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Characterisation of Type II toxins discovered through iterative guilt-by-association search

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Characterisation of Type II toxins discovered through iterative guilt-by-association search

Abstract

The classical toxin-antitoxin (TA) system is encoded as a bicistronic operon consisting of toxin and antitoxin genes. Toxin-antitoxin systems have been studied for more than 30 years and have been shown to play an important role in bacterial immunity and stress response. The most common and best-studied class of TA systems is type II, where both the toxin and the antitoxin are proteins, and the toxin is neutralized by the antitoxin via direct interaction. My thesis helps to uncover the molecular mechanisms of type II toxins found by the bioinformatics tool NetFlax (standing for Network of Flanking genes for toxins and antitoxins) via metabolic labelling assays. I characterize several toxins from different functional domains and show how the toxins affect the bacterial cell using *Escherichia coli* as the model organism.

Keywords:

Toxin-antitoxin systems, NetFlax, radioisotope metabolic labelling, translation, transcription, replication

CERCS: B230 Microbiology, bacteriology, virology, mycology

Iteratiivse seotuspõhise otsinguga leitud toksiin-antitoksiin süsteemide kirjeldamine

Lühikokkuvõte:

Bakteriaalsed toksiin-antitoksiin süsteemid koosnevad tüüpiliselt kahest geenist millest üks kodeerib kasvu inhibeerivat toksiooni ja teine seda neutraliseerivat antitoksiini. Toksiin-antitoksiin süsteeme on uuritud juba üle 30 aasta ning neid seostatakse bakteriofaagidevastase kaitse ja bakterite stressivastusega. Kõige levinum ja paremini uuritud toksiin-antitoksiinide klass on tüüp II, kus nii toksiin kui ka antitoksiin on valgud ning antitoksiin neutraliseerib toksiooni sellega seondudes. Minu lõputöö keskendub NetFlax programmiga leitud uute tüüp II

toksiinide molekulaarsete mehhanismide uurimisele kasutades selleks metaboolset märkimist. Ma iseloomustan mitmeid erinevaid toksiini domeene ja testin kuidas nad mõjutavad bakterite kasvu kasutades *Escherichia colit* mudelorganismina.

Võtmesõnad:

Toksiin-antitoksiin süsteemid, NetFlax, metaboolne märkimine raadioisotoobiga, translatsioon, transkriptsioon, replikatsioon

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

TERMS, ABBREVIATIONS AND NOTATIONS

AA – amino acid

AAA ATPase – enzymes which hydrolase ATP and the provided energy during hydrolysis leads to conformational changes which are transduced into mechanical force exerted on a macromolecular substrate

Abi – Abortive infection

CAM - Chloramphenicol

CB – Carbenicillin

FlaGs – Flanking Genes

Hyperpromiscuous - ability of an antitoxin domain to evolve to neutralize different non-cognate and non-homologous toxins

NetFlax – Network for FlaGs for toxins and antitoxins

PanA - Panacea

PanT – Panacea associated toxin

SAH – small alarmone hydrolase

SAS – small alarmone synthetase

PSK- post-segregational killing

TA – toxin-antitoxin

TCA - Trichloroacetic acid

toxSAS – toxic small alarmone synthetase

INTRODUCTION

Bacterial toxin-antitoxin systems are widespread genetic elements consisting of a toxin, that inhibits cell growth and an antitoxin, which counteracts the toxin's effect and rescues the cells. The roles of TA modules conceptually can be divided into three: plasmid maintenance, bacteriophage defence and stress response. I) TA systems can act as addiction modules: they have been found to be the culprit of the death of the bacterial cells, which have lost the TA encoding plasmid (Iqbal et al., 2015; Ogura & Hiraga, 1983). II) TA systems get induced during phage attack leading to the altruistic death of phage-infected cells to prevent the virus from spreading to the whole bacterial population (Dy et al., 2014; LeRoux & Laub, 2022). III) Lastly, toxin-antitoxin systems have been shown to get induced during stress conditions such as amino-acid starvation, high temperature, oxidative stress and so on (Hazan et al., 2004). Since some TA systems are involved in anti-phage defence, studying them helps us to improve bacteriophage therapy against bacterial infections.

Considering the increase in number of sequenced bacterial genomes and sequence sensitivity screening methods, the *in silico* methods for discovering novel TA systems have drastically improved (Akarsu et al., 2019; Fozo et al., 2010; Horesh et al., 2018; Kurata et al., 2022). The NetFlax (Network of Flags for toxins and antitoxins) program was developed to discover novel type II toxin-antitoxin systems using the bicistronic organisation of these modules and their tendency to swap toxin and antitoxin partners. The primary input for the algorithm was the hyperpromiscuous Panacea domain (Ernits et al., 2023; Kurata et al., 2022). This thesis focuses on studying molecular mechanisms of newly discovered toxins by NetFlax. The network of toxin and antitoxin systems found by NetFlax is divided into 5 zones: PanA antitoxin zone, RelA/SpoT alarmone synthetase-containing toxin zone, AAA ATPase antitoxin zone, classical type II TA system zone and ImmA/RosmerA antitoxin zone. Metabolic radioisotope labelling experiments were conducted to reveal the effects of toxins on the three major cellular processes: transcription, translation and replication. Most of the toxins tested in this work target translational machinery, which agrees with the fact that translational machinery has been thus far also reported to be the most common target of type II toxins (Jurėnas & Van Melder, 2020).

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1. LITERATURE REVIEW

1.1 Toxin-antitoxin (TA) systems

A typical bacterial toxin-antitoxin system is usually represented by two elements: a stable toxin and an cognate unstable antitoxin (Jurénas et al., 2022) (LeRoux & Laub, 2022). In the majority of toxin-antitoxin systems, a toxin is a protein, and an antitoxin is either an RNA or a protein (Jurénas et al., 2022). Bacterial TA systems were discovered in the 1980s as a set of two linked genes that promote the maintenance of conjugative plasmids (Gerdes et al., 1986, 1988; Ogura & Hiraoka, 1983). These small modules have been ubiquitously found in the genomes of bacteria (plasmids and chromosomes), archaea, and bacteriophages and have been associated with various functions such as bacterial stress response and phage defence. The importance of TA systems has been extensively discussed, although the regulation and activation mechanisms of many toxin-antitoxin systems are still unclear (Fraikin et al., 2020).

1.2 Classification of TA systems

The classification of TA systems is based on the nature of the toxin and antitoxin, as well as the mechanism of antitoxin action of toxin neutralization. Currently, TA systems have been categorized into eight types (Figure 1.). In TA systems, toxin and antitoxin genes usually form a bicistronic operon where antitoxin is located before the toxin gene. However, type I and type VIII TA systems have their own promoter, so they are exceptions to this formulation (Jurénas et al., 2022). In addition, Type II TA systems *hipBA*, *higBA* and *mqsRA* also do not follow this arrangement (S.-P. Zhang et al., 2020). The toxin is generally a protein (except for type VIII TA system, where it is an RNA), and antitoxin is either protein or small RNA which is able to neutralize toxin by directly or indirectly affecting its activity or target. Below, a short description of each type of TA system is shown and their representative example(s) have been discussed.

