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IDENTIFYING DETERMINANTS OF TARGET SPECIFICITY IN TWO RELATED BACTERIAL PEPTIDE TOXINS

By

Andrew Holmes

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to examine the thesis of Andrew Holmes
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ABSTRACT

Identifying determinants of target specificity in two related bacterial peptide toxins

Andrew Holmes

Director: Keith Weaver Ph.D.

Toxin-antitoxin (TA) systems were originally identified as two-component systems ensuring the stable inheritance of plasmids in bacterial populations. Recently, they have been identified on bacterial chromosomes where their functions remain mostly undefined. The *par* locus of *E. faecalis* plasmid pAD1 (par_{pAD1}) was the first TA system defined in a Gram-positive bacterium and a homolog encoded on the *E. faecalis* chromosome (par_{EF0409}) was later described. Related loci numbering in the hundreds have been identified throughout Gram-positive bacteria based on homology to the toxin of the system, Fst, and similarities in genetic organization and regulation. Despite their similar sequences, over-expression of Fst_{pAD1} and Fst_{EF0409} have differing effects on the host transcriptome, suggesting that sequence differences between the toxins are fine-tuned for distinct functions. Using a combination of domain swaps, as well as single and double amino acid changes, we identified key amino acid residues between Fst_{EF0409} and Fst_{pAD1} critical for triggering an Fst_{pAD1}-like response. This finding helps define the critical region of toxin specificity and will aid in determining the mechanism of action of this large family of peptide toxins.

Keywords: toxin-antitoxin systems, *E. faecalis*, plasmid pAD1, *par*EF0409

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CHAPTER 1

Introduction

Overview of Toxin-antitoxin Systems

Bacterial toxin-antitoxin (TA) systems are bipartite systems encoding a toxin that inhibits cellular processes and an antitoxin that neutralizes the effects of the toxin. The balance between toxin and antitoxin is essential; the antitoxin/toxin ratio must be high enough for the antitoxin to neutralize the toxin effects. TA systems are widespread being found in almost all phyla of bacteria and are conserved evolutionarily, suggesting that they maintain a functional role in the bacterial cell (Akarsu et al., 2019; Fozo et al., 2010). Additionally, TA systems can exist in a variety of genetic forms; they were first discovered to be encoded within F and R1 plasmids, but they also have been more recently identified on a multitude of different bacterial chromosomes such as locus EF0409 in *Enterococcus faecalis* (*E. faecalis*) strain V583 (Ogura and Hiraga, 1983; Gerdes et al., 1986; Weaver et al., 2009). The distribution of TA systems within bacteria can possibly be attributed to their genetic transfer ability, horizontally and vertically, as part of the bacterial mobilome--which is a group of mobile genetic elements (MGE) like transposons, plasmids, phages and more (Guglielmini and Van Melderen, 2011).

Along with their prevalence in bacteria, TA systems are diverse in their molecular mechanism and can be divided into groups based on their method of toxin-antitoxin interaction. TA systems have been classified into six different types. The review by Harms et al. (2018) discusses the four primary TA modules as determined by their

respective antitoxin mechanism of inhibiting the toxin: both antitoxins of type I and type III systems function via an RNA antitoxin that either blocks translation of the toxin mRNA (type I) or by direct inhibition of the protein toxin (type III); the antitoxins in type II and type IV are proteins that either inhibit toxin activity via binding the translated protein directly (type II) or by negating the toxin protein's effects in an indirect manner (type IV). Type V TA systems have been recently characterized as a system where the toxin is inhibited via a protein antitoxin that cleaves the toxin's mRNA to prevent translation (Wang et al., 2013). Aakre et al. (2013) describes type VI TA systems where the antitoxin functions as an adaptor for a protease that disrupts the toxin's function of disrupting DNA replication via collapse of the replication fork. Research is continually conducted on TA systems with increasing access to prokaryotic genomes, allowing for future possibility to discover novel TA system characteristics. Even though these systems differ in toxin regulation, bacterial species can also differ considerably in TA module composition and number. For example, *Escherichia coli* (*E.* coli) K-12 MG1655 encodes for 35 known TA systems comprised of 19 type I loci, 12 type II loci, and three type IV loci whereas *Staphylococcus aureus* has 5 identified TA systems constituted of two type I loci and three type II loci, although several TA modules resembling type III loci have been detected (Harms et al., 2018; Schuster and Bertram, 2016).

Bacterial TA systems have been accurately identified in a variety of hosts, although their biological functions are not as well-determined--especially when the system is encoded chromosomally. Nonetheless, plasmid TA modules are well understood as operons that encode for post-segregational killing (PSK) mechanisms, also known as addiction modules. TA systems that are involved in PSK operate via a

differential stability of the toxin and antitoxin; in daughter bacterial cells that fail to inherit the plasmid, the antitoxin quickly degrades and the toxin exerts its effects of inhibiting cellular processes and/or killing the cell (Gerdes et al., 1986; Unterholzner et al., 2013). The *hok/sok* system of plasmid R1 and *ccdAB* of plasmid F are two well-studied examples of PSK functions performed by TA modules (Gerdes et al., 1986; Ogura and Hiraga, 1983). Interestingly, the *hok/sok* module is a type I system and the *ccdAB* is a type II system, thus the PSK function is not bound to a certain TA-system type. A similar genetic stabilization function of TA systems is as a mediator of superintegrons, which are assemblies of genes each bordered by recombinational elements (Szekeres et al., 2007). The study by Szekeres et al. (2007) demonstrated that superintegrons and genomic dispensable regions of *E. coli* exhibited large-scale genome loss when lacking TA loci, and that incorporation of TA systems into these environments helped reduce gene loss.

TA systems have more functions than just stabilization of genetic elements, as they have been shown to be involved with bacterial immunity processes. Both chromosome and plasmid TA systems have been theorized to function in abortive infection mechanisms, an immunity process whereby a bacteriophage-infected bacterial cell performs suicide that ultimately helps protect bacterial populations against infections of invasive genetic elements (Unterholzner et al., 2013; Harms et al., 2018). For example, the chromosomal TA systems of *toxIN* and *tenpIN* families have been shown to interfere with bacteriophage infection of bacteria; the chromosomal *mazE/mazF* TA module invoked cell death via abortive infection when exposed to P1 bacteriophage (Goeders et al., 2016; Hazan and Engelberg-Kulka, 2004). Suicide of infected bacterial

cells is an altruistic mechanism that allows for decreased bacteriophage genetic replication.

Bacterial TA systems have also been demonstrated to serve a role in bacterial persistence, specifically, subpopulations of cells that are tolerant to environmental stressors like lack of nutritional resources, antibiotics and other toxic chemicals (Harms et al., 2018). This phenotypic persistence is accomplished via a conversion of the bacterial cell to a dormant state where it inactivates cellular processes that are affected by the stress condition (Harms et al., 2016). Large amounts of evidence exist for TA modules role in bacterial persisters: overproduction of the *MqsR* toxin in *E. coli* increases persister cell formation and the converse decrease in persisters occurs with deletion of the *MqsR* locus (Kim and Wood, 2010); a deletion of the *tisAB*/*istR* TA locus resulted in a significant decrease in persister cells tolerant to the antibiotic ciprofloxacin (Dörr et al., 2010); deletion of the *hipBA* TA module caused a decrease in persister cells in biofilm and stationary phase populations (Keren, 2004).

