide üheaegse aktiveerumisega selgitada persistorite tekkemehhanismi. Sarnaselt TA süsteemide ekspressioonile on antibiootikumide toimele tundetutes rakkudes tugevasti üles reguleeritud ka genoomis kõrvuti paiknevad *mqsA* ja *mqsR* geenid, mis sarnanevad oma suuruse ja organiseerituse poolest tuntud toksiinide geenidega.

Käesoleva töö üheks põhieesmärgiks oli testida, kas *Escherichia coli mqsA* ja *mqsR* geenid moodustavad uue TA süsteemi. Katsetulemused näitasid, et MqsR üle-ekspressioon põhjustab ajutise rakkude kasvu peatumise, mis on pöörduv MqsA valgu juuresolekul. MqsR aktiveerumine põhjustab rakkude väliskuju pikenemise ja paksenemise. Kõik seni kirjeldatud II tüüpi TA süsteemide geenid paiknevad järjestikku ning moodustavad ühiselt antitoksiini poolt negatiivselt reguleeritud operoni. TA operonilt sünteesitakse ühine mRNA, millel on enamasti üks ribosoomi seondumiskoht esimese geeni ees. Transkriptsiooni analüüs tuvastastas *mqsR* ja *mqsA* geenide kuulumise ühisesse operoni, mille ekspressiooni represseerib MqsA antitoksiin. Näidati, et MqsR inhibeerib tugevalt translatsiooni, kuigi täpset märklauda tuvastada ei õnnestunud. Teiste töögruppide samaaegsed uurimused näitasid, et MqsR toksiin lõikab mRNA-d ja et tema tertsiaarstruktuur sarnaneb enim uuritud RelE

toksiini superperekonnaga. *mqsRA* fülogeneesi bioinformaatilisel analüüsil täheldati alati kahe geeni koosesinemist, kuid lähedastes liikides ei olnud *mqsRA* alati esindatud. Seega võiks *mqsRA* levida edukalt horisontaalse geeniülekande kaudu. Katsed *mqsRA* geenide deletsiooni mõjust erinevate bakteritüvede biofilmi moodustumisele andsid vastakaid tulemusi. Hiljem selgus teiste gruppide uurimustest, et MqsA antitoksiinil on võime seonduda ka teistele promootor-järjestustele peale enda oma ning represseerida nende geenide avaldumist. Muu hulgas inhibeerib MqsA RNA polümeraasi sigma faktori σ^S transkriptsiooni ning seeläbi osaleb ta laialdases stressivastuse regulatsioonis, k.a. biofilmi moodustumisel. Kokkuvõtvalt, MqsRA kirjeldati kui uut TA süsteemi, millel on uudsed regulatoorsed funktsioonid.

Töö teine põhieesmärk oli uurida võimalike rist-regulatsioonide esinemist *Escherichia coli* kromosomaalsete II tüüpi TA süsteemide vahel. MazF, MqsR, HicA ja HipA toksiinide üle-ekspresseerimine indutseeris tugevalt *relBE* TA süsteemi mRNA sünteesi. Sama täheldati ka vastupidises ekperimendis, kus RelE toksiini üle-ekspressioon aktiveeris mitmete TA lookuste ekspressiooni. Füsioloogiliste tingimuste matkimiseks tekitati aminohappenälg metsiktüüpi ja *relBEF* mutantses (deletsioon) tüves ning jälgiti *mqsRA* ja *mazEF* operonide ekspressiooni tugevust mRNA-de hübridisatsioonil. Varasemalt oli teada, et aminohappenälg indutseerib tugevalt TA süsteemide transkriptsiooni. Avastati, et *mazEF* transkriptsioon aktiveerus vastusena aminohappenäljale ainult metsiktüüpi bakterites kuid mitte *relBE* defektses tüves. *mqsRA* transkriptsioon oli aktiveeritud mõlemas tüves. Seega võib järeldada, et aminohappenälja korral aktiveeritakse *mazEF* transkriptsioon ainult RelE toksiinist sõltuvalt, mis on tõestus spetsiifilisest rist-aktivatsioonist. TA süsteemide transkriptsiooni regulatsiooni arvesse võttes võib TA operoni transkriptsiooni aktiveerumisest järeldada toksiini kontsentratsiooni ülekaalu võrreldes antitoksiiniga, seega ka toksiini aktiveerumist.

Stressitingimustes lagundab ebastabiilsed antitoksiinid peamiselt Lon proteaas, mistõttu stabiilsed toksiinid vabanevad ja ka TA operonide transkriptsioon derepresseeritakse. Ühe mRNAd lõikava toksiini ekspressioon võib aktiveerida Lon sünteesi ning seeläbi aktiveerida ka teised TA süsteemid. Selle levinud hüpoteesi testimiseks mõõdeti TA transkriptsiooni rist-aktivatsiooni Lon, ClpXP ja HslVU proteaaside defektsetes tüvedes. Vastusena MazF ja MqsR ekspressioonile täheldati metsiktüübile sarnane tugev *relBEF* mRNA sünteesi induktsioon. Sellest järeldati, et TA süsteemide transkriptsiooniline ristaktivatsioon ei sõltu antud katsetingimustes proteaaside olemasolust.

Lisaks tugevale rist-aktivatsioonile täheldati toksiinide ekspresseerimisel TA mRNA-de valikulist lõikamist. TA mRNA toksiini kodeerivad lõikefragmendid akumuleerusid, samal ajal kui antitoksiini kodeerivad mRNA osad efektiivselt lagundati. *relBE* mRNA lõikekohtade täpne määramine tuvastas nn *leaderless* mRNA tekke vastusena MazF toksiini üle-ekspressioonile. Varem on kirjeldatud translatsiooni toimumist ka sellistelt mRNA-delt, millel puudub ribosoomi poolt ära tuntav seondumisjärjestus (*Shine-Dalgarno*), kuid avatud

lugemisraam on terve. Analoogse kärbitud *relBE* mRNA ekspresseerimisel plasmiidilt näidati RelE toksiini translatsiooni *in vivo*. Seega võib suurenenud toksiini kontsentratsioon vallandada rakkudes positiivse tagasiside tsükli, mis läbi teiste TA mRNA-de lõikamise ning transleerimise muudab ka toksiinide ja antitoksiinide kontsentratsioonide suhet rakus. Sellise potentsiaalse geeni-regulatsiooni mehhanismi tõendamine toetaks täiendavalt, sõltumata proteaaside aktiivsusest, TA süsteemide samaaegse aktivatsiooni teket ning seeläbi selgitaks bakteri-populatsioonide kasvu heterogeensust.

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PUBLICATIONS

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