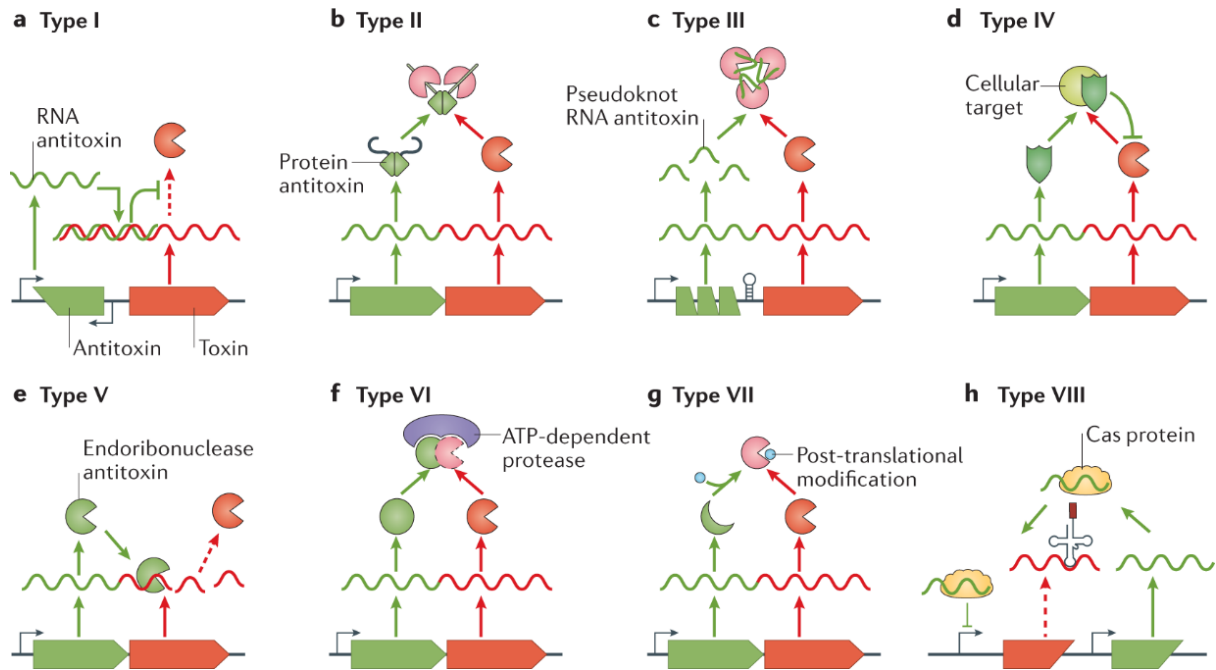


Figure 1. Types of TA systems according to current classification. As shown in (a) and (c), Type I and Type III systems have RNA antitoxins. In type I, antitoxin RNA binds to the mRNA of the toxin, resulting in the blocking of translation, whereas in type III, the RNA neutralizes the toxin by directly binding to it. In type II TA pairs (b), both the toxin and the antitoxin are proteins where the antitoxin neutralizes the toxin by directly binding to it, resulting in inactive protein complex. Type IV protein antitoxin (d) protects the toxin's target from its toxicity. Type V (e) antitoxin is RNase that counteracts toxin's activity by cleaving its mRNA. Type VI antitoxin (f) is a protein that plays an adaptor role in toxin degradation by proteases. Type VII protein antitoxin (g) neutralizes the toxin by post-translational modifications. Type VIII TA system (h) encodes toxin and antitoxin as RNA molecules. The antitoxin inactivates the toxin via CRISPR system. Adapted from (Jurėnas et al., 2022)

1.2.1 Type I

Type I TA systems consist of an RNA antitoxin and a protein toxin and the antitoxin base pairs with toxin mRNA, resulting in abrogation of toxin translation. These TA systems are either arranged as overlapping, convergently transcribed gene pairs or toxin and the antitoxin genes are located far apart and transcribed divergently (Brantl, 2012). The first discovered type I TA system is *hok/sok* from the R1 plasmid of *E. coli*, which was found due to its post-segregational killing ability (Gerdes et al., 1986). Hok toxin is a small hydrophobic protein that disrupts the bacterial cell membrane and causes cell death in the absence of Sok antitoxin (Thisted & Gerdes, 1992).

1.2.2 Type II

Type II is the most abundant and thoroughly studied class of TA systems (Fraikin et al., 2020). In this class, both toxin and antitoxin are proteins and antitoxin directly binds to the toxin and neutralizes the toxin by creating an inert protein complex (Jurėnas et al., 2022). Liberation of the toxin is usually the result of antitoxin degradation by proteases. Very often the antitoxin and/or toxin-antitoxin complex acts as the repressor of their promoter thus, reducing the total amount of synthesis (Harms et al., 2018).

The classical representative of the type II system is MazEF of *E. coli*. MazF is an endoribonuclease that cleaves single stranded RNA like mRNA and rRNA precursors at the $\sim$ ACA sequence (Mets et al., 2019; Y. Zhang et al., 2003). MazE neutralizes the toxin by directly binding to the active site; MazE and MazE-MazF complex also repress transcription from the *mazEF* operon (Chan et al., 2016). In most cases, type II antitoxins are unstable, and therefore easily degraded due to their protein folding structure or they have disarranged regions which makes them susceptible to degradation (Chan et al., 2016; Cherny et al., 2005; S.-P. Zhang et al., 2020). In type II TA systems antitoxins are often translated more rapidly than toxins, therefore ensuring excess of the antitoxin over the toxin and inhibition of toxins (G.-W. Li et al., 2014; Ruiz-Echevarría et al., 1995).

1.2.3 Type III

The third class of TA systems is represented by RNA antitoxin, which binds directly to the toxin protein and neutralizes it by creating non-toxic complexes (Short et al., 2013). *toxIN* from *Erwinia carotovora* subsp. *Atroseptica* is an example of a type III TA system, where an antitoxin RNA ToxI inhibits the protein toxin ToxN by direct interaction. *toxIN* acts as an anti-bacteriophage defense system by inducing abortive infection (Abi) of the cell in response to bacteriophage in *Erwinia carotovora* (Fineran et al., 2009).

1.2.4 Type IV

Type IV antitoxin is a protein that acts on the target of the toxin and either protects the target from the toxin or detoxifies the target (Jurėnas et al., 2022). A representative of the type IV TA system, *yeeU-cbtA* plays an essential role in bacterial stress response by inhibiting cytoskeletal proteins of *Escherichia coli*. Antitoxin YeeU protects the cell from the toxin by blocking the binding of the toxin to the MreB and FtsZ, which are target proteins of the toxin

CbtA (Masuda et al., 2012). Another example of type IV antitoxin is AtFaRel SAH hydrolase found in *Cellulomonas marina*. This antitoxin degrades the alarmone nucleotides synthesized by the toxSAS (Jimmy et al., 2020).

1.2.5 Type V

Type V antitoxins are RNases and they inhibit the accumulation of toxins by specifically cleaving their mRNAs (Jurėnas et al., 2022). *ghoST* of *E. coli* is an example of a type V TA system. Deletion of the toxin causes a decrease in the biofilm formation indicating a role in biofilm regulation (Wang et al., 2012). Antitoxin GhoS is stable, does not bind to its own promoter and does not control its own transcription (Wang et al., 2012).

1.2.6 Type VI

Type VI antitoxins act as proteolytic adaptors for the degradation of their cognate toxins by proteases. SocB toxin, a part of *socAB* found in *Caulobacter crescentus*, causes the collapse of the replication fork and induction of SOS response in the absence of SocA antitoxin (Aakre et al., 2013). SocA acts as an adaptor for toxin degradation by ATP-dependent protease, ClpXP. Although it is common for most of the TA systems that toxin is relatively stable, it is not the case in SocB toxin since it is degraded constitutively. *socAB* operon is induced in the presence of a DNA-damaging agent, which indicates that this TA system plays a role in regulating of DNA damage response (Aakre et al., 2013).

1.2.7 Type VII

In this TA system, the antitoxin enzyme neutralizes its cognate toxin by post-translational modifications (Wang et al., 2021). An example of a type VII TA system is the HEPN-MNT system found in cyanobacterium *Aphanizomenon flosaquae* (Songailiene et al., 2020). This research showed that HEPN (Higher Eukaryotes Proaryotes Nucleotidase) RNase toxin inhibits translation by cleaving the tRNA and antitoxin MNT (Minimal Nucleotidyltransferase) neutralizes the toxin by di-AMPylation of the toxin. The authors suggest that this TA system functions as a cellular ATP sensor (Songailiene et al., 2020).

1.2.8 Type VIII

This class of TA systems was discovered only recently and is thus less studied compared to other types. In type VIII toxin-antitoxin systems, both toxin and the antitoxin are RNA molecules (Jurėnas et al., 2022). *creTA* is a type VIII toxin-antitoxin system, which is located in the type I-B CRISPR-Cas cassette of *Haloarcula hispanica* and is involved in its maintenance: RNA toxin will become active if any gene of the CRISPR gene cassette is deleted (M. Li et al., 2021). CreT sequesters the rare Arginine t-RNAs (M. Li et al., 2021). This TA system is located in the intergenic region of cassette genes *cas6* and *cas8* and encodes CRISPR-like repeat genes for antitoxin RNA. The RNA antitoxin mimics the crRNA and therefore guides CRISPR to repress the toxin. Damaging the CRISPR system will cause the activation of the toxin. As a result of the action of CreT toxin the CRISPR-Cas system becomes addictive to the cell (M. Li et al., 2021).