The various physiological functions of TA systems have been tied to various bacterial pathogens important in clinical settings, particularly to those of the ESKAPE group (Fernández-García et al., 2016). Rice (2008) details the following pathogens in ESKAPE: *Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species. This group, alongside other pathogens like *E. faecalis*, are the primary causers of hospital infections, and they are becoming increasingly resistant to antimicrobial agents (Rice, 2008; Fernández-García et al., 2016). *K. pneumoniae* can cause nosocomial pneumonia and intra-abdominal infections and has hundreds of

associated type II TA loci (Fernández-García et al., 2016). The RelBE TA system is one example of a TA module relating to pathogenesis within *K. pneumoniae* as it has associated with persister cells tolerant of antibiotics like β-lactams, quinolones and aminoglycosides (Tashiro et al., 2012). *E. faecium and E. faecalis* can cause infections of the urinary tract infections, hospital bacteremia, and surgical wounds. Regarding TA systems, these enterococci species have multiple TA systems relating to stable plasmid maintenance and inheritance relating to antibiotic resistance (Fernández-García et al., 2016). Fernández-García et al. (2016) detailed a high prevalence of TA operons in vancomycin-resistant *Enterococcus* isolates relative to isolates sensitive to the antibiotic.

Fst and Fst-like Toxins

The Fst toxin encoded by the enterococcal plasmid pAD1 TA system (par_{pAD1}) is a type I TA module and was the first type I system to be described in a gram-positive bacterium (Weaver et al., 1996). Moreover, the plasmid par_{pAD1} locus in *Enterococcus faecalis* is one of the only known RNA-regulated PSK system in gram-positive bacteria (Patel and Weaver, 2006). The entire par_{pAD1} locus spans less than 400 nucleotide base pairs and is transcribed convergently to yield two interacting RNAs--RNA I_{pAD1} and RNA II_{pAD1} (Weaver et al., 1996). The antitoxin RNA II_{pAD1} binds the toxin RNA I_{pAD1} in a manner that suppresses the translation of the RNA I_{pAD1} encoded toxin Fst_{pAD1} (Greenfield et al., 2000; Patel and Weaver, 2006). The two RNAs are shown in Figure 1 where interaction initiates at the U-turn motif (green region with the labeled arrow A) and then interaction proceeds to the direct repeat sequences DRa and DRb (pink and blue regions with the labeled arrows B respectively) according to Weaver (2012). RNA-RNA

interactions at DRa and DRb sequester the initiation codon (I.C.) thereby preventing translation.

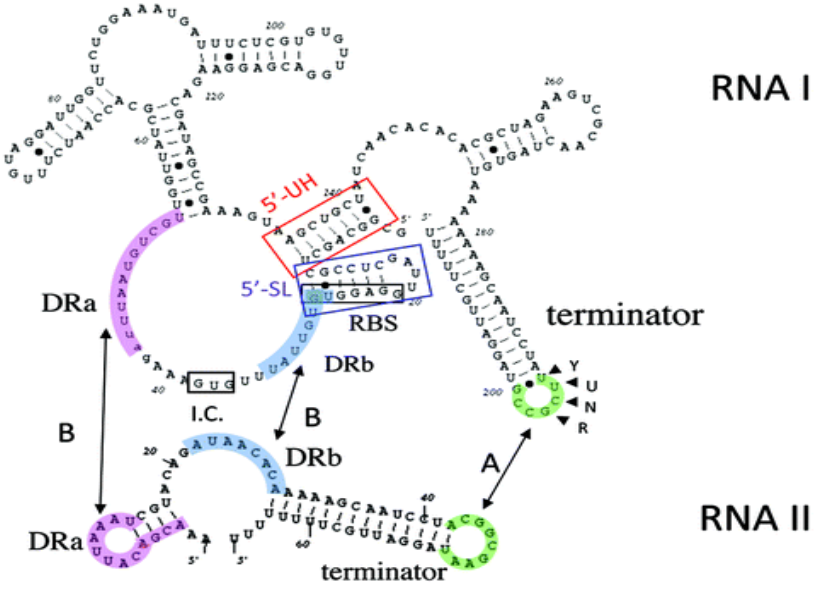

Figure 1. Interacting secondary structures of RNA I$_{pAD1}$ and RNA II$_{pAD1}$. Adapted from "The *par* toxin-antitoxin system from *Enterococcus faecalis* plasmid pAD1 and its chromosomal homologs," by K. Weaver, 2012, *RNA Biology, 9(12),* 1498-1503. Adapted with permission.

Additionally, RNA I$_{pAD1}$ has intramolecular structures critical in regulation of *par*$_{pAD1}$ Specifically, the 5' stem-loop (5'-SL) structure of RNA I$_{pAD1}$ (depicted in Figure 1 by the blue box) modulates translation and ribosome binding by sequestering the Fst$_{pAD1}$ Shine-Dalgarno sequence (Greenfield et al., 2000; Weaver, 2012). The 5' upstream helix of RNA I$_{pAD1}$ (depicted in figure 1 by the red box labeled 5'-UH) helps provide the greater stability of RNA I$_{pAD1}$ relative to RNA II$_{pAD1}$ by sequestering 5' nucleotides from cellular RNase degradation (Shokeen et al., 2008; Weaver, 2012; Greenfield et al., 2000). The lesser stability and selective degradation of antitoxin RNA II$_{pAD1}$ allows for translation of RNA I$_{pAD1}$ to Fst toxin upon plasmid loss (Greenfield et al., 2000). After translation, the

proteic Fst_{pAD1} is theorized to insert into the plasma membrane via an α-helix around amino acid residue 4 and exit around amino acid residue 26 with an unstructured, charged tail at the carboxyl-terminus (Göbl et al., 2010).

Although Fst_{pAD1} is most likely localized and active in the membrane, overexpression of this toxin can have several primary effects: condensation of the nucleoid, unequal chromosome segregation between daughter cells, and issues with cell division/peptidoglycan synthesis characterized by cellular elongation, altered septal placement, and hyperseptation (Patel and Weaver, 2006; Weaver 2012). Fst_{pAD1} also increases membrane permeability, although this was found to be a secondary effect by Patel and Weaver (2006). The specific target(s) of Fst_{pAD1} is still unknown, but evidence suggests an intracellular target since the external application of synthetic Fst_{pAD1} has no cellular effect (Weaver et al., 2003). Despite limited information on the true target(s) of Fst_{pAD1}, the amino acid residues essential for the toxic effects have been defined. Mutagenic analysis by Weaver et al. (2009) found that the predicted transmembrane domain was the most essential component for toxicity.

The toxin of the par_{pAD1} locus has a homologous putative toxin, Fst_{EF0409}, that is encoded by the par_{EF0409} locus found on the *E. faecalis* chromosome (Ibrahim et al., 2007; Weaver et al., 2009). Although Fst_{EF0409} has similar features to other toxins within the *par* family, its biological function and physiological mechanisms are not as well understood as Fst_{pAD1}. The par_{EF0409} TA system is hypothesized to modulate sugar transport because its locus is nestled between two paralogous mannitol family phosphotransferase system gene sets (Weaver et al., 2017; Weaver, 2012). A function as a PSK system is unlikely as this TA system has not been associated with any MGE that

would indicate an addictive behavior (Weaver et al., 2017). Additionally, the *par*$_{EF0409}$ locus has conserved structural features of the *par*$_{pAD1}$ locus—such as converging promotors, a stabilizing 5'-UH, a stem loop structure, direct repeats that provide RNA interacting sequences amongst other features (Weaver et al., 2009). Despite these similarities, data from Weaver et al. (2017) demonstrates that *par*$_{EF0409}$ and *par*$_{pAD1}$ can exist together and not interfere with the others function. The differences between the interacting regions of RNA molecules was theorized to allow for coexistence (Weaver et al., 2017). Moreover, Weaver et al. (2017) suggested that the plasmid pAD1 TA system may have evolved to bypass chromosomal TA systems like that of *par*$_{EF0409}$.

