Differential Expression Analysis of Type II Toxin-Antitoxin Genes of *Pseudomonas aeruginosa* PAO1 under Different Environmental Conditions

Anamul Haque

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Biological Sciences

Roderick V. Jensen, Chair
Birgit E. Scharf
Florian Schubot
Jing Chen

May 8, 2018
Blacksburg, Virginia

Keywords: Type II Toxin-antitoxin Module, Bacterial Persistence, Toxin to Antitoxin Ratio, RNA-Seq, Differential Expression Analysis

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ABSTRACT

Bacterial persistence is considered as one of the primary reason for antibiotic tolerance besides genetically acquired antibiotic resistance. Persisters are the subpopulation of a clonal bacterial population, which can survive environmental extremes and become invulnerable to stresses due to limited metabolic activities and physiological functions. Cognate toxin and antitoxin (TA) pairs, which are transcribed simultaneously from the same or different operons within the bacterial chromosomes or plasmids, play an important role for bacterial survival during stressful growth environments. *Pseudomonas aeruginosa* PAO1 is one of the most versatile microorganisms in the environment. Despite its ubiquitous presence, no studies have shown the differential expression pattern of its toxin-antitoxins, and persistence related genes. The purpose of the following study is to analyze differential expression of *P. aeruginosa* PAO1 type II toxin-antitoxins and persistence related genes under different growth conditions and to show how their stoichiometric ratio changes during different growth conditions. Differential expression analysis indicated that the toxins and antitoxin pairs behave differently under different growth conditions. In addition, the genes related to persistence presented relatively consistent differential expression pattern under different growth environment.

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GENERAL AUDIENCE ABSTRACT

Bacterial persistence is one of the main reason for antibiotic tolerance and recurrent infections. Toxin-antitoxin molecules play an important role during bacterial persistence. Change in the expression of toxin, antitoxins, and persistence related genes and the ratio of the toxin to antitoxin mRNA molecules are important for bacterial survival in stressful environments. *Pseudomonas aeruginosa* PAO1 is one of most ubiquitous bacteria and responsible for recurrent infection in patients with weaker and compromised immunity. This mRNA sequence (RNA-Seq) analysis study of *P. aeruginosa* PAO1 showed different expression levels of toxin, antitoxin, and persistence related genes in various stressful growth conditions. This expression also showed the different ratios of the toxin to antitoxin mRNA molecules under different stress conditions. These implicate the different hypothetical roles of these toxin and antitoxin molecules in different growth conditions.

DEDICATION

To my father, who died on my very first day at Virginia Tech. His passion for education has brought me to this great country for higher education.

To my mother, who has dedicated her whole life raising her six children.

To my siblings, especially my elder brother for his endless contribution to shaping the future of all the younger siblings.

To my love and best friend, my wife for her continuous care, love, and encouragement during the most challenging times.

ACKNOWLEDGEMENT

I would first like to thank my committee chair Dr. Roderick Jensen of the Department of Biological Sciences at Virginia Tech. If he would not have taken my responsibility, it would be tough for me to reach the end of the degree. Next, I would like to thank Dr. Birgit Scharf for her guidance and assistance during my tumultuous times at Virginia Tech. Without her help, it would be impossible for me to identify my strengths and weaknesses. Whenever I had any problem or question, I ran into her office. She was always there to help me. I also like to thank Dr. Florian Schubot and Dr. Jing Chen for being on my committee and providing valuable feedback on improving my research thinking and ideas. I am very grateful to my past adviser Dr. William Mather for his kind guidance through my learning stage. He introduced me to the field of Computational Systems Biology and allowed me to take courses in computer science and mathematics. His departure from Virginia Tech is heartbreaking for me.

Finally, I must express my very profound gratitude to my family members and my wife for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

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CHAPTER 1

Introduction and Literature Review

1.1. Introduction

The word bacterial persistence was first coined by Joseph Bigger, an army physician in 1944 when he first noticed the failure of Penicillin to treat *Staphylococcus aureus* infection (Wood, 2016). Subsequent studies found the broader antibiotic resistance of bacterial cells to the short-exposure of antibiotics and this resistance is not linked to the plasmid/chromosome-mediated antibiotic resistance. This phenomenon, now known as persistence, occurs for a wide variety of bacterial cells. Further studies proved that persister cells are genetically identical to normal antibiotic sensitive cells but show different phenotype under stressful antibiotic-rich environment. The molecular mechanism of bacterial persistence is not clearly understood because of its low frequency in the clonal population. Therefore, it is important to have a complete understanding of persister mechanism for the efficient use and development of antibiotics and antimicrobial agents for the treatment of chronic infection. The purpose of this literature review is to describe some aspects of bacterial persistence, intracellular toxin and antitoxin molecules and their importance in bacterial persistence.

1.2. Differential Gene Expression, Phenotypic Diversity and Formation of Persister State

Phenotypic variation in the genetically identical bacterial population can arise from intracellular noise or extracellular environmental signals. This noise or extracellular signal results in the differential expression of set of genes related to bacterial stress response and slow growth and serve as the trigger for persistence. Although it was evident that the persister state arises in part from an intracellular stochastic process, it is also triggered by environmental stress signals such as starvation, carbon source transitions, oxidative stress, quorum sensing, SOS response, heat response and the presence of antibiotics (Cohen, et al., 2013).

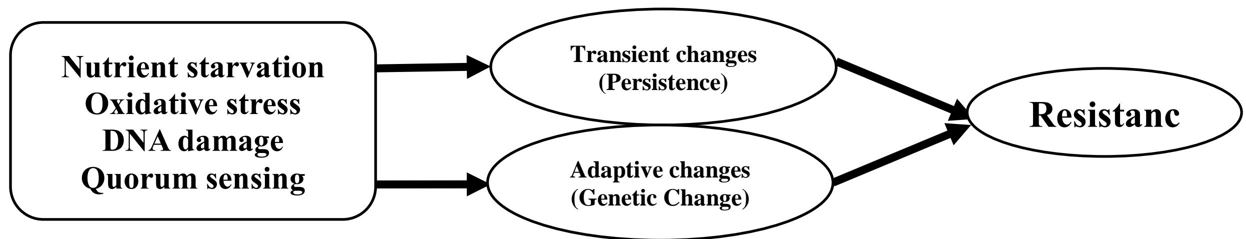

Figure 1.1. Stress responses link persistence, adaptive evolution and resistance (Cohen, et al., 2013).

Diversity in phenotypic response is a strategy for survival in an adverse environment. Genetically identical cells leverage a random phenotype, which fits the environment. This is very similar to an insurance policy or bet hedging (Ratcliff, et al., 2015). Persistence is one such bet-hedging process during the times of low stress or no stress. If cells cannot form a persister phenotype during their favorable growth phases, then the entire population dies if any sudden stress is applied. On the other hand, if all cells go to persister state, the population would cease to grow and thus miss a potential opportunity to replicate normally. It is very similar to the decision-making steps like other bacterial survival strategies (i.e. bacterial sporulation) (Schultz, et al., 2009).

Persister cells are divided into two types based on the mechanism of their entry into the persistent state. This classification is based on the distinct growth phases where persister cells enter into the persister phenotype (Balaban, et al., 2004). In term of the bacterial growth phase, type I persister cells are formed in the stationary phase whereas the type II persisters formed in the exponential phase. Type I persisters population is the part of the normal bacterial population which are produced during the stressful environment. The contributing factors for persister phenotype are normally kept suppressed and do not contribute to persistence during normal physiological conditions. Nutrient starvation plays a major role in the phenotypic variability that leads towards the triggering of bacterial persistence for type I. On the other hand, type II persisters arise randomly

without stress and from a homogenous normal population. Persisters become tolerant to antibiotic or other assaults, due to their slow growth rate and regain normal growth after the remission of the antibiotic exposure. They use their slow metabolic activity as the bet-hedging process to survive the harsh environment like antibiotic treatment, thermal stress, and nutrient deficiency. Slow growth of the persister cells make them less fastidious for essential growth factors and therefore they can maintain a very low metabolic profile. This study is focused on type I persister cells.

1.3. Toxin and Antitoxin Systems

Toxin-antitoxin systems (TA) systems were discovered around 1980 for a different reason in bacteria than bacterial persistence (Bahassi, et al., 1999). Scientist found that TA modules are required for the maintenance of plasmid and the post cell division killing of bacteria, which does not have any plasmid copy inherited from the parent strain. This toxin-antitoxin module later identified as the *ccdBA* toxin-antitoxin modules. Multiple TA modules have found for model organism *E. coli* and they are now considered as the main molecular player of bacterial persistence. TA molecules work as a cognate pair and sometimes found under the control of the same promoter. The cognate pairs were described as modules and present in all bacteria in different numbers. For example, there are at least thirty-seven known types of Toxin-Antitoxin (TA) modules in the model organism *E. coli* while *Mycobacterium tuberculosis* has more than eighty TA modules. Only a few of these TA modules are biochemically characterized, i.e. for *E. coli* eleven TA modules have been thoroughly studied (Deter, et al., 2017). Further studies showed that these TA modules work in pairs consisting of a stable toxin and unstable antitoxin. TA modules are classified into six types based on the mechanisms that antitoxin uses to neutralize the deleterious effect of the toxin on bacterial metabolism (Page and Peti, 2016). Type I and III TA modules consist of a small RNA antitoxin that counteracts the toxins at the translational (antisense RNA) or posttranslational levels

(direct toxin binding). Type II TA modules consist of a protein antitoxin that neutralizes the protein toxin by direct protein-protein interaction. In type IV TA modules, the antitoxins indirectly inhibit the toxin by counteracting the function of toxin. Type V antitoxins are site-specific endoribonucleases which cleave toxin mRNAs to inhibit its further processing into a toxin. Type VI TA pairs are discovered recently where the antitoxin acts as an adapter molecule for the degradation of toxin molecule.