1.3 Mechanism of action of TA systems

Toxins of bacterial TA systems affect different processes and targets in the bacterial cell, such as replication, translational machinery, transcription, and cell membrane. One of the most common examples of replication targeting TA system is the type II *ccdAB* system from *E. coli*, which targets gyrase and results in the double-stranded DNA breaks, leading to induction of SOS response (Bernard et al., 1993; Bernard & Couturier, 1992). The second example is FicT toxins from the *ficTA* system family, which inhibit replication by adenylation of DNA gyrase and topoisomerase IV (Harms et al., 2015). Another intriguing example of replication targeting toxin is type IV toxin DarT of *Thermus aquaticus*, which directly acts on the DNA by mediating ADP-ribosylation of ssDNA, causing DNA damage and induction of SOS response (Jankevicius et al., 2016).

The most common target of Type II toxins is protein synthesis (Fraikin et al., 2020). Endoribonuclease activity is the most common mechanism of action for translation targeting toxins (Jurėnas & Van Melder, 2020). Some well-known toxins cleaving mRNA are MazF, MqsR and RelE and rRNA cleaving is VapC (Mets et al., 2017; Winther & Gerdes, 2011). The degree of specificity in mRNA cleaving or tRNA modifying varies in different families, sometimes within the family of toxins. For example, although both AtaT and TacT are GNAT (GCN5-related *N*-acetyltransferases) domain toxins, TacT toxin of *Salmonella typhimurium* targets different types of tRNAs, while AtaT of *E. coli* specifically acetylates initiator tRNA^{fMet} (Cheverton et al., 2016; Harms et al., 2018; Jurėnas et al., 2017). Another

translation-targeting toxin is Doc of *E. coli*, which is a kinase that phosphorylates the translation elongation factor EF-Tu and therefore hinders the tRNA binding to the ribosome (Castro-Roa et al., 2013).

Type I TA systems typically contain a transmembrane domain and cause membrane disruption, resulting in the decline of intracellular ATP (Brantl, 2012; Fozo et al., 2008; Gerdes et al., 1986). In addition to type I toxins, some type II toxin families also target bacterial cell membrane: upon induction of PanT toxin from *Escherichia coli* and PanT toxin from *Helicobacter* sp. membrane depolarization occurs (Kurata et al., 2022).

1.4 Biological roles of TA systems

The diversity and mobility of TA systems have led to many discussions in the TA field. Moreover, because these small modules are not highly conserved, yet are also found in the chromosomal DNA of bacteria has led to possibilities of different adaptation mechanisms through evolution. Below, some of the main suggested biological functions of toxin-antitoxin systems are discussed.

1.4.1 TA-s as plasmid addiction modules

The first identified TA system, which is a type II system from *E. coli* named *ccdAB* plays a role in plasmid maintenance (Ogura & Hiraga, 1983). The antitoxin CcdA is unstable and needs to be continuously synthesized to neutralize toxin CcdB. If the cell loses the plasmid which carries the *ccdAB* system, antitoxin cannot be anymore synthesized and is rapidly degraded while the stable CcdB becomes active and leads to cell death (Bernard & Couturier, 1992; Van Melder et al., 1994). This phenomenon is known as post-segregational killing (PSK). Another fascinating example of TA-s mediating plasmid addiction is the type I *hok/sok* TA system found in the R1 plasmid of *E. coli*, the daughter cells which failed to carry this plasmid die as a result of PSK mediated by the Hok toxin (Thisted & Gerdes, 1992). Due to many examples of TA systems found mediating plasmid maintenance or genomic regions addiction features, it has been hypothesized that these elements increase the fitness of the plasmids that competes for the same host (Jurėnas et al., 2022).

1.4.2 TA-s as anti-addiction modules

It has been suggested that chromosomal toxin-antitoxin systems selectively protect their host organism against plasmid borne TA systems, which mediates post-segregational killing (Melderen & Bast, 2009). In short, the chromosomal TA system provides the antitoxins to inhibit the toxins from the plasmid, thus freeing the bacteria from addiction. A remarkable example of this phenomenon is the chromosomal *ccd_{Ech}* TA system of *Erwinia chrysanthemi*, which defends the host cell against *E. coli* plasmid borne TA system *ccd_F*. Antitoxin of *ccd_{Ech}* neutralizes the *ccd_F* toxin and prevents PSK upon plasmid loss (Saavedra De Bast et al., 2008). Thus, anti-addiction might be the role of some chromosomal TA systems (Melderen & Bast, 2009).

1.4.3 TA-s role in stress modulation

The bacterial chromosomes encode several TA systems, which play an essential role in bacterial stress response. For example, AA (amino acid) starvation induces *relBE* transcription in *E. coli* (Christensen et al., 2001). During metabolic stress, the RelE toxin hinders translation by cleaving mRNA in the ribosomal A site (Pedersen et al., 2003). In non-stress conditions, RelB antitoxin is expressed more compared to RelE and rescues cells by overexpressing tmRNA, whereas during stress conditions antitoxin gets degraded by Lon protease (Christensen et al., 2001). Another example is MqsA antitoxin from *E. coli* shown to be degraded by Lon protease during oxidative stress (Wang et al., 2011). In non-stress conditions, MqsA represses the production of RpoS, which is an important regulator for stress-related genes. Therefore, the de-repression of *rpoS* by MqsA degradation results in the transcription of genes for resistance to oxidative stress, low pH, heat shock, etc.

1.4.4 The role of TA systems in phage defence

TA systems have been found to play an important role in phage defence via different strategies. For example, CapRel system encoded by the *Salmonella* temperate phage SJ46 gets activated during SECΦ27 phage infection; the antitoxin domain of CapRel interacts with phage capsid protein which triggers activation of the TA system, and infected cells commit suicide via inhibiting translation (T. Zhang et al., 2022). This phenomena is known as “abortive infection”, where infected cells get killed by themselves in order to protect the whole bacterial population. In the case of the CapRel TA system, toxin inhibits the translation

by tRNA pyrophosphorylation and therefore bacterial protein synthesis machinery shuts down, which leads to the cell death before phage propagation (T. Zhang et al., 2022). Another well-studied example of TA system mediated bacteriophage defence is type II TA system *rnlAB* from *E. coli* K12 strain. This system protects bacteria against T4 bacteriophage *dmd* mutant, with ability to decrease plaquing in *dmd* lacking phage (LeRoux & Laub, 2022). When T4 *dmd* mutant infects the *E. coli*, unstable RnlB antitoxin gets degraded, which is accompanied by liberation of endoribonuclease RnlA toxin (Koga et al., 2011).

The phages have evolved several mechanisms for fighting against bacteria's immunity elements (including the TA systems), in other words, these systems are called "Anti-anti-phage defence systems". As mentioned earlier, *rnlAB* system only gets activated by the infection of *dmd* lacking T4 phages, because *dmd* protein mimics the RnlB antitoxin and neutralizes the toxin by binding directly to it (Otsuka & Yonesaki, 2012). This is so-called "antitoxin mimicking" ability which phages gained in the ongoing war between phages and bacteria (LeRoux & Laub, 2022). Another mechanism against defensive elements is blocking the activation of toxin-antitoxin system. Compared to other well-known elements (CRISPR and Restriction-modification systems) of bacterial immune system, toxin-antitoxin systems have higher specificity towards certain phages and they have more diversity in the mechanism of action.

1.5 Distribution of TA systems

TA-s are highly variable modules among different phylae of prokaryotic life and this diversity and variability is linked to their relationship with mobile elements such as prophages, plasmids, transposons etc. In addition to dynamicity of this modules, one study showed that, even though TA systems are abundant in free living organisms, almost none or very few TA pairs have been found in obligate intracellular species. Authors associates this scarcity with the fact that, intracellular bacteria mostly live in stable environment and evolutionarily do not have to adapt to changing environments (Pandey & Gerdes, 2005; Xie et al., 2018).