Besides the two related *par*$_{pAD1}$ and *par*$_{EF0409}$ TA modules, Weaver et al. (2009) identified seven further Fst$_{pAD1}$-like systems and Kwong et al. (2010) discovered more than 200 additional modules that code for Fst-like toxins through the utilization of homology searches and the NCBI nucleotide sequence database. Overall, this *par* family of Fst-like toxins were identified in numerous gram-positive bacterial species and were found to be encoded by chromosomal, plasmid, and phagic genetic elements (Kwong et al., 2010). The most prominent commonality amongst all of the Fst-like toxins is the characteristically short polypeptide length, ranging between 27 to 35 residues (Kwong et al., 2010). Similar to the results from the earlier study by Weaver et al. (2009), these Fst-like toxins were predicted to have a hydrophobic transmembrane domain (Kwong et al., 2010).

Analysis of the peptide sequences of the Fst-like toxins also yielded characteristics that apply to most, if not all, of the toxins (Weaver et al., 2009; Kwong et al., 2010). Kwong et al., found that all Fst-like toxins exhibited similar expression

sequences, containing both the mRNA toxin and an antisense RNA antitoxin (2010). Furthermore, the DNA sequence analysis indicated that all loci possess a potential promoter with transcriptional machinery resembling the σ_{70} factor, in addition to a bidirectional terminator sequence close to the culmination of the toxin expression sequence similar to the prototypical *par*$_{pAD1}$ locus as shown in Figure 2(a) (Kwong et al., 2010). The duplex terminator of these toxins yields a stable, complementary SL structure similar to one shown in Figure 1 (Kwong et al., 2010; Greenfield et al., 2000). Previous work done by Greenfield et al. (2001) has shown that the resulting SL structures are crucial for inhibiting the Fst toxin in the original Fst$_{pAD1}$ system; therefore, these loop interactions may also be important for the negation of their corresponding toxins for the rest of the familial Fst-like toxins. Additionally, complementary regions of direct repeats (DRa and DRb) were readily distinguishable in these toxins (Kwong et al., 2010). The direct repeats can be visualized in Figure 2(a) where they overlap the start codons and are near the bidirectional terminator (Kwong et al., 2010). Furthermore, these related toxins contained sequences for the 5' stem-loop (5'-SL) and 5' upstream-helix (5'-UH) of RNA I as shown in Figure 2(b), providing further evidence of a possible shared regulatory mechanism as the prototypical *par*$_{pAD1}$ locus (Kwong et al., 2010). Both Kwong et al. (2010) and Weaver et al. (2009) hypothesized that although these features were highly conserved, the great variability in their DNA sequence, particularly in the regions encoding RNA-RNA interaction sites, would suggest that the TA systems would exhibit specificity in their targets.

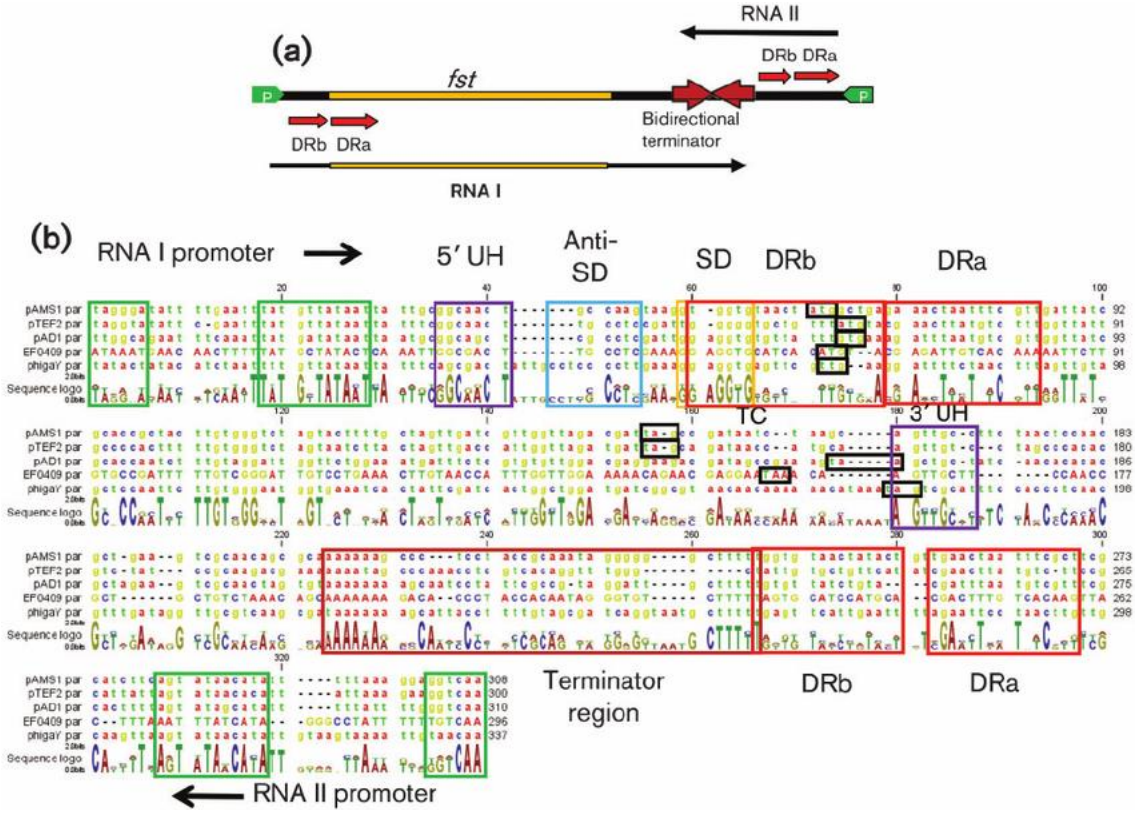

Figure 2. Structural features conserved within Fst-encoding *par*-like loci. Figure 2(a) represents the organization of *par*$_{pAD1}$ and 2(b) demonstrates the conserved features of Fst toxins within *par*-like loci. Adapted from "Identification and characterization of a family of toxin-antitoxin systems related to the *Enterococcus faecalis* plasmid pAD1 par addiction module," by K. Weaver, 2009, *Microbiology*, *155(Pt 9)*, 2930-2940. Adapted with permission.

A recent study done by Weaver et al. (2017) found that the two similar Fst toxins, Fst$_{pAD1}$ and Fst$_{EF0409}$ had both shared and unique effects on the transcriptome of their native host, *E. faecalis*. The two related Fst toxins predominately affected genes encoding for surface proteins (Weaver et al., 2017). Their analysis suggested that the two toxins have a shared effect on plasma membrane organization while still having the capability to exert their function on distinct targets within the cell, although no clear interactional mechanism was proposed other than their differing amino acid composition.