Table 1.1. Cellular targets of selected well-characterized type II toxins (Unterholzner, et al., 2013).

Toxin	Antitoxin	Toxin activity	Cellular process
CcdB	CcdA	Inhibition of DNA gyrase	Replication
ParE	ParD	Inhibition of DNA gyrase	Replication
MazF	MazE	Ribosome-independent mRNA and 16S rRNA cleavage	Translation
Kid	Kis	Ribosome-independent mRNA cleavage	Translation
HicA	HicB	Ribosome-independent mRNA cleavage	Translation
RelE	RelB	Cleavage of ribosome-bound mRNA	Translation
VapC	VapB	Cleavage of $tRNA^{fMet}$	Translation
Doc	Phd	Binds to the 30S ribosomal subunit	Translation
HipA	HipB	Phosphorylation of EF-Tu	Translation

This study will focus on the Type II Toxin-antitoxin system, which is the most widely studied group of TA molecule. Type II Toxin-antitoxins modules are mostly encoded by consecutive DNA fragments, where the antitoxin gene under the control of the same promoter precedes the toxin in the same operon. In normal physiological conditions, there is always an excess production of antitoxin than toxin to keeping cellular metabolism uninterrupted. During stress, the antitoxins are degraded by the cellular proteases (ClpXP/ ClpAP/ Lon), toxin becomes liberated from the antitoxins, starts to interrupt cellular metabolism, inhibits cellular metabolism and switches into the persister state. Table 1.1 presents the cellular target for some of type II TA module.

Toxins can interfere with the cellular translation machinery in three different ways: interacting with the ribosome, direct interaction with the target mRNA and interaction with translational machinery. As an example of the first category, RelE toxin cleaves the ribosomal A site between the second and third base in a translation-dependent manner (Goeders, et al., 2013). Some Type II toxins found to cleave the ribosomal RNA without any specificity. For example, YoeB cleaves between second and third codon of mRNA, where YafQ works at the AAA-lysine codon (Armalyte, et al., 2012; Goeders, et al., 2013; Hurley and Woychik, 2009; Prysak, et al., 2009). The second category directly interacts with mRNA targets before any active translation. The most common of this group of TA module is the MazF toxin molecule, which cleaves mRNA in three, five or seven base recognition sites (Zhu, et al., 2009). Another E.coli toxin MqsRA cleaves the mRNA GC (U/A) site (Christensen-Dalsgaard, et al., 2010; Yamaguchi, et al., 2009). For the third category of the translation hindering mechanism, Doc toxin interacts with the transcription factor EF-Tu by phosphorylation (Castro-Roa, et al., 2013) . The most studied toxin HipA is also a kinase, which interferes with translation by phosphorylating the uncharged tRNAGlu (Germain, et al., 2013; Schumacher, et al., 2009). Bacterial cell wall formation is also a targeted by the PezT toxin.

The PezT toxin inhibits the cell wall synthesis by phosphorylating the UDP-N-Acetyl glucosamine (UNAG) (Tan, et al., 2011). The phosphorylated UNAG results in the inhibition of MurA, which is the first step of cell wall peptidoglycan biosynthesis. Most of the toxins from type I are hydrophobic are hydrophobic in nature and contains a cytoplasmic domain which results in the generation of a pore and charge disruption leading to cell lysis. Toxin CbtA, is known for the GTP-dependent polymerization of FtsZ and ATP-dependent polymerization of MreB (Mutschler, et al., 2011). The phosphorylated groups of CbtA causes inhibition of cellular segregation and results in elongated cells.

1.4. Mechanism of Hindering Bacterial Persistence

Toxins slow down cellular metabolism by targeting cellular processes starting from DNA replication to cell wall synthesis. Most methods of hindering bacterial persistence are strategized either by targeting toxin targets or cellular machinery that degrades antitoxins There are multiple methods has been developed to hinder bacterial persistence exploiting active metabolism or targeting the cellular machinery which interferes with the function of toxin and antitoxin. Activating the ClpP protease by chemical acyldepsipeptide (ADEP4) results in decreased bacterial persistence in *Staphylococcus aureus* (Conlon, et al., 2013). This over activation results in the removal of the bottleneck in the protein degradation process and results in removal of the proteolytic queue (Conlon, et al., 2013). Another mechanism popular for the inhibition of *Pseudomonas aeruginosa* quorum sensing is BHL ([*N*-butyryl]-l-homoserine lactone), and OdDHL (*N*-[3-oxo-dodecanoyl]-l-homoserine lactone) (Smith and Iglewski, 2003). Synthetic quorum-sensing (QS) inhibitors derived from natural brominated furanone compounds isolated from *Delisea pulchra* known for its inhibition of quorum sensing and, biofilm inhibition and persistence inhibition (Hentzer and Givskov, 2003). Sometimes synthetic peptides known such as

dodecamer peptide termed 1018 was reported to label the alarmone (p)ppGpp for degradation resulting as the same result as above (Andresen, et al., 2016). Since bacterial toxin to antitoxin maintains certain stoichiometric ratio in the cell, changing the ratio may serve as an alternative method for hindering bacterial persistence. Some synthetic biology methods shown to change the frequency of persister cells using the same principles (Vazquez-Laslop, et al., 2006).

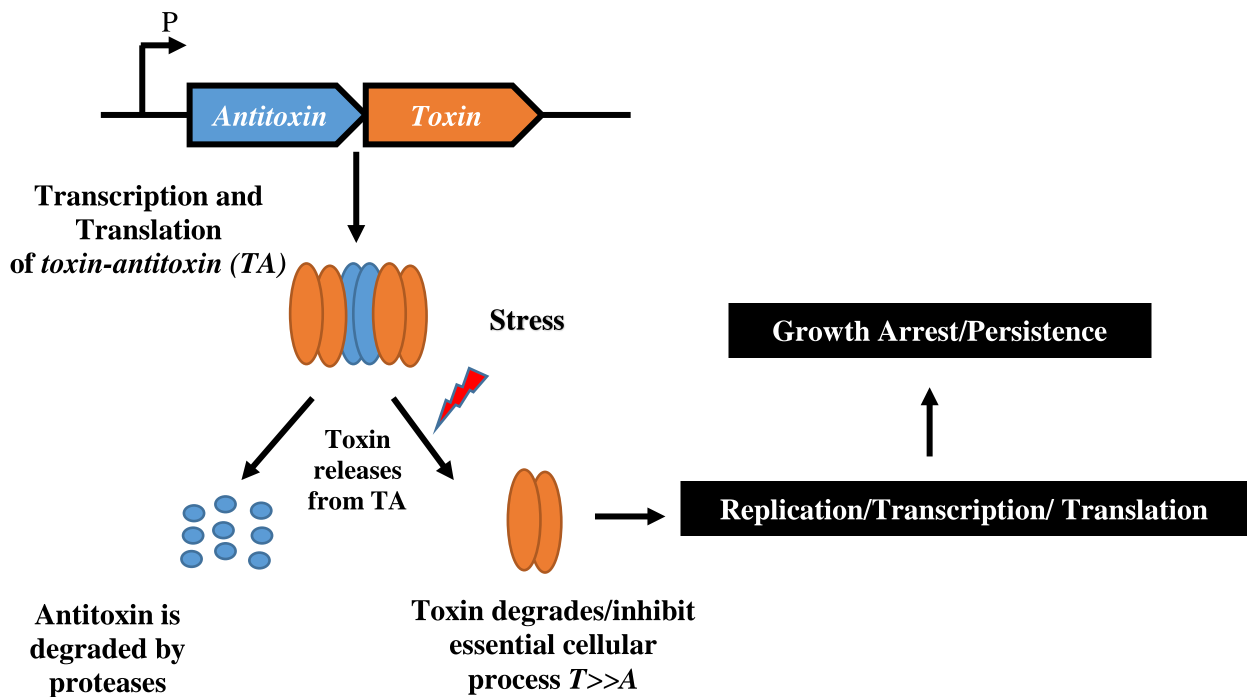

Figure 1.2. Mechanism of bacterial growth arrest and persistence under stress.

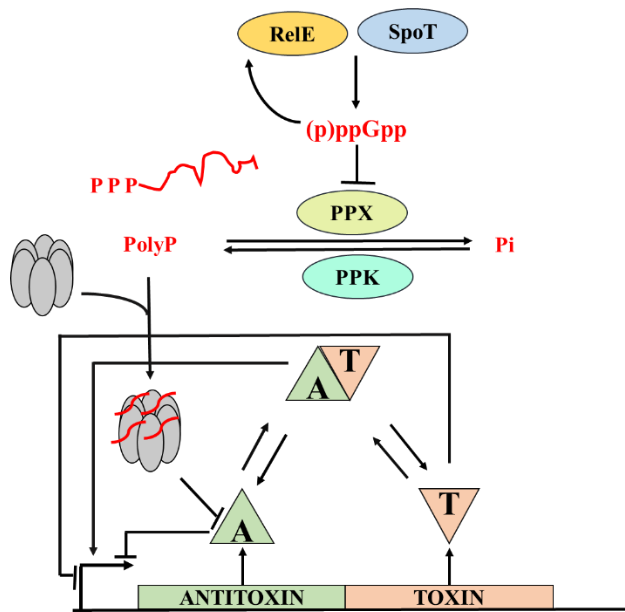

Figure 1.3. Role of ppGpp, RelA and SpoT in bacterial persistence (Maisonneuve and Gerdes, 2014).