1.5.1 Tools for discovering new TA systems

Experimentally, shotgun cloning has been used for the identification of toxin-antitoxin systems in bacterial cells (Sberro et al., 2013). With increasing genome sequencing data, bioinformatics tools have become more and more popular in recent years. The conserved

bicistronic operon structure of TA systems allows us to analyse conserved gene neighbourhoods and possibly find new toxin-antitoxin modules. Also, the role of TA systems in phage immunity opened new gates to discover novel systems (Millman et al., 2022; Vassallo et al., 2022).

1.5.2 NetFlax

It has been shown that TA systems relocate through evolution by horizontal gene transfer and specifically type II TA-s swap their partners (Akarsu et al., 2019; Leplae et al., 2011; Makarova et al., 2011). Based on this property of type II toxin-antitoxin systems, our group discovered many potential TA systems using different bioinformatics approaches. Panacea antitoxin domain (named after the Greek goddess of universal remedy) is a striking example of how extensively TA system can exchange their partners (Kurata et al., 2022). The antitoxins containing PanA (Panacea) domain is able to neutralize multiple non homologous and non-cognate toxins just with two substitutional mutation, which suggests this domain has intrinsic properties enabling to neutralize dozens of diverse domains containing toxins (Kurata et al., 2022). After discovery of the Panacea, NetFlax was developed based on FlaGs (standing for flanking genes; gene neighbourhood analysis program, <https://server.atkinson-lab.com/webflags>), which aimed to uncover new toxin-antitoxin network/systems with focus on Panacea (Ernits et al., 2023; Saha et al., 2021). TA system is used as the input for the program which searches for all of the possible homologs of toxins and antitoxins in the database and checks whether they are in conserved bicistronic operons. If the TA member is found with a different partner, it is considered a hit (if this gene is conserved in at least 8 different genomes) and the algorithm will use the new partners as the basis of a new run. The cycle will continue until no new partners are found. The final result will be a network of interconnected TA systems (Figure 2.).

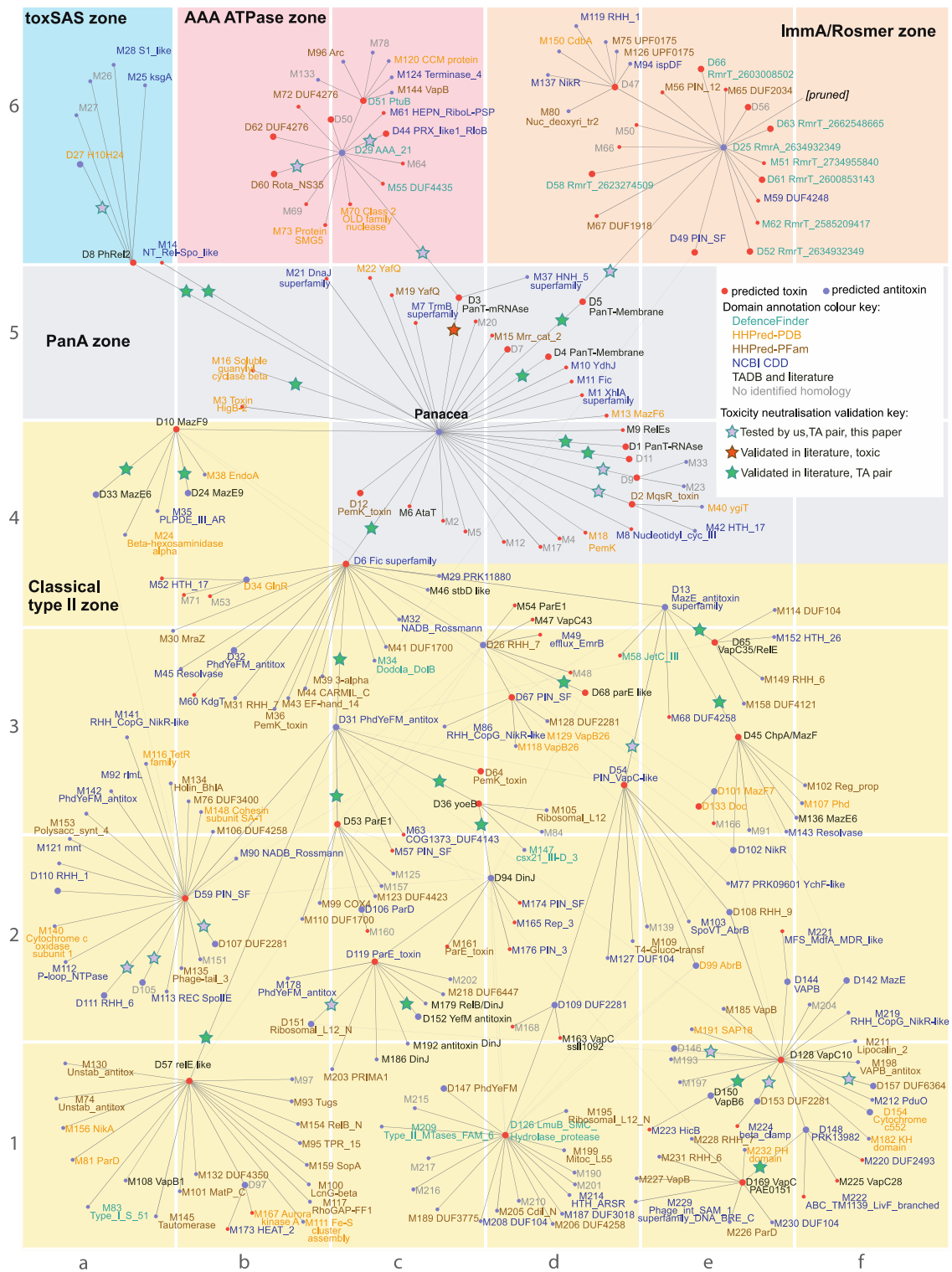


Figure 2. The final NetFlax network. In the above centre the starting input hyperpromiscuous Panacea antitoxin domain is shown. The predicted toxins are marked with red circles and predicted antitoxins are illustrated with blue circles. The formerly validated toxins are marked with orange stars and green stars show previously verified TA systems (Ermitis et al., 2023; Kurata et al., 2022).

In addition to NetFlax, AlphaFold2 was utilized to unveil structural fold of toxin and antitoxins. By exploring 24,479 representative genomes, the NetFlax algorithm revealed a core network of TA-like bicistronic modules (Ernits et al., 2023).

The final proteinaceous NetFlax network is composed of 278 nodes: 107 of these are predicted as toxins and the remaining 171 nodes are antitoxin like proteins. The TA pairs, which has been found to be at least in 8 genomes are named as D (D stands for central Domain) nodes (Ernits et al., 2023). Following the less strict final guilt by association step, Mininodes shortly called as M nodes have been annotated in the network. Functionally, the network was divided into 5 zones:

1. The anti-toxSAS zone – toxins from this zone are related to RelA/SpoT alarmone synthetase domains and are expected to modify tRNA (Kurata et al., 2021) resulting in the inhibition of the translation.
2. AAA ATPase zone – antitoxins from this zone contain AAA ATPase domain and they use the energy provided by ATP hydrolysis to neutralize the toxin by inducing conformational changes (Yedidi et al., 2017).
3. ImmA/Rosmer zone – antitoxins located in this zone have ImmA protease domain and this domain recently has been found in several RosmerTA systems, which has been shown to mediate important role in phage defence (Millman et al., 2022; Vassallo et al., 2022). ImmA domain has been shown to cleave repressor ImmR of Integrase by cleaving HTH (Helix-Turn-Helix) domain (Bose & Grossman, 2011).
4. PanA zone – This zone contains TA systems which their antitoxins directly carry Panacea domain.
5. Classical Type II TA systems zone – Being the largest zone of NetFlax network, we rediscovered many canonical type II toxins with alternate antitoxin partners.

2. THE AIM OF THE THESIS

The aim of my bachelor thesis was to study the mechanism of actions of toxins discovered by the bioinformatics tool NetFlax.

More specifically, the aims were:

- I) test the toxicity of the toxin in *E. coli*.
- II) to unveil which major cellular process - replication, transcription or translation - is inhibited by the toxin.

3. EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strains, antibiotics, and media

In this work I used *E. coli* BW25113 as standard bacterial strain (Datsenko & Wanner, 2000) I transformed IPTG inducible pMG25::ampR (Jaskólska & Gerdes, 2015) (empty vector, does not carry the antitoxin gene) and pBAD33::camR (Guzman et al., 1995) arabinose inducible (carries appropriate toxin gene) plasmids into *E. coli* BW25113 chemical competent cells. Carbenicillin (CB) (Fisher Bioreagents) and chloramphenicol (CAM) (PanReac Applichem ITW reagents) were used for plasmid selection. The construction of plasmids was done by supervisors Toomas Mets and Tetiana Brodiazhenko.