To investigate the underlying mechanism that causes different gene expression in response to the two toxins, various mutants of Fst_{pAD1} and Fst_{EF0409} were constructed. These toxins utilized the pheromone-responsive plasmid vector, designated as pCIE, which ultimately allowed the movement of the mutant toxin from *E. coli* to *E. faecalis* (Weaver et al., 2017). Vector pCIE allowed for tight regulation of the toxin constructs in response to nanogram amounts of cCF10 pheromone (Weaver et al., 2017). After introduction into *E. faecalis*, these mutants were assessed and compared for expression of multiple genes when induced with ccf10 pheromone to determine which amino acid residue substitutions were responsible for creating the distinct transcriptomic effects seen between the two related bacterial toxins.

CHAPTER 2

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

All bacterial strains used in this study are shown in Table 1. All constructs of mutant Fst toxin and primers are shown in Table 2. All *E. faecalis* cultures (OG1RF) for growth curves and RNA preparation were grown in M9YEG medium that consists of 96% M9 Base (M9 Salts, casamino acids, and yeast extract), 1% 0.1M magnesium sulfate ($MgSO_4$), 1% 0.01M calcium chloride ($CaCl_2$), and 2% 1.0M glucose. Cultures were grown overnight and diluted to one to two percent into new M9YEG medium and were grown with the antibiotic chloramphenicol (Sigma-Aldrich) if necessary, to ensure plasmid selection. All *E. faecalis* cultures for plasmid electroporation experiments were grown in Todd Hewitt Broth made of 30g of Todd Hewitt powder (Sigma-Aldrich) per one liter of distilled water. Luria-Bertani (LB) medium was used for all *E. coli* growth with the following formulation per one liter: 10g peptone, 5g yeast extract, and 5g sodium chloride (NaCl). The antibiotic concentration of chloramphenicol routinely used was either 10µg/mL (Cm-10) or 25µg/mL (Cm-25) for culture growth when plasmid selection of pCIE was desired. The antibiotic ampicillin (amp) was also used at a concentration of 100µg/mL. All liquid cultures were grown at 37°C with rotary shaking at either 25rpm for *E. faecalis* or 250 rpm for *E. coli*. Any growth on solid medium utilized the respective concentrations with the addition of 17g Agar (Research Products International) per one liter of medium.

Strain or plasmid	Relevant phenotype
Strains	
DH5α	*E. coli* intermediate host for cloning
OG1RF	*E. faecalis* used for all testing
Plasmid	
pCIE	Cm-resistant *E. coli* - *E. faecalis* shuttle expression vector with cCF10-regulateable promoter *prg*Q

Table 1. Bacterial strains and plasmid used in this study

The peptide pheromone cCF10 (H-LVTLVFV-OH) used previously in Weaver et al. (2017) was dissolved in dimethylformamide and stored at -20°C. For experiments that involved induction with pheromone, cultures were grown overnight in M9YEG-Cm25 for optimal plasmid pCIE construct retention. Overnight cultures would then be diluted at 1 to 2% in M9YEG-Cm10 and grown for 1 hour prior to cCF10 pheromone addition. Pheromone cCF10 was used at multiple working concentrations to assess phenotypic toxicity of strains: 0ng/mL, 1ng/mL, 5ng/mL, 10ng/mL, or 50ng/mL. Strains with 0ng/mL induction experiments had an equivalent volume of dimethylformamide added.

Plasmid Construction, DNA Purification and Manipulation

Mutant constructs encoding varying Fst_{pAD1} and Fst_{EF0409} derivatives as shown in Table 2 were commercially synthesized and delivered in plasmid pUCminusMCS (Blue Heron Biotech, LLC). The mutant toxin alleles within the pUCminusMCS vector and the pCIE expression vector contained flanking BamHI and SphI restriction enzyme sites. Mutant toxins were cut from pUCminusMCS and cloned into plasmid pCIE through restriction digest and ligation products purchased through New England BioLabs or Promega according to provided protocols. A post-ligation cut was performed with SalI

(New England BioLabs) that eliminated any pCIE plasmid without the necessary cloned construct. Mutant toxin-containing pCIE plasmids were transformed into competent *E. coli* DH5α cells per manufacturer instructions (Invitrogen) and spread onto LB-cm25 plates. Bacterial colonies were then selected and tested for ampicillin resistance by picking colonies onto LB-Amp plates to ensure the absence of pUCminusMCS. Plasmid purification from DH5α colonies with the desired plasmid mutants was performed through using the Quantum Prep plasmid miniprep kit (Qiagen) according to their instructions. Mutant inserts were tested for proper base pair length via restriction enzyme digests and agarose gel electrophoresis. Mutant pCIE plasmids were inserted into *E. faecalis* cells through electroporation of plasmids into OG1RF cells treated with lysozyme as described by Cruz-Rodz and Gilmore (1990) and electroporation conditions described by Brinkman et al. (2012). To assess if the proper mutations were emplaced, plasmids were isolated from the plasmid containing OG1RF cells via a modified Quantum Prep plasmid miniprep kit protocol. ON cultures were diluted 50% into new M9YEG media containing 3% glycine and were grown for 90 minutes. Cultures were pelleted, washed in water, and again pelleted. Pellets were resuspended in 25% sucrose containing lysozyme at a 30mg/mL concentration and incubated until a slight separation was seen between a clear supernatant and cells. Samples were then pelleted and run through the Quantum Prep plasmid miniprep kit. Constructs were again tested for correct size inserts via restriction enzyme digests and agarose gel electrophoresis. Plasmids that contained the proper insert size were then sequenced by Eurofins Genomics LLC to determine correct DNA sequence of the emplaced mutant toxins.

Mutant Fst toxin or Primer	Sequence
Mutant Fst Toxin	Amino Acids
pAD1 wild-type	VKDLMSLVIAPIFVGLVLEMISRVLDEEDDSRK
pAD1 E19K	VKDLMSLVIAPIFVGLVL**K**MISRVLDEEDDSRK
pAD1 L7K-E19K	VKDLMS**K**VIAPIFVGLVL**K**MISRVLDEEDDSRK
pAD1EF0409	VKDLMSLVIAPIFVGLVLEMISRVL**EKQNEE**
pAD1ter2	VKDLMSLVIAPIFVGLVLEMISRVLDEEDDS
pAD1ter5	VKDLMSLVIAPIFVGLVLEMISRVLDEE
EF0409 wild-type	MYEIVTKILVPIFVGIVLKLVTIWLEKQNEE
EF0409 K19E	MYEIVTKILVPIFVGIVL**E**LVTIWLEKQNEE
EF0409pAD1N6-K19E	M**KDLMSL**ILVPIFVGIVL**E**LVTIWLEKQNEE
EF0409 K7L-K19E	MYEIVT**L**ILVPIFVGIVL**E**LVTIWLEKQNEE
EF0409pAD1	MYEIVTKILVPIFVGIVLKLVTIWL**DEEDDSRK**
EF0409pAD1:13	MYEIVTKILVPIFVGIVLK**MISRV**L**DEEDDSRK**
EF0409pAD1:14	MYEIVTKILVPIFVGIVL**EMISRV**L**DEEDDSRK**
EF0409ter2	MYEIVTKILVPIFVGIVLKLVTIWLEKQN
EF0409ter5	MYEIVTKILVPIFVGIVLKLVTIWLE
Primer	Nucleotides
pCIE-EF0409 FWD	GTATACAGTTCATGTATATGTTCCC
pCIE-EF0409 REV	TGTGATGCACCTCCTTTC
10493 FWD	CAGATGACGGCTCAATTCAAAC
10493 REV	CAGCGGTACTTCCTTCAATCA
10301 FWD	GCACGATGTCTGGTGATGAT
10301 REV	CTTCGCTCCTAAATCCGCTAAG
MgtA FWD	AAAGGTGCGGTTGAAGAAATG
MgtA REV	TGACGCAGTGTCTCTGTTAAG
CelA3 FWD	AGAAGATCGTGGCATGGAAG
CelA3 REV	TGAAACGAACTTGTGGACCTAA
GyrB FWD	ACCAACACCGTGCAAGCC
GyrB REV	CAAGCCAAAACAGGTCGCC

Table 2. Mutant Fst toxins with encoded amino acid residue sequence All amino acid sequences are shown from the amino-terminus to carboxyl-terminus with the bolded residues indicating a mutation. Primers are listed from the 5’ to 3’ end.