1.5. (p)ppGpp and other Molecular Players of Bacterial Persistence

Secondary messenger molecules are released by the cell to trigger physiological changes such as cell division, differentiation, migration, survival. In combination with the bacterial sensory molecules, they play an important role in bacterial persistence (Maisonneuve, et al., 2013). Among the three secondary messenger molecules (cAMP, c-di-GMP and (p)ppGpp), (p)ppGpp plays an important role in bacterial persistence. Guanosine 3'-5'- bisdiphosphate (ppGpp) function as one of the major modulators of bacterial growth rate general metabolism; contributing to the growth rate control by inhibiting ribosomal RNA production.

(p)ppGpp serves as the alarmone, an intracellular signal for nutrient starvation, amino acid starvation and other stresses. It is synthesized by RelA and SpoT and inhibits the production exopolyphosphate (PPX) (Maisonneuve, et al., 2013). PPX is the enzyme, which hinders the constitutive expression of inorganic polyphosphate (PolyP). Absence of PolyP keeps Lon protease

inactive and hence enough antitoxin is available for the inhibition of toxin function (Chowdhury, et al., 2016). Signaling pathway connecting TA activity to ppGpp, PolyP and Lon protease is central to the persistence.(Maisonneuve and Gerdes, 2014). Moreover, positive feedback on ppGpp synthase RelA and SpoT by ppGpp and negative feedback of toxin on the TA promoter helps the cell to exit the persister state. When the ratio of either the toxin or antitoxin decreases in the cell, conditional cooperativity helps to balance the concentration of toxin and antitoxin transcripts and their degradation rate (Cataudella, et al., 2012).

1.6. Interaction of the TA module with Biofilm formation

Many bacteria use the biofilm for their survival in the adverse environments like the host immune system and low nutrients. The biofilm is the harbor for persister cells since the outer layer of the biofilm consists of the metabolically active planktonic cells and persisters stay in the inner core. Upon antibiotic treatment, the planktonic outer surface cells die, providing the protection and nutrients to the inner core organisms (Lewis, 2005). On the other hand, the inner persister core can survive the treatment of antibiotic and after the decrease of the antibiotic dosage; these persisters later changed into normal cells (Toyofuku, et al., 2016). There are some TA modules found to be directly involved in the formation of planktonic cells in the *E.coli* b3022 cell line. The TA pair involved in the biofilm formation is the MqsRA TA pair, which interacts with the Autoinducer -2- dependent quorum sensing system and regulates cell motility. MqsA is found to repress the expression of the general stress response regulator RpoS and CsgD, leading to the reduced formation of signaling molecule c-di-GMP, and ultimately in the reduction in biofilm formation. Triggered by oxidative stress, Lon protease directly degrades the antitoxin MqsA (Gonzalez Barrios, et al., 2006).

Another study found that the deletion of five TA modules (MazF/MazE, RelE/RelB, YafQ/Dinj and ChapB) in *E.coli* results in the reduction of persister formation after hours of antibody (or antibiotic?) treatment and then subsequent increase (Wang and Wood, 2011). However, *Streptococcus mutans* mutants lacking MazF/MazE do not have any effect on biofilm formation (Wang and Wood, 2011).

Beside the chromosomal TA modules, cryptic phage TA modules have found to influence biofilm formation. For example, the TA pair YpjF-YfjZ in *E.coli* K-12 found from cryptic phage CP4-57. Deletion of this cryptic phage toxin YpjF results in the decrease in bacterial persistence. Another cryptic phage TA system RelBE found from the prophage Qin shows the same result for persistence after its deletion. Quorum sensing, a cell to cell communication system in bacterial the biofilm formation, also has interaction with some toxin-antitoxin molecules (Wang, et al., 2010).

1.7. Systems Biology Approaches to Study Bacterial Persistence

The study of persister metabolism is limited by several factors such as the absence of normal growth phase, temporary nature of the persister state, small subpopulation of persisters (Prax and Bertram, 2014). Few groups tried to study persister metabolism using methods like Phenotypic Microarray Methods (Orman and Brynildsen, 2013) and Carbon Isotope Analysis (Prax and Bertram, 2014) . The mixture of Viable but Non-Culturable Cells (VBNC) limits the former and the later only measures the relative abundance of the metabolites. Though not ideal, the ^{13}C is one of the useful methods for studying the metabolism. In this technique, ^{13}C isotope fed to the cell culture and the intermediates of the metabolism are determined in a time-dependent manner. Studies required for the long-term behavior of the persister cell is very hard to study. Another tough part of the persister study is the persister exit mechanism. Although a lot of research have

done for the persister entry state, less is known about the exit mechanism due to cells stochastic behavior during the exit process.

Synthetic biology enables the control of biological systems in a tunable manner. Using synthetic biology, scientists design biological systems to take input (i.e. stress) as biochemical signal to yield desired output. Generally, these output signals are cleaner than the native genetic circuits. These input-output systems are very similar to the switch and gate behavior of electrical circuits. Synthetic biology approaches are being used for the persister phenotype (Vazquez-Laslop, et al., 2006). Cellular toggle switch based on these synthetic circuits can be used for the study of persister behavior. Moreover, these tunable systems are used for increasing the persister frequency and for the study of phenotypic variation. Beside these synthetic approaches, native gene circuit architecture can also be used for modelling of persister phenotype (Koh and Dunlop, 2012).

Studying microorganisms for their collective behavior has been practiced for long time in classical microbiology. This kind of collective behavior study may be performed in two ways: looking for the change of gene expression and for the change in phenotypic response. With the improvement of the modern sequencing techniques, it is now easier to look for changes in overall gene expression patterns under different environmental stress. Modern techniques like Microarray and Next Generation Sequencing (NGS) make the study of overall gene expression patterns easier. Further analysis of these gene expression profiles using modern computational biology methods enables the identification of group of genes, which are expressed under certain environmental stresses. For example, gene expression data along with further bioinformatics analysis enables better classification of bacterial toxin and antitoxin modules (Deter, et al., 2017).

1.8. Bioinformatics Methods for the Identification of the TA modules

Most of the bacteria have multiple toxin-antitoxin systems. Some of them have similar functions as well as homologous nucleotide and amino acid sequences. Therefore, TA modules can be a fascinating subject to computational biologists. A few bioinformatics methods have developed for the identification of new TA modules and for the prediction of their functions. In addition to these methods, development of RNA-Seq and Ribo-Seq makes the study of the TA systems at the post-transcriptional and translation levels more feasible. These techniques provide functional insight for the identification and classification of new groups of TA modules (Pandey and Gerdes, 2005). The first data mining for the identification of TA modules has used BLASTP and BLASTN methods for the identification of potential TA modules in both chromosome and plasmid. Use of simple database searching like BLASTP and BLASTN have their limitation because they only search for homologous nucleotide and protein sequences in sequences in databases rather than looking for any functional domains (Pandey and Gerdes, 2005). The most popular method for the identification of the unknown TA module is the RASTA (Rapid Automated Scan of Toxin and Antitoxin) (Sevin and Barloy-Hubler, 2007). The RASTA method relies on a three-step algorithm, the presence of common TA modules, identification of small genes, and the pairing of one ORF with another. For the prediction of the bi-cistronic operon in prokaryotic genomes, another method is used which is known as the 'Cluster of Orthogonal Genes (COGs) method (Makarova, et al., 2009). The COGs method requires known TA families for the queries of PSI-BLAST. PSI-BLAST identifies TA modules even with a very small functional similarity in their function. There is another recent method built for the identification of the Type II TA modules. This method uses the idea that toxin and antitoxin may flank another antitoxin and toxin gene. All these methods lead to

the development of a database known as the toxin-antitoxin database (current version 2.0) (Shao, et al., 2011).

While most of the bioinformatics methods designed towards the identification of Type II TA modules, few are invested in the bioinformatics methods for the identification of Type I and III TA module (Sberro, et al., 2013). This is partly due to the structural differences of the toxin an antitoxin molecule of Type I and III TA modules. While for Type II both the toxin and antitoxin molecules are proteins but for others types, only the toxin molecules are proteins. Simply database searching is not a good idea for searching type I and types III TA module since one of them consists TA systems with one small hydrophobic toxin protein (<70 amino acids) and another consist of antisense mRNA toxins. One of this algorithm first identifies experimentally identified toxins of Type I using PSI-BLAST. TBLASN, then look for transmembrane proteins less than 70 amino acids, selection of intergenic regions higher than 400 nucleotides from upstream gene and 250 nucleotides from the downstream genes and then excluding the pseudogenes. Then small antisense mRNAs are searched for in the bacterial transcriptome. The development of the shotgun cloning and genome sequencing has also been used for the identification of unknown TA modules. The shotgun sequencing is helpful but has some difficulties. Some of the small mRNA antitoxins are so small that cannot be identified using the shotgun sequencing. The shotgun sequencing is limited to the identification of TA modules only from *E.coli* because the random cloning of genes from other organism was only done by cloning into *E. coli*. Moreover, shotgun sequences may end up giving wrong TA pairs.

Most recently, yet another method has been developed for the classification of the type II TA modules based on the differential expression of type II TA modules. This method based on the on RNA-Seq, Ribo-Seq, and Ribosome Binding Site Calculation (RBS Calculator v2.0) for the

classification of type II TA module based on their protein production (Deter, et al., 2017). In this method the authors classify type II toxin and antitoxins based on DNA sequence and mRNA product, determine protein synthesis rate on Ribo-Seq and analyzed differential protein expression rate based on Translation Initiation Calculator (TIR)

1.9. Objective:

Differential expression analysis of Type II toxin and antitoxin genes of *Pseudomonas aeruginosa* PAO1 under different environmental conditions.