The composition of liquid nutrient (MOPS) (Neidhardt et al., 1974) media presented in the next tables (Table 1 and 2).

Table 1. Composition of 1 % Glucose MOPS medium (for 100 ml)

Fresh MilliQ	< 80 ml
Casamino acid	0,1 g
10x MOPS	10 ml
132 mM K ₂ HPO ₄	1 ml
2 mg/ml Thiamine	5 µl
40 % Glucose	2.5 ml

To prepare the 1% glucose MOPS I mixed the components in Table 1. and adjusted the volume up to 100 ml and pH to 7.2 using HCL. Next, I sterilized the media using a 0.2 µm filter. I prepared and sterilized the medium next to the Bunsen burner flame.

Table 2. Composition of 0.5 % Glycerol MOPS medium (for 500 ml)

Fresh MilliQ	< 360 ml
10x MOPS	50 ml
132 mM K ₂ HPO ₄	5 ml

2 mg/ml Thiamine	25 µl
10x AA sol. (25 mg/ml of each AA in 10x solution, -Met)	50 ml
50 % Glycerol	5 ml

To prepare the 0.5% glycerol MOPS I mixed the components in Table 2. and adjusted volume to 500 ml and pH to 7.2 using HCL, also sterilized the media using 0.2 µm filter. I prepared and sterilized the medium next to the Bunsen burner flame.

I used glucose containing MOPS media for overnight bacterial (2 ml) culture and glycerol containing media for culture used next day for metabolic labelling experiment (15 ml) with appropriate antibiotics.

3.1.2 Chemically competent cell preparation

I inoculated single colony of *E. coli* BW25113 in 2 ml of LB (BD Difco™ LB broth, Lennox) medium and let it grow overnight at 37° C, shaking at 200 RPM (Centrifuge 5415 R, Eppendorf). The next day, I added 0.2 ml of overnight culture into 20 ml of LB in 250 ml Erlenmeyer flask and let it grow until early log phase (until OD₆₀₀ = 0.2). Next, I aliquoted 1 ml of culture into 1.5 ml tubes and centrifuged the cells at 5000 RPM (Centrifuge 5415 R, Eppendorf) for 5 mins at 4° C. I discarded the supernatant and resuspended the pellets in 200 µl of cold TSB (Chung & Miller, 1988) (Table 3). After this step the cells are chemically competent and ready for transformation, also they can be frozen using dry ice-ethanol or liquid nitrogen bath and stored at -80° C.

Table 3. Composition of TSB buffer

LB broth	100 ml
PEG	10 g (MW = 3.350)
DMSO (100%)	5 ml
1 M MgCl ₂	2 ml

3.1.3 Bacterial Transformation

I took competent *E. coli* BW25113 cells from -80° C freezer and appropriate plasmids from -20° C and put them on the ice to melt for 10 min. Next, I added 0.5 µl (> 50 ng/µl) of

pBAD33 carrying a toxin gene and empty pMG25 vector to the 50 µl of competent cells into 1.5 ml tube. After the incubation of mixture of plasmids and competent cells on ice for 25 minutes, I performed a heat shock for 35 second. Immediately after heat shock, I put the mixture on ice and incubated for 3-5 minutes. I added 950 µl of 0.2% glucose LB medium to the mixtures and put the transformants to 37° C shaker machine at 750 RPM (Thermomixer compact, Eppendorf) for 1 hour. After recovery, I centrifuged the cells for 5 min at 4.5 RPM (Centrifuge 5415 R, Eppendorf) to get the cell pellets and removed the supernatant so that 100-200 µl of media was still left in the tube. Finally, I resuspended the pellets in remaining supernatant and plated on non-induction (25 µg/ml CAM, 100 µg/ml CB, 0.2% glucose) and induction (25 µg/ml CAM, 100 µg/ml CB, 1mM IPTG, 0.2% arabinose) LA (BD Difco™ LB Agar, Lennox) plates for overnight. As a result, we end up with *E. coli* BW25113 cells transformed with an appropriate plasmid pair that contain toxin needed to be tested (pBAD33 vector, the toxin is expressed under the control of L-arabinose and empty vector where usually antitoxin is located (pMG25 derivatives, IPTG-inducible promotor).

3.1.4 Toxicity validation assay

As a first step, I co-transformed appropriate plasmids into *E. coli* BW25113 cells as described above. Following, I inoculated single colony of transformant into 2 ml of LB broth containing 25 µg/ml CAM and 100 µg/ml CB and 0.2 % glucose and grew the culture at 37° C overnight. The next day, I diluted overnight culture to the OD₆₀₀ = 1.0 with LB medium. For preparing dilutions I used sterile 96-well Falcon plate, I added 90 µl of LB broth to the needed wells next to the Bunsen burner flame. Next, I added 10 µl of diluted culture to the 1st row of wells, resuspended it and trashed the pipette tip. I performed a series of 10x serial dilutions by transferring 10 µl of culture to the next row of wells (from 10⁻¹ to 10⁻⁸). Afterwards, I spotted 5 µl of dilutions to the non-induction (25 µg/ml CAM, 100 µg/ml CB, 0.2% glucose) and induction (25 µg/ml CAM, 100 µg/ml CB, 1mM IPTG, 0.2% arabinose) plates using a multi-channel pipette. I dried the plates by the flame. The plates were incubated at 37° C for overnight. The picture of the induction and non-induction plates have been taken next morning using the scanner (Epson Expression 1680 Pro).

3.1.5 Metabolic labelling

I did metabolic labelling using next isotopes: ³⁵S-methionine, ³H-uridine or ³H-thymidine. For metabolic labelling experiments, *E. coli* BW25113 strains co-transformed with pBAD33 derivatives (for L-arabinose-inducible expression of toxins) as well as the empty pMG25

vector were first plated out on LB plates supplemented with 100 µg/ml carbenicillin, 25 µg/ml chloramphenicol and 0.2 % glucose (to suppress the leaky expression of the toxin). Next, I used fresh *E. coli* colonies for inoculation into 2 ml liquid cultures were prepared in defined Neidhardt MOPS minimal media (Table 1.) supplemented with appropriate antibiotics, 0.1% of casamino acids, and 1% glucose, and grown overnight at 37°C with shaking. Next day, I diluted overnight culture to OD₆₀₀ 0,05 into 15 ml of MOPS medium supplemented with 0.5% glycerol, 25 µg/ml of 19 amino acids (lacking methionine), and appropriate antibiotics. These cultures grown at 37°C with shaking up to of OD₆₀₀ 0.2. At this point, one 1-ml aliquot (the pre-induction zero time-point) was transferred to 1.5 ml Eppendorf tube containing 10 µL of radioisotope—either ³⁵S-methionine (4.35 µCi, Hartman), or ³H-uridine (0.65 µCi, Hartman) or ³H-thymidine (2 µCi, Hartman)—and transferred to the heat block at 37°C. Immediately after that, I induced the toxin in the remaining culture by addition of L-arabinose (final concentration of 0.2%). Throughout the toxin induction time course, 1-ml aliquots were taken from induced culture and transferred to 1.5 ml Eppendorf tubes containing 10 µl of radioisotope (³⁵S-methionine /³H-uridine /³H-thymidine) (Figure 3.). The incorporation of radioisotopes was stopped after 8 minutes of incubation at 37°C by adding 200 µl of ice-cold 50% trichloroacetic acid (TCA) to 1 ml cultures. In parallel with taking the time-points for labelling, 1 ml aliquots were taken for OD₆₀₀ measurements. The samples were filtrated using GF/C (Whatman) filters and the filter with the sample was washed twice with 5 ml 5 % TCA and 95 % Ethanol. The filters were dried for at least 2 hours at room temperature. In the end, 5 ml of scintillation cocktail (EcoLite (+)™) per vial was added to the samples, after 15 minutes of shaking, using the TRI-CARB 4910TR 100 V scintillation counter radioactivity levels were measured. Isotope incorporation was quantified by normalising radioactivity counts (CPM) to OD₆₀₀, with the pre-induction zero time-point set as 100%. All experiments were performed in three biological replicates (i.e., using three independent cultures inoculated from three different colonies). For statistical analyses of the results Microsoft Excel was used and standard deviation for 3 biological replicas were calculated using SDV.P formula in Microsoft Excel.