RNA Purification and Manipulation

For any incubation period, *E. faecalis* cultures were grown at 37°C and 25rpm. Cultures were grown overnight in M9YEG-Cm25 and then inoculated in fresh M9YEG-Cm10 at a 2% dilution. These cultures were then grown for 1 hour before being induced with cCF10 at 1ng/mL or 5ng/mL; uninduced samples were given 5µL of dimethylformamide. After induction, cultures were grown for one more hour before harvesting. Cultures were harvested by centrifuging, resuspending the bacterial pellets in M9 Base and Bacteria RNA Protect (Qiagen), incubating for 5 minutes at room temperature, centrifuging, and then freezing the pellets at -80°C.

For RNA preparation, pellets were thawed, resuspended in 200µL of Tris/EDTA-mutanolysin-lysozyme solution (10mM Tirs [pH 8.0], 1.0mM EDTA [pH 8.0], mutanolysin [500 units/mL], lysozyme [30mg/mL]), and incubated for 10 minutes at 37°C in a water bath. 700µL of Buffer RLT/β-mercaptoethanol (1mL Buffer RLT [Qiagen RNeasy minikit] with 10µL β-mercaptoethanol) was added to each resuspension and vortexed to mix. RNA purification was then performed with the Qiagen RNeasy minikit per the provided instructions within the kit. All the RNA from each sample was incubated with 2µL of TURBO DNase and 10µL of TURBO DNase Buffer (Ambion/Thermo Scientific) at 37°C for 30 minutes; an additional 1µL of TURBO DNase was added and the samples were incubated for 30 more minutes at the same temperature. The DNase enzyme was removed through the RNA Clean & Concentrator-25 (Zymo Research) according to their provided protocol. 1µg of RNA was then mixed with 1 µL of Random Primer 9 (New England BioLabs), 1 µL of 10mM dNTP Mix (New England BioLabs), and a corresponding amount of DEPC-treated water to achieve a total

14 µL mixture, before being incubated at 65°C for 5 minutes. Reverse transcription was performed with SuperScript III Reverse Transcriptase (ThermoFisher Scientific) per the manufacturer's instructions. The reversely transcribed samples were diluted 10-fold by the addition of water and then were stored at -80°C. 5 µL of cDNA samples were mixed with specific forward and reverse primers listed in Table 2 and with PerfeCTa SYBR Green SuperMix (Quanta-Bio) to a final 25µL qPCR total mixture in 96-well Thermo Scientific PCR Plates. qPCR was performed on sample triplicates and measured through an ABI 7300 system (Applied Biosystems [Life Technologies]) using the standard amplification cycle with a melting-curve analysis. cDNA results were compared to a standard curve created against purified OG1RF genomic DNA and the relevant primers for each gene of interest. The final Ct results of the RT-qPCR samples were corrected for efficiency through standard curves with r_2 values above 0.99. The Ct values utilized the standard curves to generate estimated ng amounts of cDNA, and these were normalized using the ratio of the average nanogram cDNA amounts of the desired gene to the average nanogram cDNA amounts of *gyrB*, a housekeeping gene specific for *E. faecalis*. Each sample had at least three biological replicates.

CHAPTER 3

Results

Determinants of target specificity between the two toxins were assessed through qRT-PCR of induced mutant samples at pheromone concentrations of 0ng/mL, 1ng/mL, and 5ng/mL. The amino acid sequence of constructed Fst mutants is shown in Table 2 for reference. Multiple genes of *E. faecalis* were utilized to examine and compare the critical domains of the toxins: (1) *10493* (OG1RF_RS02610 in the study done by Weaver et al. [2017]) which is theorized to encode a copper P-type ATPase transport and showed the largest difference between Fst_{pAD1} and Fst_{EF0409}; (2) *10301* (OG1RF_RS01655) which is theorized to encode for an ATP binding cassette (ABC) transporter and is linked to par_{EF0409} (3) *MgtA* (OG1RF_RS12145) which is theorized to encode for a P-type ATPase that imports magnesium ions and exhibits an induction response for both toxins; and (4) *CelA3* (OG1RF_10750) which is theorized to encode a phosphotransferase system involved in sugar transport and exhibits a repression response for both toxins. The wild-type Fst_{EF0409} and Fst_{pAD1} toxins exhibited a graded response to cCF10 pheromone for all assessed genes as shown in Figure 3. These two wild-type toxins had similar effects on *10301*, *MgtA*, and *CelA3*. However, there was a significant difference in *10493* induction between the two toxins at a 5ng/mL cCF10 concentration that was also seen by Weaver et al. (2017).