Rationale: Bacterial persistence arises from the differential expression of toxin and antitoxin genes and their relative abundance under different environmental stress. The stoichiometry of the ratio of toxin to antitoxin is an important factor during the diminished cellular function, which is essential for bacterial persistence. In addition to the ratio of toxin to antitoxin, the expression level of the persistence related genes changes with bacterial growth environments.

Aims: Determine trends in the relative expression of toxin and antitoxin and persister related genes across different growth conditions of *Pseudomonas aeruginosa* PAO1.

Approaches: The hypothetical toxin and antitoxin genes will be determined using the bioinformatics tools. The RNA-Seq data will be downloaded from the NCBI Gene Expression Omnibus (GEO) database and the relative abundance of these genes will be quantified using the Geneious pro software. Persistence related genes will be identified using current literature and quantified using the same analysis.

Pitfalls: However, ultimately, the level of gene expression alone cannot be used for finding correlation with the persister frequency. It is the actual protein level, which shows strong

correlation of toxin and antitoxin which persistence phenotype. In addition, there is not enough studies, which show clear relationships between transcript level and protein level in the cell for the study of bacterial persistence. Even the most current studies do not show any clear evidence how the toxin-antitoxin transcript level changes during bacterial persistence partly due to the low number of cells in the persister population. Therefore, a best the measurements of the gene expression levels can be used for indicating candidates for differentially expressed TA modules that could provide theoretical insight and suggest future experiments.

CHAPTER 2

Differential Expression Analysis of Type II Toxin-Antitoxin Genes of *Pseudomonas aeruginosa* PAO1 Under Different Environmental Conditions

2.1. Introduction

Bacterial persistence is a phenotypic trait shown by a small population of bacteria, which has characteristics of slow growth and diminished metabolism. This phenotype may develop due to the presence of stress in the growth environment or spontaneously in a subgroup of a homogenous population. Bacterial persistence involves a cascade of events that consists of a network of cellular macromolecules (Wood, 2016). Central to this network are the toxin and antitoxin molecules. The toxin and antitoxin are cognate molecules and work as a pair and may reside under the control of the same or different promoters. They are expressed simultaneously. The toxin hinders normal cellular metabolism (Page and Peti, 2016), while the antitoxins tend to minimize the detrimental effect of toxin by binding to them. Under normal physiological conditions, antitoxins are expressed at sufficient levels to bind to the toxin to inhibit its detrimental role in cellular metabolism. There are around 40 toxin and antitoxin pairs have discovered in the model organism *E. coli*. Despite this discovery, only few of them are well characterized and thoroughly studied. These toxin and antitoxins pairs are classified based on the mode of action of the antitoxin. These modes of action classify toxin-antitoxin pairs into six different groups. Among these six groups, the type II toxin-antitoxin pairs are the most studied group of toxin-antitoxin molecules (Unterholzner, et al., 2013). The type II toxin-antitoxin pairs, where both the toxin and antitoxins are proteins, interact with other cellular macromolecules, like bacterial protease, to facilitate entry into the persister stage by selective degradation of the antitoxins to liberate free toxins. Under normal physiological growth conditions, the type II toxin and antitoxin molecules interact with each other and maintains a stoichiometric ratio. Therefore, the cells does not have excess toxins, which may interfere with normal cell physiology. However, in an adverse environment or due to environmental stress, the ratio of the toxin to antitoxins increases to provide more free toxins allowing its interaction with

the essential cellular machinery (Muthuramalingam, et al., 2016). This results in the change of antibiotic efficiency and generates persister cells, which are resistant to antibiotics without acquiring any antibiotic resistant traits.

The regulation of the toxin-antitoxin pairs is one of the most interesting field in the research of bacterial persistence because of its further role as a mechanism of antibiotic resistance. The most studied mechanism of the toxin and antitoxin regulation is named as the conditional cooperativity (Cataudella, et al., 2012). This regulatory mechanism only works when the toxin and antitoxins in the same operon bind to each other and to their promoters in a certain orientation and in certain stoichiometric ratios of the antitoxin to toxins (Cataudella, et al., 2012). This cooperative binding facilitates the transcription of a cognate toxin-antitoxin pair at low toxin concentration, minimum expression at a medium toxin level, and again high level of transcription at a high toxin level.

Pseudomonas aeruginosa is one of the ubiquitous microorganisms in the environment and an opportunistic pathogen. This seemingly harmless bacterium becomes pathogenic in immunocompromised cystic fibrosis patients and is a major etiological agent in hospital acquired infections (Attila, et al., 2008). *P. aeruginosa* PAO1 is one of the metabolically diverse strains of *P. aeruginosa* and is considered the most opportunistic pathogen in this group. Despite its relatively lower rate of pathogenicity, *P. aeruginosa* also has a unique feature that is not present in most other pathogenic organisms. It is considered to be the model organism for the study of biofilm. *P. aeruginosa* has a cell-to-cell communication system known as quorum sensing and this aids in the formation of complex biofilms and persister cells (Mulcahy, et al., 2014). The phenomenon of persistence has been used to describe the cells in biofilm a long ago (López, et al., 2010), but no detailed study has been done yet to determine the role of toxin-antitoxin molecules in those persister cells. Using bioinformatics methods, four different toxin and antitoxin pairs and

four putative toxin antitoxin pairs of Type II have been predicted but no detailed study has been done so far to biochemically confirm the function or relative expression of these toxin and antitoxin molecules.

High throughput methods like RNA-Seq are one of newest method in the field of genomics. In combination with high throughput computational analysis, RNA-Seq methods enables the characterization of transcriptional landscape of any microorganism at different points of time in their growth. This method enabled the detailed study of transcriptional response of microorganism under different growth conditions (Costa-Silva, et al., 2017). Use of high throughput computation like differential expression analysis, enables scientists to identify the set of genes, which are upregulated or downregulated under different stress conditions.

The purpose of the following study was to identify the gene expression patterns of the type II toxin and antitoxin genes in *P. aeruginosa* under different growth conditions. I will also compare the expression pattern of additional genes and their transcriptional landscape changes in bacterial persistence. This will enable us to compare the ratio of toxin to antitoxin and their relation to conditional cooperativity.

2.2. Methods and Materials

2.2.1. Identification of Putative Type II Toxin and Antitoxin Pairs in *P. aeruginosa PAO1*

Bioinformaticians use different methods for the identification of unknown toxin-antitoxins (TA) pairs in bacteria. Though most of those methods rely on homology search, some of the methods are more reliable because of the use of multiple levels of homology identification (Shao, et al., 2011). For this study, I used the TA database (http://bioinfo-mml.sjtu.edu.cn/TADB2/), which stores a database for all putative bacterial type II, TA pairs. The TA database identified four

confirmed type II TA pairs and four putative type II TA pairs in *P. aeruginosa* PAO1. The first four TA pairs (Table 2.1) have been identified using the toxin-antitoxin database (TADB). On the other hand, the four putative TA modules identified using the Rapid Automated Scan for Toxins and Antitoxins (RASTA) tool, which utilizes rpsBLAST search algorithm with common features of searching TA modules such as the presence of two genes co-directed modules that code for small proteins (Sevin and Barloy-Hubler, 2007). Cutoff for RASTA-Bacteria TA hit was set to greater than 70% and 60%, which shows candidate TA modules with very high confidence.

Table 2.1. Confirmed toxin and antitoxin pairs of *P. aeruginosa* PAO1 predicted by TADB.

Toxin	**Antitoxin**	**Location**	**TA gene family**	**Protein Domain**
PA0124	PA0125	143567 - 144072	*relBE*	RHH-RelE
PA1030	PA1029	1116213 - 1117390	*Unclassified*	Xre-COG5654
PA1878	PA1879	2048570 - 2049791	*vapBC*	Xre-PIN
PA3270	PA3269	3658248 - 3659756	*Unclassified*	Xre-GNAT (Acetyltransferase)

XRE : Xenobiotic Response Element. **RHH :** Right Handed Helix.

Table 2.2. Putative toxin and antitoxin pairs in *P. aeruginosa* PAO1 predicated by RASTA.

Toxin	Protein Domain	Antitoxin	Protein Domain
PA5404	Unclassified	PA5403	HTH_XRE/HTH_XRE/HTH_3
PA3056	HTH_XRE/HTH_XRE/ HipB/HTH_3	PA3055	HipB/HTH_XRE/HTH_XRE
PA2781	Unclassified	PA2780	HTH_XRE/HTH_XRE/HTH_3/HipB
PA0125	Unclassified	PA0124	ParE/Plasmid_Stability

XRE : Xenobiotic Response Element. **HTH :** Helix Turn Helix

2.2.2. Selection of other genes related to persistence and stress sigma factors in *P. aeruginosa* PAO1

Beside the toxin-antitoxin molecules, some other genes are involved in bacterial persistence. Previous studies have shown the importance of the genes listed in Table 2.3 during persistence or harsh environmental conditions. Some studies also scored these genes based on their importance in survival in harsh environment. Using their dataset of *E. coli (Wu, et al., 2015)*, I made the comprehensive list of genes other than toxin and antitoxins, which are also known to fluctuate among bacterial growth conditions. Most of the genes listed are encoding transcription factors or their genes products are involved in the cellular metabolism.

Table 2.3. Important genes related to persistence (Wu, et al., 2015).