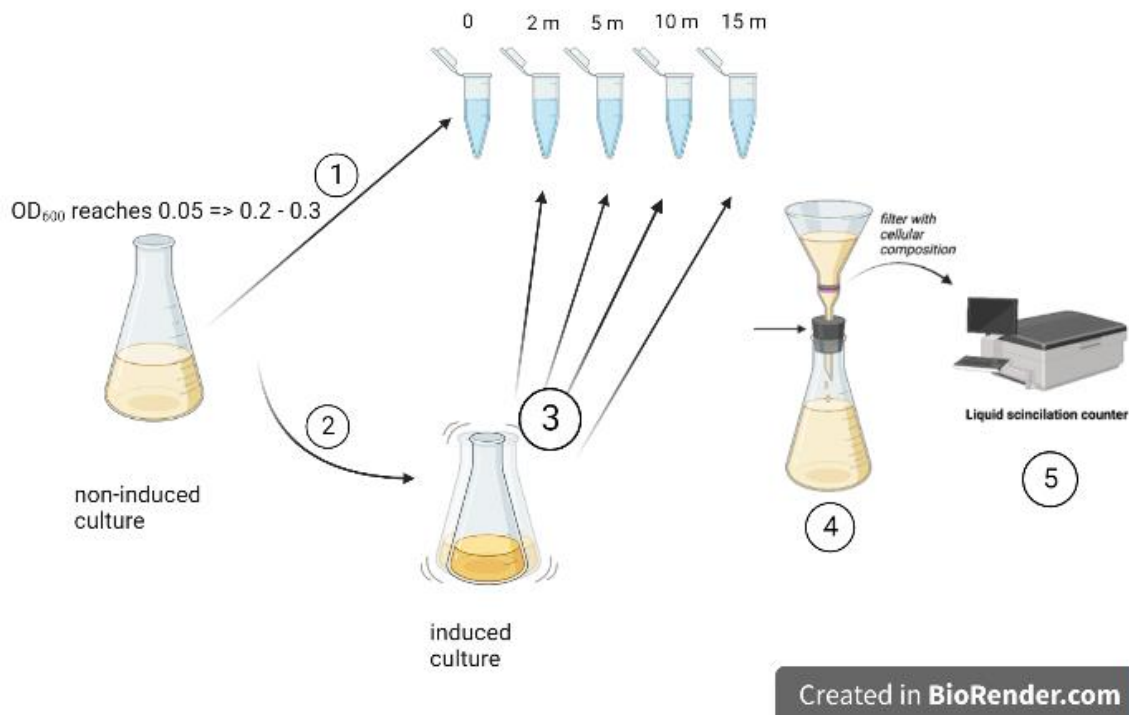


Figure 3. Metabolic labelling experiment. 1) The radioisotope incorporation starts when the OD₆₀₀ of bacterial culture reaches 0.2-0.3. Firstly, 1 ml of bacterial culture is transferred to 0 min Eppendorf tube containing the appropriate isotope solution. 2) Secondly, the bacterial culture containing the toxin plasmid gets induced by the addition of Arabinose at a final concentration of 0.2. 3) Next, at 2, 5, 10, 15 minute time points 1 ml of bacterial culture is added to the tube containing isotope solution. The isotope incorporation reaction for all time point samples lasts for 8 minutes and the reaction is stopped by adding ice-cold 50 % TCA to the tube. 4) In fourth step, the samples were filtrated using 1.2 µm filters and left to dry for at least 2 hours. 5) In the final step, 5 ml of scintillation cocktail was added on the sample per vial and using TRI-CARB 4910TR liquid scintillation counter radioactivity levels were estimated. The figure is created with BioRender.com

3.2 RESULTS AND DISCUSSION

3.2.1 AAA ATPase antitoxin zone toxins

The first TA pair of this zone which was tested, is found in the gram-negative bacteria *Acinetobacter guerrae*, where antitoxin is shown in the center of AAA ATPase zone and the toxin is named as AarT. The AAA ATPase domain is found in several phage defence systems mediating abortive infection, such as Gabija (Cheng et al., 2021) and Septu (Doron et al., 2018). Therefore, we suspect that the new TA pairs which contain AAA ATPase domain in their antitoxin gene also may play a role in the phage defence.

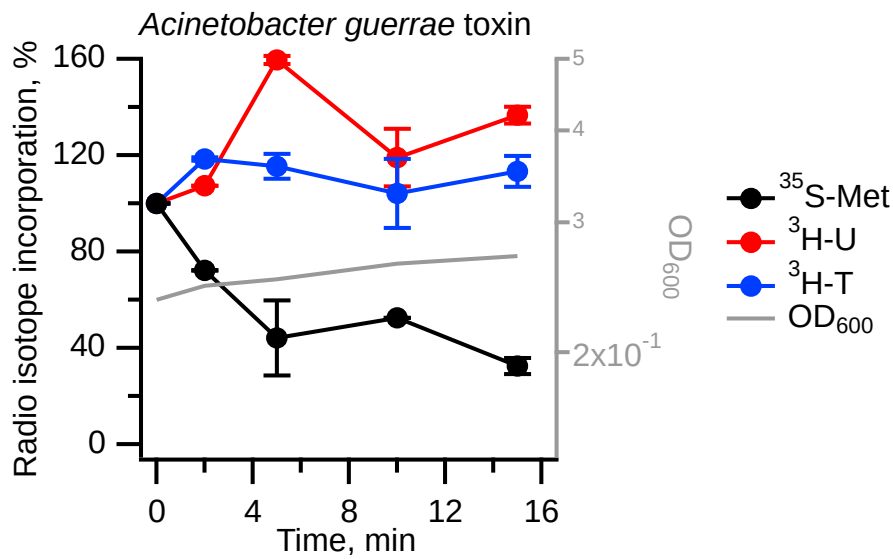


Figure 4. Metabolic labelling of *Acinetobacter guerrae* toxin found in AAA ATPase zone. *E. coli* BW25113 was used as the host. The cells were grown in MOPS glycerol 0.5% media until OD₆₀₀ = 0.2, toxin expression was induced by adding L-Arabinose 0.2% final concentration. 1 ml of sample was collected at timepoints 0' (uninduced), 2', 5', 10' and 15'; radioisotope incorporation was performed for 8 minutes at 37°C and stopped. Samples were filtered and analysed with liquid scintillation counter.

My colleagues found that the toxin of this module has the same fold structure as mRNA interferases, which is quite common for type II toxin-antitoxin systems. Therefore, this domain was named as AarT (AAA-associated RNase-like toxin) (Ernits et al., 2023). As expected, RNase-like *Acinetobacter guerrae* toxin inhibits translation, as we can see from the ³⁵S-methionine incorporation in Figure 4. Interestingly, the rate of transcription increases during the inhibition of translation which is indicated by ³H-uridine incorporation. This phenomenon is mostly likely due to a decline in ATP consumption which was also observed

before upon the effect of antibiotics or other translation-targeting toxins (Jimmy et al., 2020; Kurata et al., 2021).

The second toxin from this zone is found in the gram-positive bacterium *Streptococcus agalactiae*. Here, antitoxin has the same domain as *Acinobacter guerrae* antitoxin and the toxin is named as TOPRIM_OLDD₆₀. TOPRIM stands for topoisomerase-primase endonuclease, and it is associated with OLD – Qvercome Lysogenisation Defect nuclease families. Metabolic labelling result showed that (Figure 5.) the translation rate is inhibited up to 20% based on ³⁵S-methionine incorporation, which is an anticipated result for an endoribonuclease.