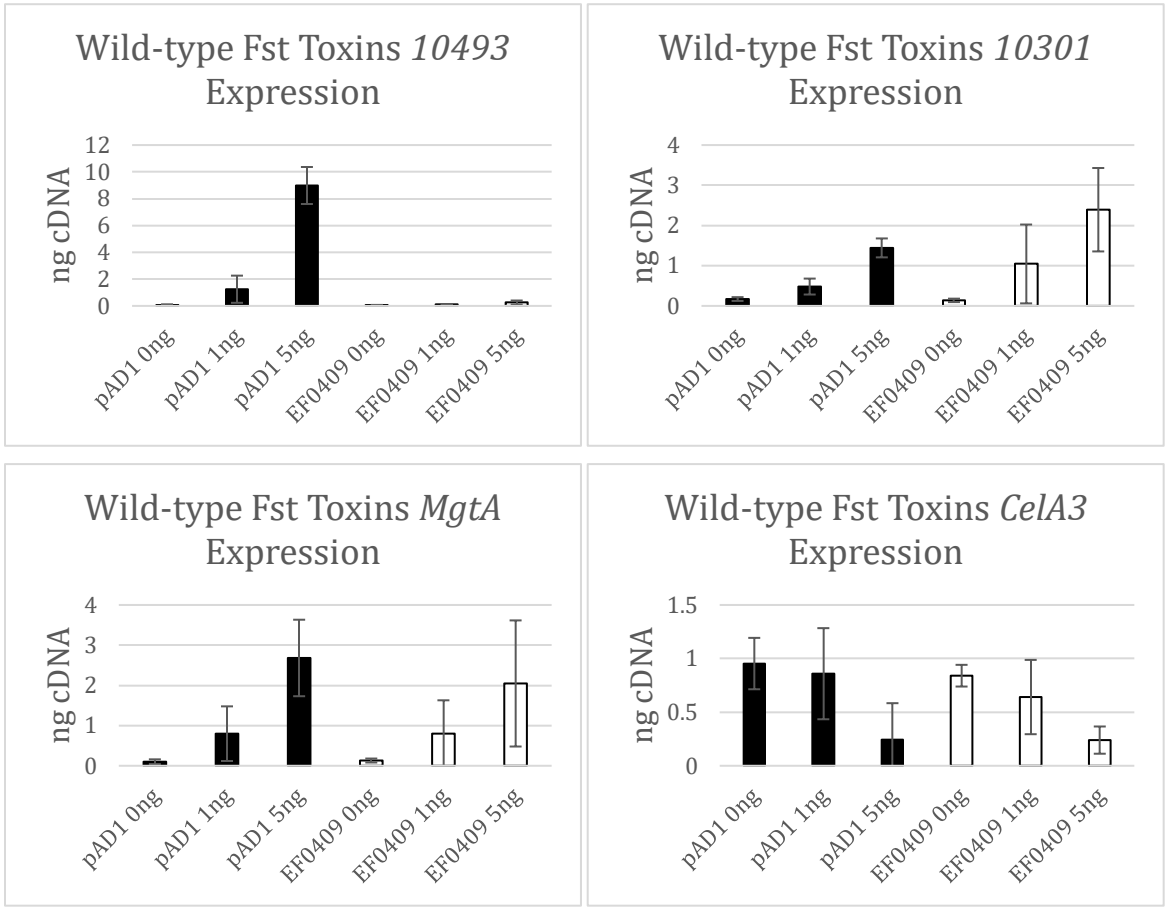

Figure 3. Fst_{pAD1} and Fst_{EF0409} wild-type toxins on selected genes. Black bars indicate Fst_{pAD1} and white bars indicate Fst_{EF0409}. Error bars represent 95% confidence intervals.

To identify the determinants of the specificity between Fst_{pAD1} and Fst_{EF0409}, mutated Fst toxins of each were constructed and are shown in Table 2. These toxins' effects on *10493* expression are exhibited in Table 3. A loss of Fst_{pAD1} function was observed when any amino acid(s) swap was made or when a truncation was made at the C-terminus. However, incorporating one or two amino acids from Fst_{pAD1} into the corresponding positions of Fst_{EF0409} residues (EF0409 K19E and EF0409 K7L-K19E respectively) demonstrated a gain-of-function resembling Fst_{pAD1}. Clearly, the leucine

residue at position 7 and the glutamic acid residue at position 19 in Fst_{pAD1} are important for this toxins specificity in inducing *10493*.

Fst Sample	ng cDNA (avg.)	Standard deviation	Significance
pAD1	8.983	1.221	
pAD1 E19K	2.360	1.888	*
pAD1 L7K-E19K	0.075	0.015	*
pAD1EF0409	0.149	0.057	
pAD1ter2	3.525	0.619	*
pAD1ter5	0.195	0.062	*
EF0409	0.253	0.151	
EF0409 K19E	1.933	0.341	*§
EF0409 K7L-K19E	3.779	1.505	*§
EF0409pAD1	0.186	0.059	
EF0409pAD1:13	0.502	0.240	
EF0409pAD1:14	2.162	0.439	*§
EF0409ter2	0.104	0.030	
EF0409ter5	0.039	0.004	*

Table 3. Fst toxins expression of *10493* when induced with 5ng/mL cCF10. The symbol "*" next to a sample indicates a significant difference compared to their respective wild-type Fst toxin. The symbol "§" next to a sample indicates toxins that demonstrated an Fst_{pAD1}-like gain of function.

The amino acid swap mutations did not have any induction effects on the other assessed genes. However, Fst_{pAD1} has other amino acid residues that are essential for triggering the induction of *10493*. The truncated Fst_{pAD1} toxins, pAD1ter2 and pAD1ter5, exhibit significant decreases in *10493* expression with their lack of residues at the carboxyl-terminus as shown in Figure 4. Although important, more than just the carboxyl-terminus tail of Fst_{pAD1} is necessitated for triggering induction of *10493* as shown by the failure of EF0409pAD1 in Table 3. For the EF0409pAD1 hybrid, it is probable that

addition of the leucine and glutamic acid residues at positions 7 and 19 respectively may have conferred a nearly full Fst_{pAD1}-like response of *10493*.

A commonality amongst all genes assessed was the importance of the carboxyl-terminus tails of both Fst_{EF0409} and Fst_{pAD1}. The truncated Fst_{EF0409} and Fst_{pAD1} toxins resulted in a decreased response of induction or repression for all genes. With these truncated toxins, genes *10493*, *MgtA* (as shown in Figure 5), and *10301* exhibited decreased induction at 5ng/mL cCF10 pheromone whereas gene *CelA3* demonstrated less repression when compared to the wild-type Fst toxins (data not shown).

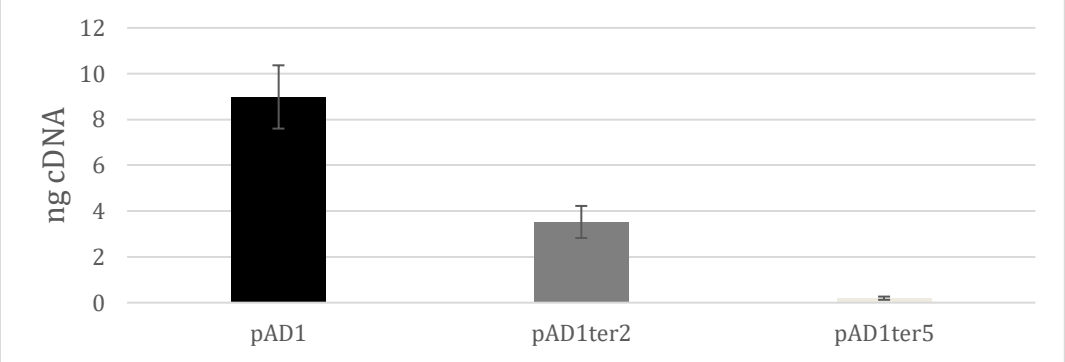

Figure 4. Fst_{pAD1} and truncated Fst_{pAD1} toxins expression of *10493* when induced with 5ng/mL cCF10 pheromone.

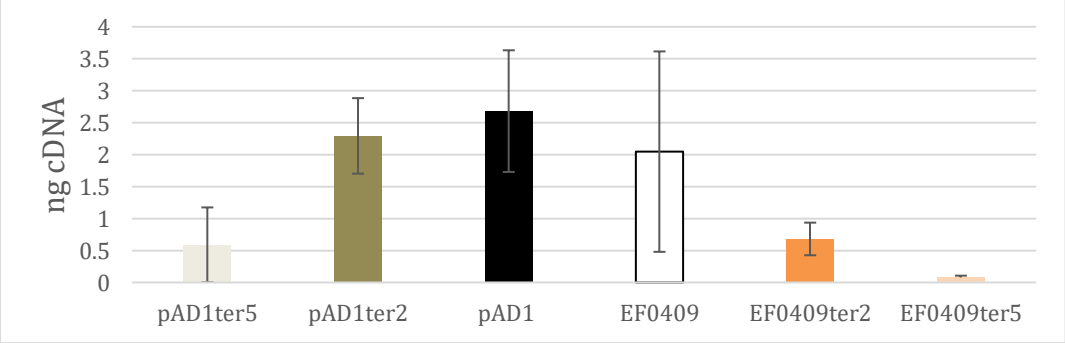

Figure 5. Fst_{pAD1} and Fst_{EF0409} and truncated toxins expression of *MgtA* when induced with 5ng/mL cCF10 pheromone.