Gene Name	**Function**
oxyR (PA5344)	Regulation of response to reactive oxygen species
dnaK (PA4761)	Global regulator/Heat Shock Protein Signature/Protein Folding
sucB (PA1586)	Energy production/TCA Cycle
relA (PA0934)	Stringent response/ ppGpp biosynthesis/purine metabolism
rpoS (PA3622)	Sigma factor/ transcription factor activity, sequence-specific DNA binding
clpP (PA1801)	Serine-type endopeptidase activity
clpB (PA4552)	ATP binding/ATPase activity
clpX (PA1802)	ATP binding/Unfolding protein binding
recA (PA3617)	DNA-dependent ATPase activity/DNA repair
lon (PA1803)	ATP-dependent peptidase activity
phoU (PA5365)	Cellular phosphate ion homeostasis
smpB (PA4768)	Trans-translation/RNA binding
glpD (PA3584)	Oxidoreductase activity/Glycerol-3-phosphate dehydrogenase activity
uvrA (PA4234)	SOS response/excinuclease ABC activity/ATPase activity
soxR (PA2273)	Response to oxidative stress
marC(PA5205)	Membrane protein, multiple antibiotic resistance

2.2.3. RNA-Seq Datasets

For this study, datasets were obtained from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI – GEO) database. All the data downloaded as FASTA format, which served as the input for the bioinformatics software Geneious. In the study GSE71880, *P. aeruginosa* PAO1 were continuously grown at 37°C in 150 ml synthetic cystic fibrosis medium in duplicate, their growth rate tested (2.4 hour and 9.8 hour doubling times) under two different growth conditions (microaerophilic, mO2 and anaerobic, AnO2). The *P. aeruginosa* PAO1 strain contains *pel* and *psl* unmarked deletions which results in the decrease of biofilm forming capability. In the study GSE73323, *P. aeruginosa* PAO1 was grown under anaerobic denitrifying conditions (0 mg N/L). Addition of Free Nitrous Acid (FNA, 0.1 mg-N/L) results in the temporarily halt of growth. Finally, in the study GSE107758, all *P. aeruginosa* PAO1 cultures were grown in M9-casamino acids at 37°C with 250 rpm shaking and samples were collected at log and stationary phase for RNA extraction.

Table 2.4. List of the experiments and sequencing runs used in this study.

GEO Accession No	**Sequence Read Archive (SRA)**	**Description**
GSE71880	SRX1140450	2.4h_mO2_1
	SRX1140451	2.4h_mO2_2
	SRX1140452	9.8h_mO2_1

	SRX1140453	9.8h_mO2_2
	SRX1140454	2.4h_AnO2_1
	SRX1140455	2.4h_AnO2_2
	SRX1140456	9.8h_AnO2_1
	SRX1140457	9.8h_AnO2_1
GSE73323	SRX1273291	0 mg N/L
	SRX1273292	0 mg N/L
	SRX1273293	0.1 mg N/L
	SRX1273294	0.1 mg N/L
	SRX1273295	0.1 mg N/L
GSE107758	SRX3449588	wild-type log-phase replicate 1
	SRX3449589	wild-type log-phase replicate 2
	SRX3449590	wild-type log-phase replicate 3
	SRX3449603	wild-type stat-phase replicate 1
	SRX3449604	wild-type stat-phase replicate 2
	SRX3449605	wild-type stat-phase replicate 3

2.2.4. Reference Sequence

For mapping the reads, the complete genome of the *P. aeruginosa* PAO1 (Accession Number: NC_002516.2) was downloaded from NCBI Genebank in .gb format.

2.2. 5. Mapping and Alignment

Mapping the raw reads to the reference sequence NC_002516.2 was performed using the Geneious software. For my datasets, I used the Geneious algorithms fast mapping method, which is relatively slower than other command line based methods but has higher accuracy than other methods like Kallisto and Bowtie2.

2.2.6. Expression Analysis

Calculation of the expression level was also performed by Geneious software. For normalizing the raw read count, I used the TPM method for comparing the differentially expressed genes, where the normalization factor is the sum over all genes of the (number of reads mapped to gene/gene length). All normalized counts (TPM) was converted into log2 for accommodating its wider range.

$$\textbf{TPM of Gene} = \frac{\textbf{(Number of reads mapped to a gene / gene length)} \times 10^6}{\textbf{Normalization Factor}}$$

For differential expression analysis, I used Microsoft Excel for preparing two dimensional correlation graphs to show how the ratio changes over time and to show the shift of ratio in toxin to antitoxin in different growth conditions.

2.2.7. Statistical Analysis

For the statistical analysis, I performed the t-test and only chose the datasets which significant difference between experimental conditions ($p < 0.05$). Error bars were calculated using the

Standard Error of Mean (SEM), where SEM is the ratio of standard deviation to the square root of the number of observation.

2.2.8. Heatmap Analysis

Heatmap analysis was performed using the tool ClustalVis (https://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015). This website provides a simple graphical user based interface for clustering and Heatmap analysis.

2.3. Results and Discussion

2.3.1. Raw Read Assembly to the Reference Sequence

The raw read counts from each of these sequencing run experiments (SRXs) were mapped against the *P. aeruginosa* reference sequence NC_002516.2. Geneious software successfully aligned 80% to 99% of the raw reads from each experiment run (Table 2.5). For expression analysis, I converted the raw read counts into TPM value for normalization using the built in methods in Geneious.

Table 2.5. Percentage of raw reads assembled to reference sequence NC_261500.

Experiment Number	Total Reads	Mapped Reads	% Reads Mapped
SRX1140450	5,367,285	5,121,235	95.42
SRX1140451	5,775,328	5,389,888	93.33
SRX1140452	5,904,702	5,832,273	98.77
SRX1140453	7,876,096	7,726,181	98.10
SRX1140454	5,333,081	5,293,525	99.26

SRX1140455	5,887,971	5,845,567	99.28
SRX1140456	6,598,795	6,548,129	99.23
SRX1140457	6,647,803	6,594,200	99.19
SRX1273291	12,560,216	12,080,084	96.17
SRX1273292	11,034,592	10,525,102	95.38
SRX1273293	12,034,388	11,303,939	93.93
SRX1273294	12,212,058	11,550,442	94.58
SRX1273295	10,946,316	10,506,161	95.98
SRX2915111	11,615,614	11,313,223	97.40
SRX2915112	11,615,614	11,274,034	97.06
SRX2915113	11,535,698	11,330,773	98.22
SRX2915114	12,312,782	11,893,875	96.60
SRX3449588	26,212,322	22,670,652	86.49
SRX3449589	25,696,396	21,051,599	81.92
SRX3449590	29,740,056	25,601,033	86.08
SRX3449603	24,819,864	18,092,303	72.89
SRX3449604	35,846,342	29,263,682	81.64

SRX3449605	34,554,884	28,182,185	81.56

2.3.2. Ratio of Toxin and Antitoxin and Their Differential Expression

After expression analysis, I found that only six pairs of toxin and antitoxin had significant levels of expression. To make the comparison consistent across multiple growth condition we chose those six toxin and antitoxin pairs for the study. For the data set GSE107758 (Figure 2.1 and Figure 2.2), we compared the expression ratios of toxin to antitoxin between exponential and stationary phase of bacterial growth. Four of the toxin and antitoxin pairs had lower toxin to antitoxin ratios in the stationary phase than the exponential phase. On the other hand, for two other toxin and antitoxin modules (PA1879:PA178 and PA2781:PA2780), the toxin to antitoxin ratio increased during stationary phase. Here, both the toxin (PA1878 and PA2780) and the antitoxin (PA179 and PA2781) is overexpressed, but the fold change in toxin overexpression is higher for toxin than antitoxin. This increased ratio indicates the overexpression of toxins compared to the antitoxin.

For the Free Nitrous Acid Stress (GSE73323, Figure 2.3 and 2.4), ratio of the toxin to antitoxin for most of the toxin and antitoxin pairs increased, except PA0124:PA0125 and PA1878:PA1879 pairs, where the ratio decreased due to the higher fold change in the expression of the antitoxin (PA0125 and PA1879) than the toxin (PA0124 and PA1878)

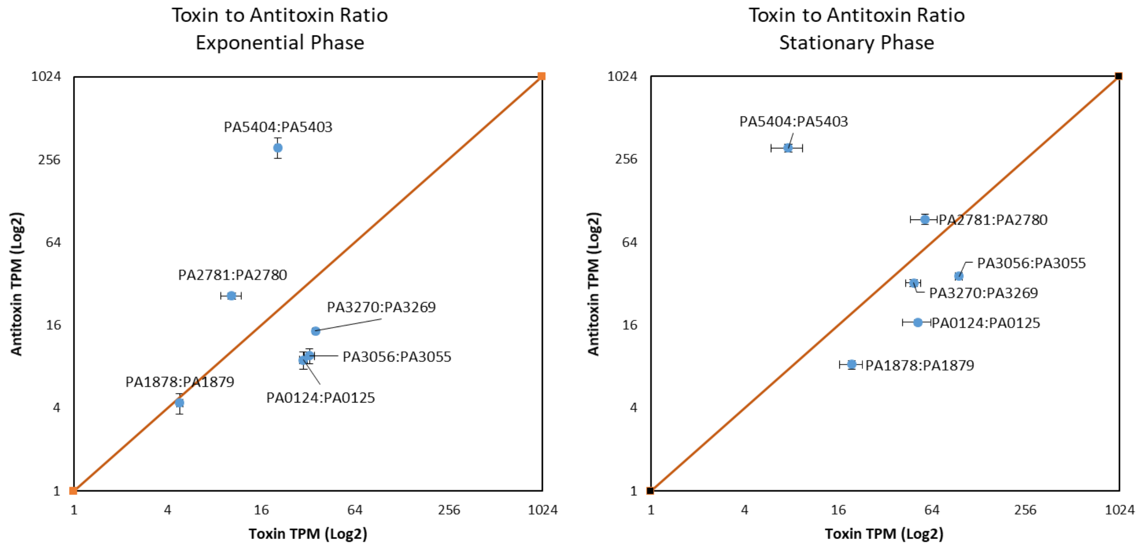

Figure 2.1. Differential expression of toxin and antitoxin pairs between exponential and stationary growth phase [GSE107758].