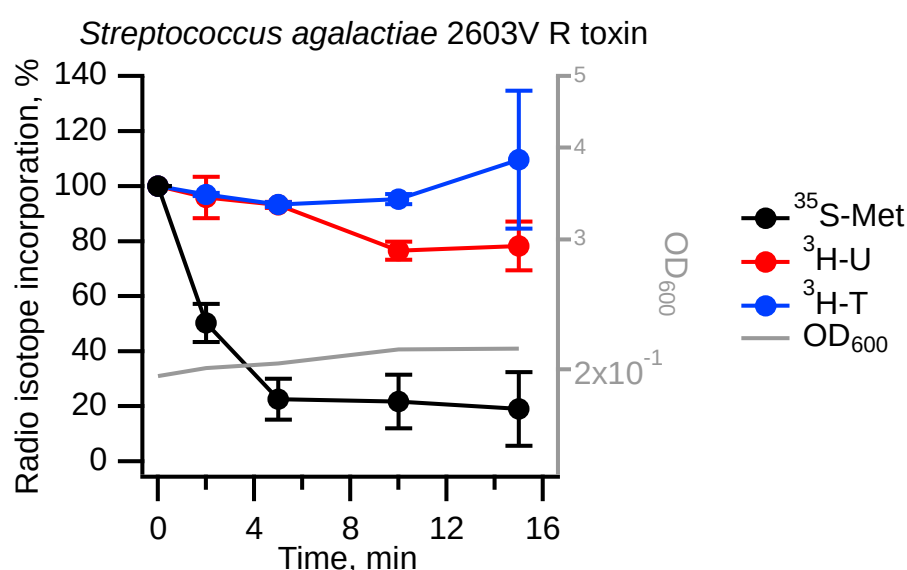


Figure 5. Metabolic labelling of *Streptococcus agalactiae* toxin from AAA ATPase zone. Labelling was conducted as described in the Figure 4. legend.

3.2.2 ImmA/RosmerA zone toxins

Antitoxins from the ImmA zone belong to the ImmA/IrrE family of metallo-endoropeptidase, which are predicted to break down nonterminal peptide bonds of amino acids by the participation of metal ions (Glucksman et al., 1992). Also, ImmA protease-containing systems have been detected in RosmerTA systems, where different toxins (RmrTs) are paired with the protease domain-containing antitoxin (RmrA) (Ernits et al., 2023). A novel example of a toxin located in this zone is from ESKAPE pathogen bacterium *Acinetobacter baumannii*, where antitoxin contains the RmrA domain, and the toxin domain is unknown. Unfortunately, the metabolic labelling experiment of this toxin (Figure 6.) did not give us a clear result. That

may indicate that this toxin acts for a longer period or does not target or affect any of the processes we tested.

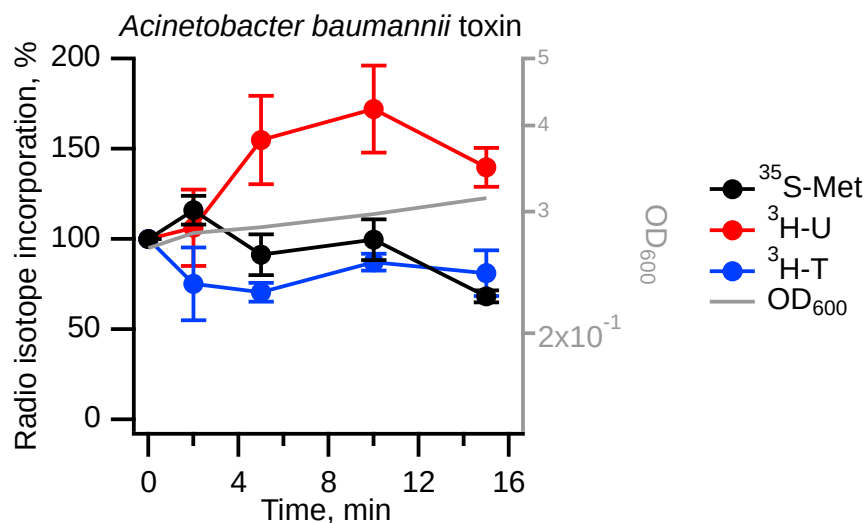


Figure 6. Metabolic labelling of toxin found in *Acinetobacter baumannii* toxin from ImmA/RosmerA zone. Labelling was conducted as described in the Figure 4. legend.

Another example toxin from this zone was found in *Vibrio sinensis* species and its antitoxin also contains the RmrA domain; however, the toxin contains the DUF5986 domain, (DUF stands for Domain of Unknown Function) (Ernits et al., 2023). Interestingly, the metabolic labelling results of this toxin were inconclusive as well (Figure 7).

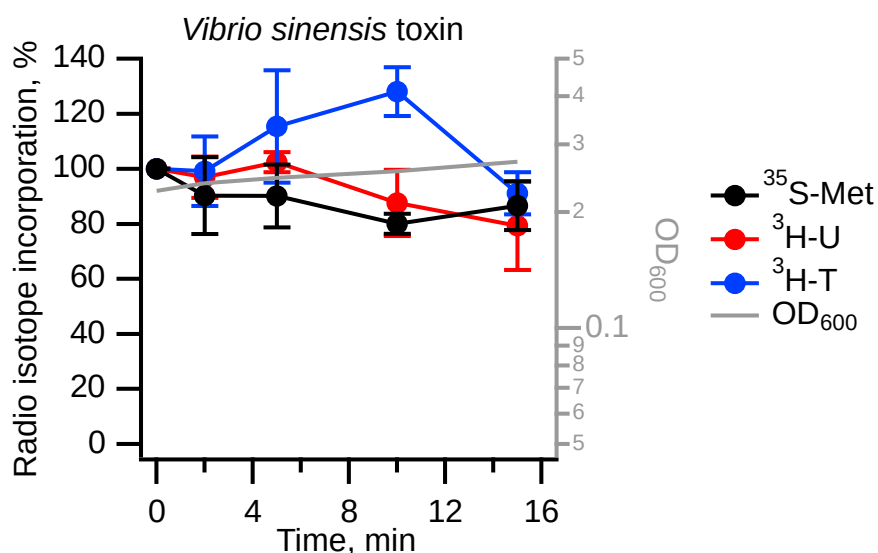


Figure 7. Metabolic labelling of toxin found in *Vibrio sinensis* toxin from ImmA/RosmerA zone. Labelling was conducted as described in the Figure 4. legend.

After getting indeterminate labelling results from *Acinetobacter guerrae* toxin, we speculated that the toxin gene might be mutated and thus acted differently. Therefore, we performed Toxicity assay for *Acinetobacter guerrae* toxin using empty vector containing bacterial cells as a negative control and *Vibrio sinensis* toxin containing cells as a positive control (Fig 8.). In the end, my results were similar to the results which my colleagues obtained in Lund University.

10-fold dilutions	Non-induced								Induced							
	-1	-2	-3	-4	-5	-6	-7	-8	-1	-2	-3	-4	-5	-6	-7	-8
Vector control																
<i>Acinetobacter baumannii</i> Toxin																
<i>Vibrio sinensis</i> Toxin																

Figure 8. Toxicity assay for *Acinetobacter baumannii* toxin. *E. coli* BW25113 cells transformed with pBAD33 and pMG25 empty plasmids as a vector control. *E. coli* BW25113 *Acinetobacter baumannii* toxin contains pBAD33 with toxin gene plasmid and empty pMG25. *E. coli* BW25113 *Vibrio sinensis* toxin contains pBAD33 with toxin gene plasmid and empty pMG25. Bacterial cells carrying different plasmids sets were plated on LA containing 25 µg/ml of chloramphenicol, 100 µg/ml of carbenicillin and either 0.2 % glucose for non-induction condition or 0.2% L-arabinose and 1 mM IPTG for induction condition.

Therefore, we can conclude that our toxin construct is valid and that the targeted process might be not tested in the labelling experiment. This toxin might affect the cell division or target SOS system which need to be checked in the future. Also, these two toxins are from nodes that are located at the termini of the NetFlax generated network where the uncertainty of predictions is high, so they might not constitute a TA pair at all (Ernits et al., 2023).

3.2.3 Classical Type II toxins

In addition to other zones, NetFlax tool found many classical type II toxins paired with alternative antitoxin partners. The first TA example from this zone is from *Thiomicrospira cyclica* and toxin contains classical endoribonuclease PIN VapC-like domain and was therefore predicted to disrupt global translation in the cell. Indeed, as we can see from metabolic labelling experiment, translation rate is clearly disrupted (Fig. 9).