CHAPTER 4

Discussion and Conclusions

Substantial evidence exists for the physiological roles of TA systems in bacteria—such as addiction modules that stabilizes inheritance of MGE on chromosomes and plasmids, abortive infection mechanisms to prevent infection of foreign phagic elements, and as mediators of persister cell formation that may provide tolerance to antimicrobial agents. A multitude of TA systems have been inextricably linked to pathogenic properties in certain nosocomial bacteria like the ESKAPE group and *E. faecalis* that cause most hospital infections, ultimately warranting investigation of their properties and roles to combat infection. Moreover, multidrug resistant nosocomial bacteria are increasingly becoming prevalent, so TA systems may present a unique target that can be exploited for the treatment of infections. Future research is necessitated to better characterize TA systems and their diverse function(s), genetic organization, and antitoxin regulation mechanisms, amongst other pertinent aspects of TA morphology.

The par_{pAD1} TA system was the first type I module described in the gram-positive bacterium *E. faecalis*. The par_{pAD1} locus stabilizes its host plasmid, pAD1, by a PSK mechanism dependent upon the relatively small Fst_{pAD1} peptide toxin that localizes within the cytoplasmic membrane. Expression of Fst_{pAD1} has been shown to condense nucleoid structures, cause unequal segregation of chromosomes in replicating cells, and affect cell division at the septum by impacting peptidoglycan synthesis (Patel and Weaver, 2006; Weaver 2012). Further investigations of Fst-like toxins by Kwong et al (2010) and Weaver et al (2009) identified over two hundred Fst-like toxins that share locus

organization and secondary structure features between the interacting RNA toxins. Despite structural conservation within the *par* family, toxins exhibit both general and specific interactions with their target(s) as indicated by the respective shared and variant gene expression response to overexpression of Fst_{pAD1} and Fst_{EF0409}. The unshared levels of specific gene induction between Fst_{EF0409} and Fst_{pAD1} demonstrates the existence of determinant regions of each toxin critical for triggering their unique response.

Through the utilization of a pheromone-responsive expression vector, the data presented here demonstrates the determinants of Fst_{pAD1} and Fst_{EF0409} toxicity function. The carboxyl-terminal tail for both toxins was determined to be important for responses in all genes assessed, regardless of whether the gene was induced or repressed due to toxin overexpression. However, the carboxyl-terminal tails do not explain the distinct effects of Fst_{pAD1} and Fst_{EF0409} on targets related to expression of *10493*. The hybrid toxins EF0409pAD1:13 and EF0409pAD1:14 produced significant differences in induction of *10493* and only deferred by one amino acid at position 19. These results provoked the idea to swap the Fst_{Ef0409} lysine for the Fst_{pAD1} glutamic acid at position 19. Incorporating the corresponding Fst_{pAD1} amino acid residue at position 19 instead of the normal residue of Fst_{EF0409} resulted in a partial Fst_{pAD1}-like response of *10493*. Furthermore, pairing the position 19 swap with the incorporation of another Fst_{pAD1} position 7 amino acid reside in Fst_{EF0409} generated an even greater response akin to the effect of Fst_{pAD1} on *10493*. Therefore, the amino acid residues at positions 7 and 19 in Fst_{pAD1} are important for triggering the distinct expression of its *10493* target.

In summary, determinants of the critical regions of toxins Fst_{pAD1} and Fst_{EF0409} were identified to be key amino acid residues and the carboxy-terminus tail. Both toxins

exhibit a shared need for the carboxyl-terminus tail for certain intracellular targets, demonstrating a generalized mode of interaction. Specificity of targets between Fst_{pAD1} and its chromosomal homologue Fst_{EF0409} is dictated by the variant amino acid residue sequence, especially at residue positions 7 and 19. One possible explanation is that the negatively charged glutamic acid residue at position 19 of Fst_{pAD1} has repulsive electrostatic interactions with the negatively charged phospholipid head of the plasma membrane. In theory, this would allow for greater mobility of Fst_{pAD1} to interact with targets relating to *10493*. Moreover, the glutamic acid residue at position 19 of Fst_{pAD1} may facilitate an electrostatic interaction with proteins or other targets that are associated with *10493*. The oppositely charged lysine residues at positions 7 and 19 of Fst_{Ef0409} perhaps create a lysine clamp with the negatively charged phospholipid head groups of the membrane, ultimately hindering or restricting the movement of Fst_{EF0409}. In addition, the positive charge of the lysine residue at position 19 in Fst_{EF0409} may repel certain protein targets, thus also creating specificity. Future experiments will need to be conducted to tease out the nature of these interactions. These results help elucidate the fine-tuned responses of these toxins and suggest that other Fst-related toxins have similar specific targets determined by their peptide sequence. Encouraging future investigations of Fst-like toxin specificity will be useful for further understanding how this related family of toxins interact with their targets and their physiological function.

REFERENCES

Aakre, C. D., Phung, T. N., Huang, D., & Laub, M. T. (2013). A bacterial toxin inhibits DNA replication elongation through a direct interaction with the β sliding clamp. Molecular cell, 52(5), 617–628. https://doi.org/10.1016/j.molcel.2013.10.014

Akarsu, H., Bordes, P., Moise Mansour, D. B., Genevaux, P., & Falquet, L. (2019). TASmania: A bacterial Toxin-Antitoxin Systems database. Retrieved from https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1006946

Brinkman, C. L., Bumgarner, R., Kittichotirat, W., Dunman, P. M., Kuechenmeister, L. J., & Weaver, K. E. (2012). Characterization of the Effects of an rpoC Mutation That Confers Resistance to the Fst Peptide Toxin-Antitoxin System Toxin. Journal of Bacteriology, 195(1), 156-166. doi:10.1128/jb.01597-12

Cruz-Rodz AL, Gilmore MS. 1990. High efficiency introduction of plasmid DNA into glycine treated Enterococcus faecalis by electroporation. Mol Gen Genet 224:152–154. https://doi.org/10.1007/BF00259462.

Dörr, T., Vulić, M., & Lewis, K. (2010). Ciprofloxacin Causes Persister Formation by Inducing the TisB toxin in Escherichia coli. PLoS Biology, 8(2). doi:10.1371/journal.pbio.1000317

Fernández-García, L., Blasco, L., Lopez, M., Bou, G., García-Contreras, R., Wood, T., & Tomas, M. (2016). Toxin-Antitoxin Systems in Clinical Pathogens. Toxins, 8(7), 227. https://doi.org/10.3390/toxins8070227

Fozo, E. M., Makarova, K. S., Shabalina, S. A., Yutin, N., Koonin, E. V., & Storz, G. (2010). Abundance of type I toxin–antitoxin systems in bacteria: Searches for new candidates and discovery of novel families. *Nucleic Acids Research, 38*(11), 3743-3759. doi:10.1093/nar/gkq054

Gerdes, K., Rasmussen, P., Molin, S. (1986). Unique Type of Plasmid Maintenance Function: Postsegregational Killing of Plasmid-Free Cells. *Proceedings of the National Academy of Sciences, vol. 83, no. 10*, 1986, pp. 3116–3120., doi:10.1073/pnas.83.10.3116.