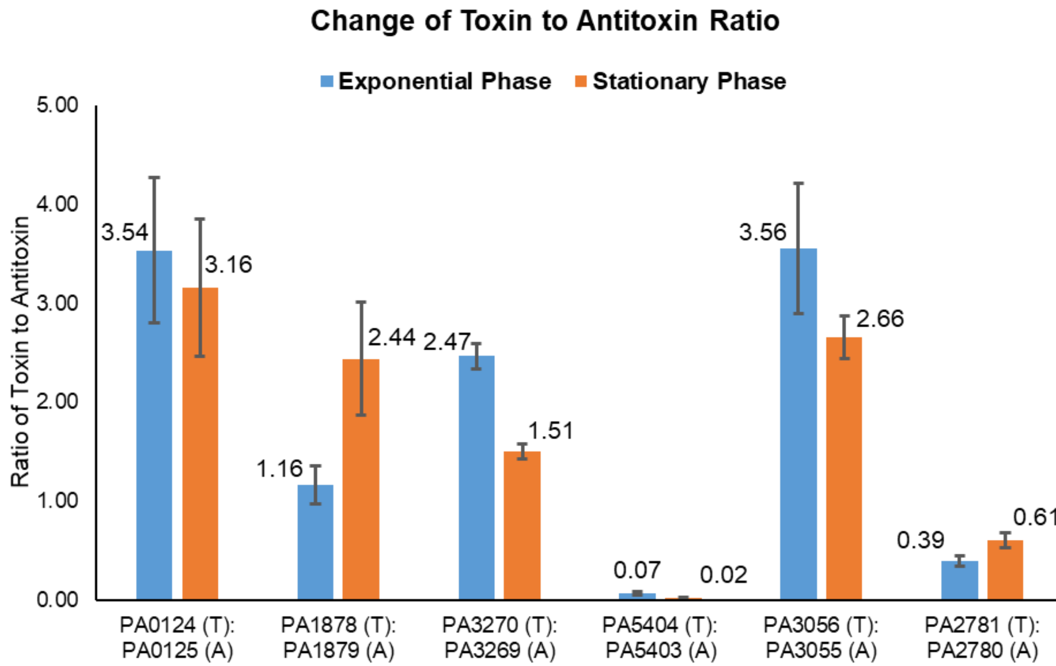

Figure 2.2. Change of the ratio of toxin to antitoxin between exponential and stationary phase of growth [GSE107758].

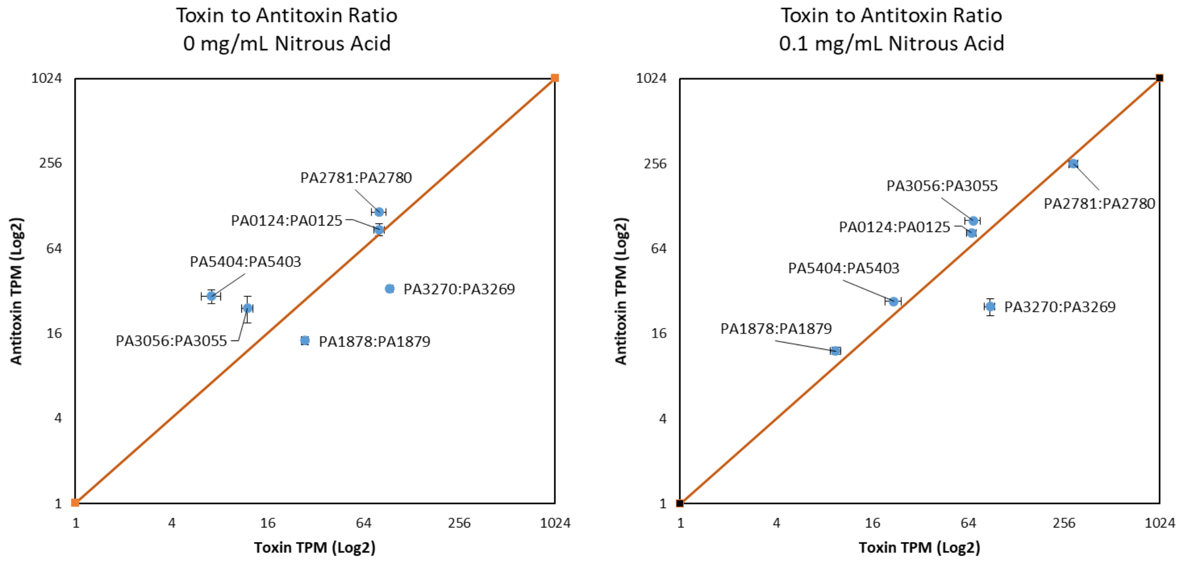

Figure 2.3. Differential expression of toxin and antitoxin pairs between anaerobic denitrifying conditions and Free Nitrous Acid stressed growth [GSE73323].

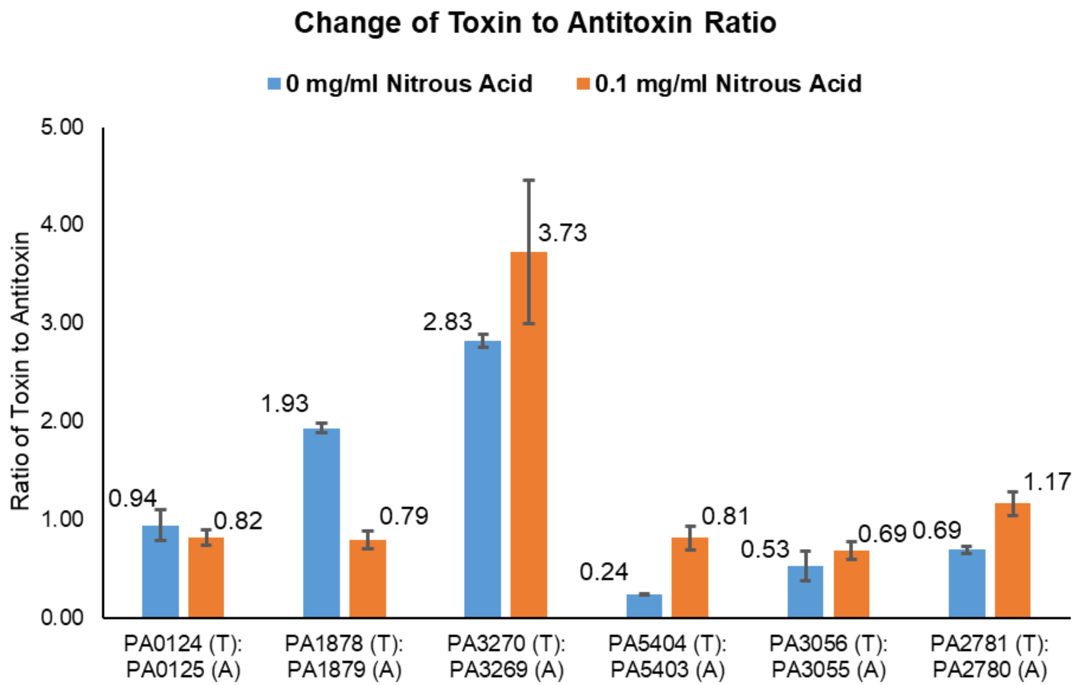

Figure 2.4. Change of the ratio of toxin to antitoxin between denitrifying conditions and Free Nitrous Acid stressed growth [GSE73323].

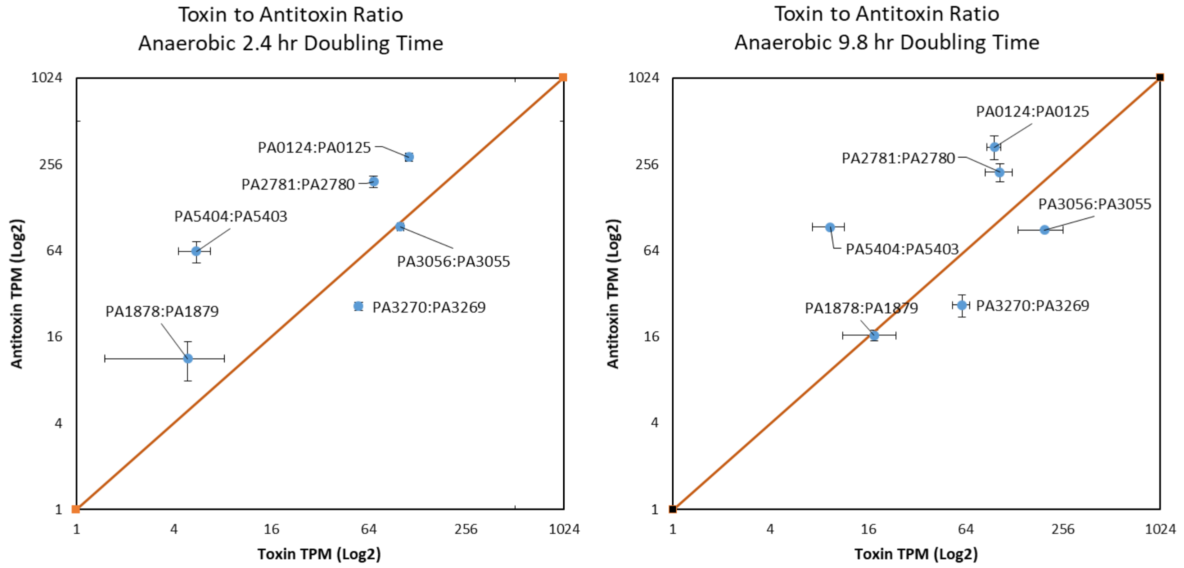

Figure 2.5. Differential expression of toxin and antitoxin pairs between 2.4 and 9.8 hour doubling time under anaerobic growth environment [GSE71880].