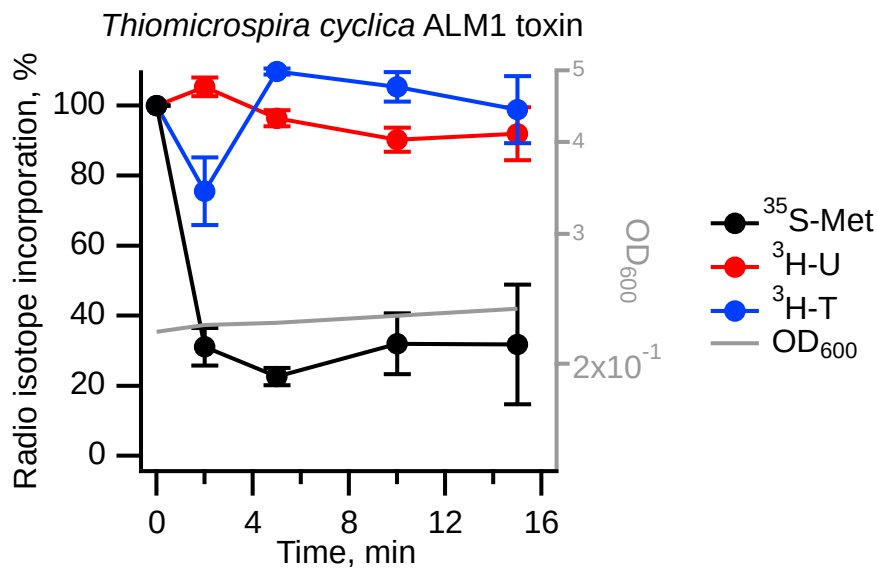


Figure 9. Metabolic labelling of toxin from *Thiomicrospira cyclica* toxin from classical type II TA zone. Labelling was conducted as described in the Figure 4. legend.

The final example from this zone we tested is from *Desulfonatronovibrio magnus* species that contains toxin with YafQ domain, which is a sequence specific mRNA endoribonuclease. From metabolic labelling data I showed that translation is inhibited upon expression of the toxin (Fig. 10).

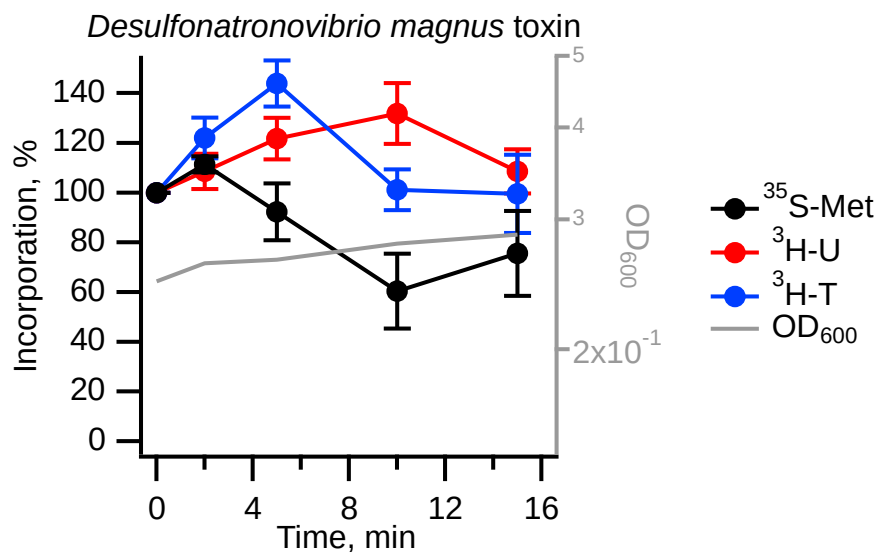


Figure 10. Metabolic labelling of *Desulfonatronovibrio magnus* toxin from classical type II TA zone. Labelling was conducted as described in the Figure 4. legend.

3.2.4 The anti-toxSAS zone toxins

The TA system I tested from this zone is found in an anaerobic bacterium *Clostridium hylemonae*. The toxin contains PhrE2 domain which has been previously shown to inhibit translation via modifying tRNA, therefore we expected similar effect for this toxin. (Kurata et al., 2021). The antitoxin of this system is named PAD1 (standing for Panacea Associated Domain 1) (Kurata et al., 2022). Remarkably, although this antitoxin does not carry the Panacea domain, it is still able to neutralize its PhrE2 toxin, proving that the PAD1 domain alone is able to neutralize toxins (Ernits et al., 2023). The toxin did not have any clear effect in my metabolic labelling assay (Figure 11.). As the *E. coli* seems to easily select for suppressor mutants against this toxin (data not shown) the experiments should be repeated.

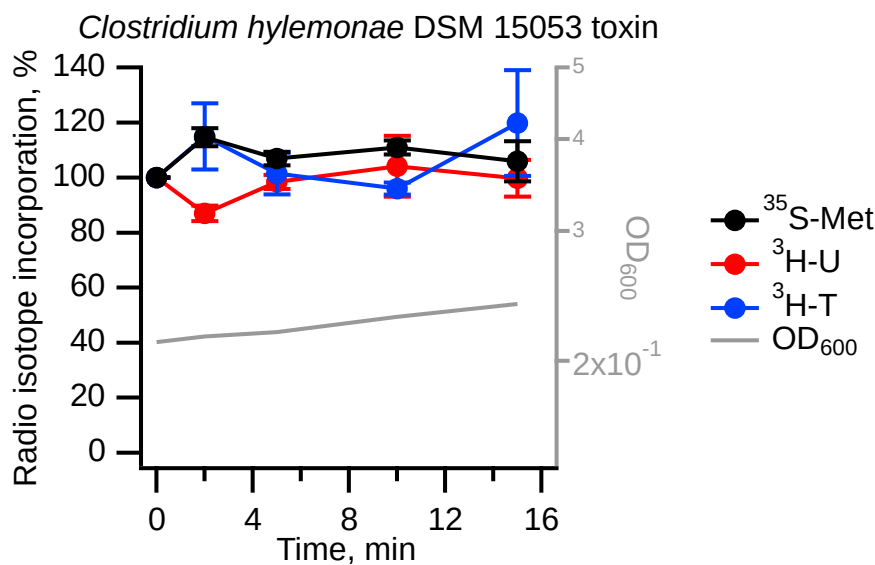


Figure 11. Metabolic labelling of *Clostridium hylemonae* toxin from classical type II TA zone.

Labelling was conducted as described in the Figure 4. legend.

SUMMARY

Toxin-antitoxin systems are involved in bacterial immunity. Nowadays, bacteriophage therapy is a promising alternative strategy against antibiotic multi resistance bacterial pathogens. Thus, studying TA systems helps us to improve bacteriophage therapy. Our group developed a bioinformatics tool NetFlax to search for novel type II TA pairs. The program leverages on the fact that the type II TA systems are in a bicistronic configuration and often swap partners. Our approach resulted in network of TA systems which contained many novel predicted TA pairs. The findings of NetFlax were functionally divided to five topological zones: 1) a zone with toxins related to RelA/SpoT alarmone synthetases 2) the classical type II TA system zone 3) The AAA ATPase antitoxin domain containing zone 4) a zone with ImmA/RosmerA antitoxins 5) a zone of TA systems which contain Panacea antitoxin domain. This thesis focuses on studying the mechanisms of action of the toxins from the newly discovered TA systems using metabolic labelling. I tested the incorporation of different radioisotopes within *E. coli* BW25113 cells following the induction of toxins of interest. The labelling experiment allowed me to test the effects of toxin replication, transcription and translation. Most of the novel toxins inhibited translation in the bacterial cell. Induction of *Acinetobacter guerrae* toxin from AAA ATPase zone and *Desulfonatovibrio magnus* toxin from Classical type II zone in both cases led to inhibition of translation and to increase of transcription. Only translation was affected by *Streptococcus agalactiae* toxin from AAA ATPase zone and *Thiomicrospira cyclica* toxin from classical type II TA zone. The studied toxins from ImmA/RosmerA zone did not have any clear effect on the tested cellular processes, which suggests that they either affect other cellular processes or do not constitute TA pairs. Finally, the *Clostridium hylemonae* toxin from the anti-toxSAS zone also failed to inhibit any of the tested processes in our conditions. Suppressor mutants against the toxin appear rapidly which could affect the results our experimental results. My initial results are the basis for further experiments to study the mechanism of the tested toxins.

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