Göbl Christoph, et al. Solution Structure and Membrane Binding of the Toxin Fst of TheparAddiction Module. *Biochemistry*, vol. 49, no. 31, 2010, pp. 6567–6575., doi:10.1021/bi1005128.

Goeders, N., Chai, R., Chen, B., Day, A., & Salmond, G. (2016). Structure, Evolution, and Functions of Bacterial Type III Toxin-Antitoxin Systems. Toxins, 8(10), 282. doi:10.3390/toxins8100282

Greenfield, T. J., Ehli, E., Kirshenmann, T., Franch, T., Gerdes, K., & Weaver, K. E. (2000). The antisense RNA of the par locus of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. Molecular Microbiology, 37(3), 652-660. doi:10.1046/j.1365-2958.2000.02035.x

Greenfield T. J., Franch T., Gerdes K., Weaver K. E.. 2001; Antisense RNA regulation of the par post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RNAII and its target, RNAI. Mol Microbiol42:527–537. Retrieved from https://doi.org/10.1046/j.1365-2958.2001.02663.x

Guglielmini, J., & Van Melderen, L. (2011). Bacterial toxin-antitoxin systems: Translation inhibitors everywhere. *Mobile genetic elements*, 1(4), 283–290. https://doi.org/10.4161/mge.18477

Harms, Alexander, Brodersen, Ditlev E., Mitarai, Namiko, Gerdes, Kenn. (2018). Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Molecular Cell, vol. 70*, no. 5, 2018, pp. 768–784., doi:10.1016/j.molcel.2018.01.003.

Harms, A., Maisonneuve, E., & Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. Science, 354(6318). doi:10.1126/science.aaf4268

Hazan, R., & Engelberg-Kulka, H. (2004). Escherichia coli mazEF-mediated cell death as a defense mechanism that inhibits the spread of phage P1. Molecular Genetics and Genomics, 272(2), 227-234. doi:10.1007/s00438-004-1048-y

Keren, I., Shah, D., Spoering, A., Kaldalu, N., & Lewis, K. (2004). Specialized Persister Cells and the Mechanism of Multidrug Tolerance in Escherichia coli. Journal of Bacteriology, 186(24), 8172-8180. doi:10.1128/jb.186.24.8172-8180.2004

Kim, Y., & Wood, T. K. (2010). Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in Escherichia coli. Biochemical and Biophysical Research Communications, 391(1), 209-213. doi:10.1016/j.bbrc.2009.11.033

Kwong, Stephen M., Jensen, Slade O., Firth, Neville. (2010). Prevalence of Fst-like Toxin–Antitoxin Systems. *Microbiology, vol. 156*, no. 4, 2010, pp. 975–977., doi:10.1099/mic.0.038323-0.

Ogura, T., Hiraga, S. (1983). Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proceedings of the National Academy of Sciences of the United States of America, August 1, vol. 80, no. 15,* pp. 4784-4788, Retrieved from https://doi.org/10.1073/pnas.80.15.4784

Patel, S., & Weaver, K. E. (2006). Addiction Toxin Fst Has Unique Effects on Chromosome Segregation and Cell Division in Enterococcus faecalis and Bacillus subtilis. Journal of Bacteriology, 188(15), 5374-5384. doi:10.1128/jb.00513-06

Rice, L. (2008). Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. The Journal of Infectious Diseases, 197(8), 1079-1081. doi:10.1086/533452

Schuster, C. F., & Bertram, R. (2016). Toxin-Antitoxin Systems of Staphylococcus aureus. *Toxins*, *8*(5), 140. https://doi.org/10.3390/toxins8050140

Shokeen, S., Greenfield, T. J., Ehli, E. A., Rasmussen, J., Perrault, B. E., & Weaver, K. E. (2008). An Intramolecular Upstream Helix Ensures the Stability of a Toxin-Encoding RNA in Enterococcus faecalis. Journal of Bacteriology, 191(5), 1528-1536. doi:10.1128/jb.01316-08

Szekeres, S., Dauti, M., Wilde, C., Mazel, D., & Rowe-Magnus, D. A. (2007). Chromosomal toxin-antitoxin loci can diminish large-scale genome reductions in the absence of selection. Molecular Microbiology, 63(6), 1588–1605. doi: 10.1111/j.1365-2958.2007.05613.x

Tashiro, Y., Kawata, K., Taniuchi, A., Kakinuma, K., May, T., & Okabe, S. (2012). RelE-mediated dormancy is enhanced at high cell density in Escherichia coli. Journal of bacteriology, 194(5), 1169–1176. https://doi.org/10.1128/JB.06628-11

Unterholzner, S. J., Poppenberger, B., & Rozhon, W. (2013). Toxin–antitoxin systems. Mobile Genetic Elements, 3(5). doi: 10.4161/mge.26219

Wang, X., Lord, D. M., Cheng, H. Y., Osbourne, D. O., Hong, S. H., Sanchez-Torres, V., Quiroga, C., Zheng, K., Herrmann, T., Peti, W., Benedik, M. J., Page, R., & Wood, T. K. (2012). A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nature chemical biology, 8(10), 855–861*. https://doi.org/10.1038/nchembio.1062

Weaver, Keith. (2012). The par toxin-antitoxin system from Enterococcus faecalis plasmid pAD1 and its chromosomal homologs. RNA Biology, 9(12), 1498-1503. doi:10.4161/rna.22311

Weaver, Keith. (2015). The Type I toxin-antitoxin par locus from Enterococcus faecalis plasmid pAD1: RNA regulation by both cis- and trans-acting elements. Plasmid, 78, 65–70. https://doi.org/10.1016/j.plasmid.2014.10.001

Weaver, K. E., Chen, Y., Miiller, E. M., Johnson, J. N., Dangler, A. A., Manias, D. A., . . . Dunny, G. M. (2017). Examination of Enterococcus faecalis Toxin-Antitoxin System Toxin Fst Function Utilizing a Pheromone-Inducible Expression Vector with Tight Repression and Broad Dynamic Range. Journal of Bacteriology, 199(12). doi:10.1128/jb.00065-17

Weaver, Keith E., Jensen, Kristi, Colwell, Amy, Sriram, Saileela. (1996). Functional Analysis of the Enterococcus Faecalis Plasmid pAD1-Encoded Stability Determinant Par. *Molecular Microbiology, vol. 20, no. 1,* 1996, pp. 53–63., doi:10.1111/j.1365-2958.1996.tb02488.x.

Weaver, K. E., Reddy, S. G., Brinkman, C. L., Patel, S., Bayles, K. W., & Endres, J. L. (2009). Identification and characterization of a family of toxin-antitoxin systems related to the Enterococcus faecalis plasmid pAD1 par addiction module. Microbiology (Reading, England), 155(Pt 9), 2930–2940. https://doi.org/10.1099/mic.0.030932-0

Weaver, K. E., Weaver, D. M., Wells, C. L., Waters, C. M., Gardner, M. E., & Ehli, E. A. (2003). Enterococcus faecalis Plasmid pAD1-Encoded Fst Toxin Affects Membrane Permeability and Alters Cellular Responses to Lantibiotics. Journal of Bacteriology, 185(7), 2169-2177. doi:10.1128/jb.185.7.2169-2177.2003

Wen, Yurong, Ester, & Bart. (2014, April 01). Toxin–Antitoxin systems: Their role in persistence, biofilm formation, and pathogenicity. Retrieved from https://academic.oup.com/femspd/article/70/3/240/567411