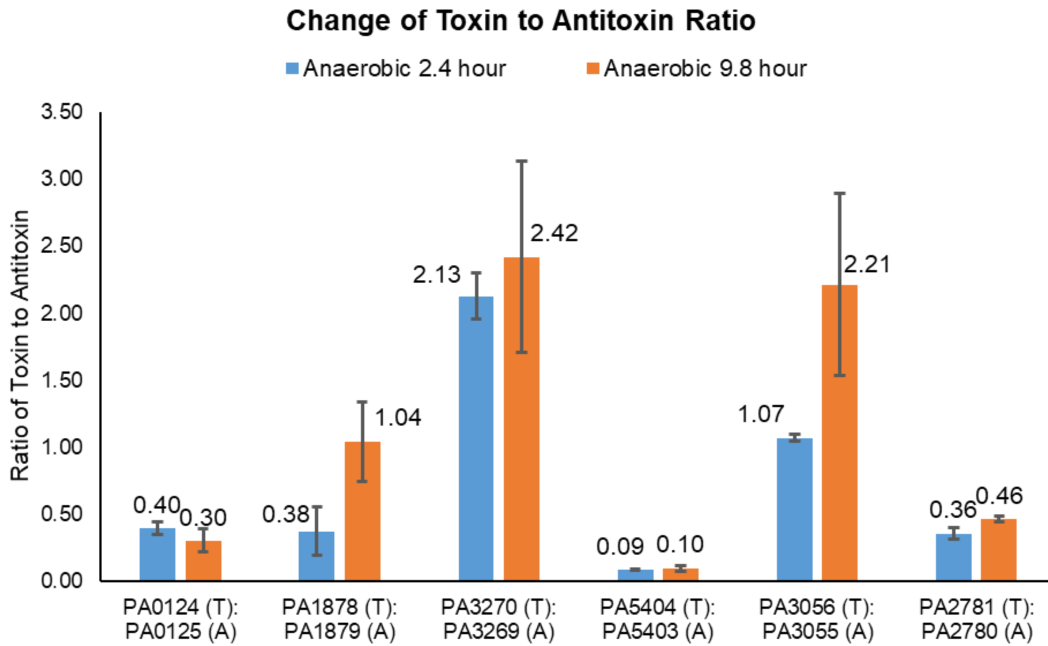

Figure 2.6. Change of the ratio of toxin to antitoxin between 2.4 and 9.8 hour doubling time under anaerobic growth environment [GSE71880].

For the anaerobic growth condition (GSE71880, Figure 2.5 and Figure 2.6), ratio of the toxin to antitoxin increased for all five TA pair except the first pair PA0124:PA0125. For PA0124:PA0125, the ratio decreased due to the overexpression of antitoxin PA0125. Different patterns of ratio change were found for the microaerophilic growth (GSE71880, Figure 2.7 and Figure 2.8) where the last two TA modules PA3270:PA3269 and PA5404:PA5403 have a decreased ratio compared to the normal doubling time. These two ratios decreased because of the decreased expression of toxin PA3270 and PA5404 in the 9.8 hour doubling time data set.

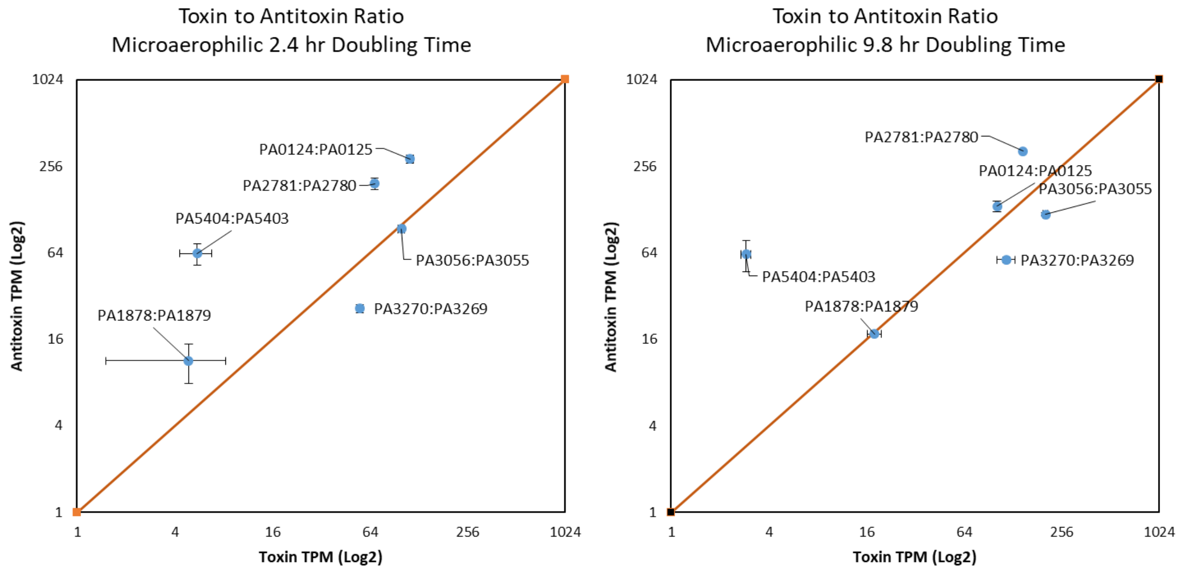

Figure 2.7. Differential expression of toxin and antitoxin pairs between 2.4 and 9.8 hour doubling time under microaerophilic growth environment [GSE71880].

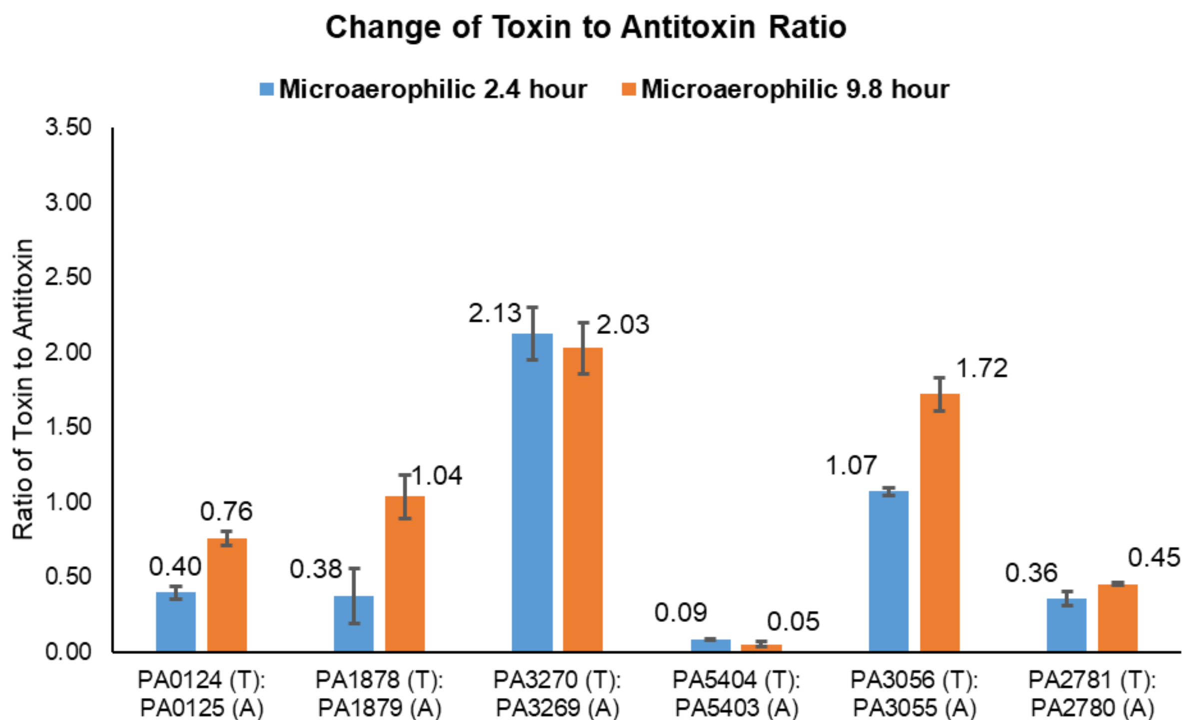

Figure 2.8. Change of the ratio of toxin to antitoxin between 2.4 and 9.8 hour doubling time under microaerophilic growth environment [GSE71880].

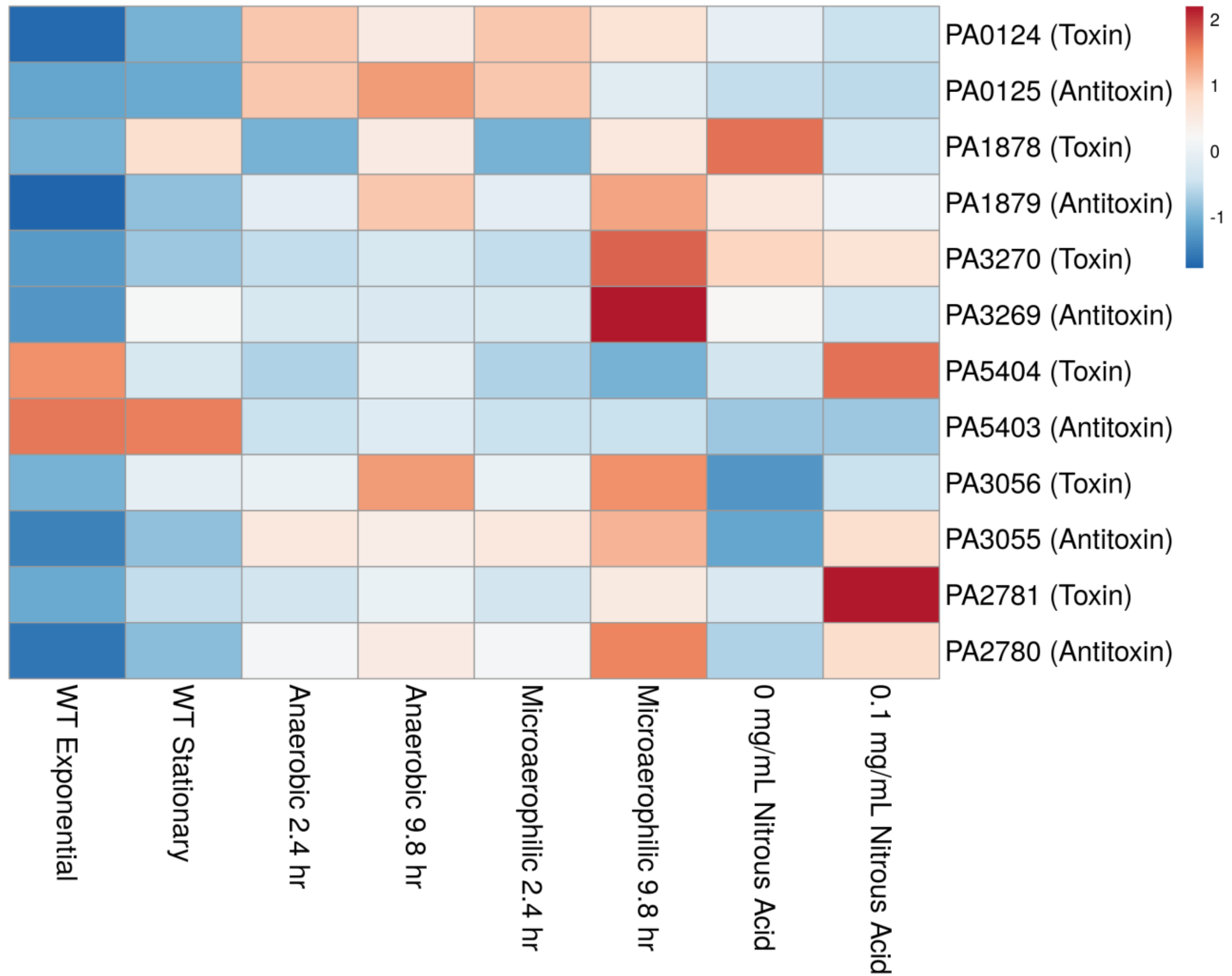

Figure 2.9. Heat map showing the relative expression level of toxin and antitoxin in different environmental conditions. Anaerobic 2.4 hour and Microaerophilic 2.4 hour are same samples used as control for the anaerobic and microaerophilic experiment.

The heat map (Figure 2.9) showed the comparison of the expression level for the toxin and antitoxin in all those eight environmental conditions. Antitoxin PA5403 overexpressed in WT *P. aeruginosa* PAO1 grown exponentially in M9-Casamino acid medium. Toxin PA5404 and PA2781 overexpressed in WT *P. aeruginosa* PAO1 grown in Free Nitrous Acid stress in GLYM9 medium. Under microaerophilic, slow growth conditions (9.8 hour doubling time), most of the toxin and antitoxin genes (PA3056, PA1879, PA3055, PA2780, PA3270, PA3269) are

overexpressed in *psl* and *pel* deleted *P. aeruginosa* PAO1 strains. Majority of the toxin and antitoxin molecules expressed at very low levels in the wild type *P. aeruginosa* PAO1 during exponential growth in M9-Casamino acid medium.

2.3.3. Change in the Expression Level of Other Persister Related Genes

Besides the toxin and antitoxin genes, several other genes are involved in bacterial stress tolerance. They play a very important role in bacterial survival under extreme environments. Most of these genes are involved in either bacterial energy transport or stringent response regulation. The genes analyzed below are important for modulating persistence in *E. coli* and looked for their importance in persistence for *P. aeruginosa* PAO1.

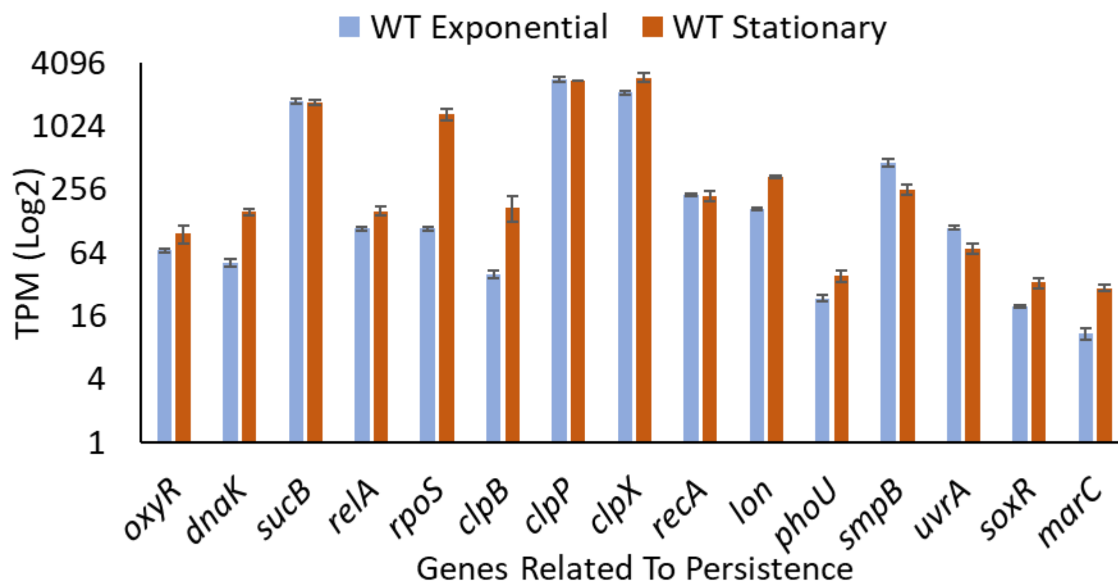

Figure 2.10. Differential expression of persister related genes between exponential and stationary growth phases of *P. aeruginosa* PAO1 [GSE107758].

During the stationary phase, expression level of most of the genes is increased in comparison to the exponential phase. Among the three proteases, expression levels of only chaperon protein *clpB*

and ATP-binding protease *clpX* increased during the stationary phase. For some genes like *clpP*, *recA*, and *smpB* the expression levels decreased in stationary phase. The *clpP* gene product has endopeptidase activity, *recA* gene product involves in DNA repair, and *smpB* gene product involves in the rescue of ribosomal stalling activity (Giudice, et al., 2014).

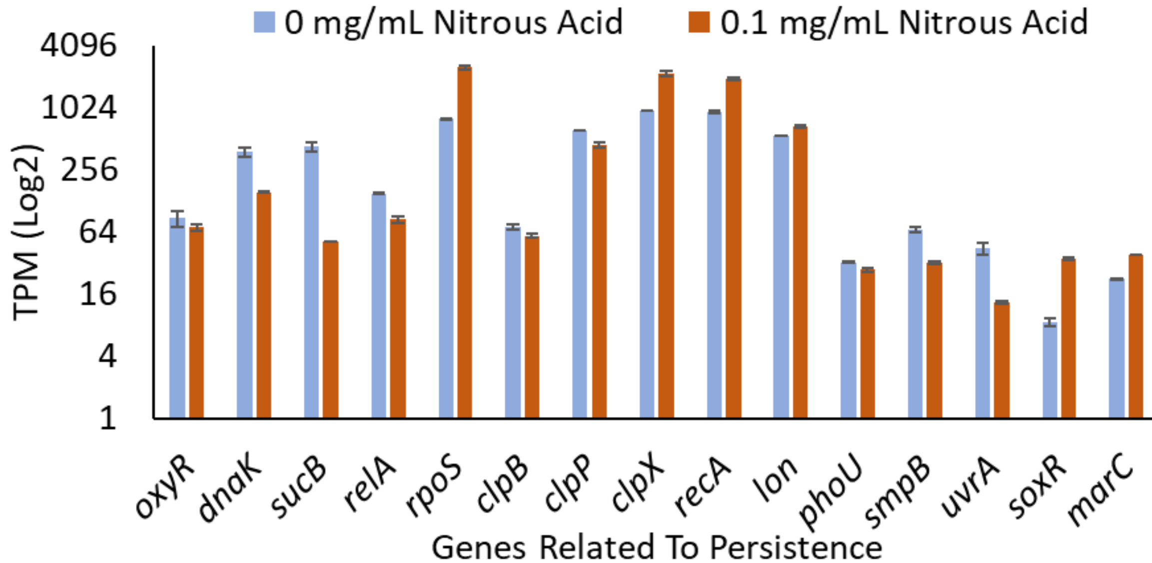

Figure 2.11. Differential expression of persister related genes between anaerobic denitrifying conditions and Free Nitrous Acid stressed growth [GSE73323].

In the presence of metabolic inhibitor, Free Nitrous Acid (FNA), a different pattern of gene expression of the persistence related genes observed. However, expression level for most of the persister related genes had decreased under FNA stress but the expression level of six of the genes had increased under stress. The genes *rpoS*, *clpX*, *lon soxR* and *marC* increased their expression levels. These genes are important for survival under stress conditions and their elevated expression is required for bacterial survival. *oxyR* and *soxR* overexpression is required for the survival under oxidative stress. Expression of ATP dependent proteases *clpX* and *lon* also had increased in level

during stress. These proteases are responsible for the degradation of type II antitoxins and results in the increased toxin concentration and dormancy of cell.

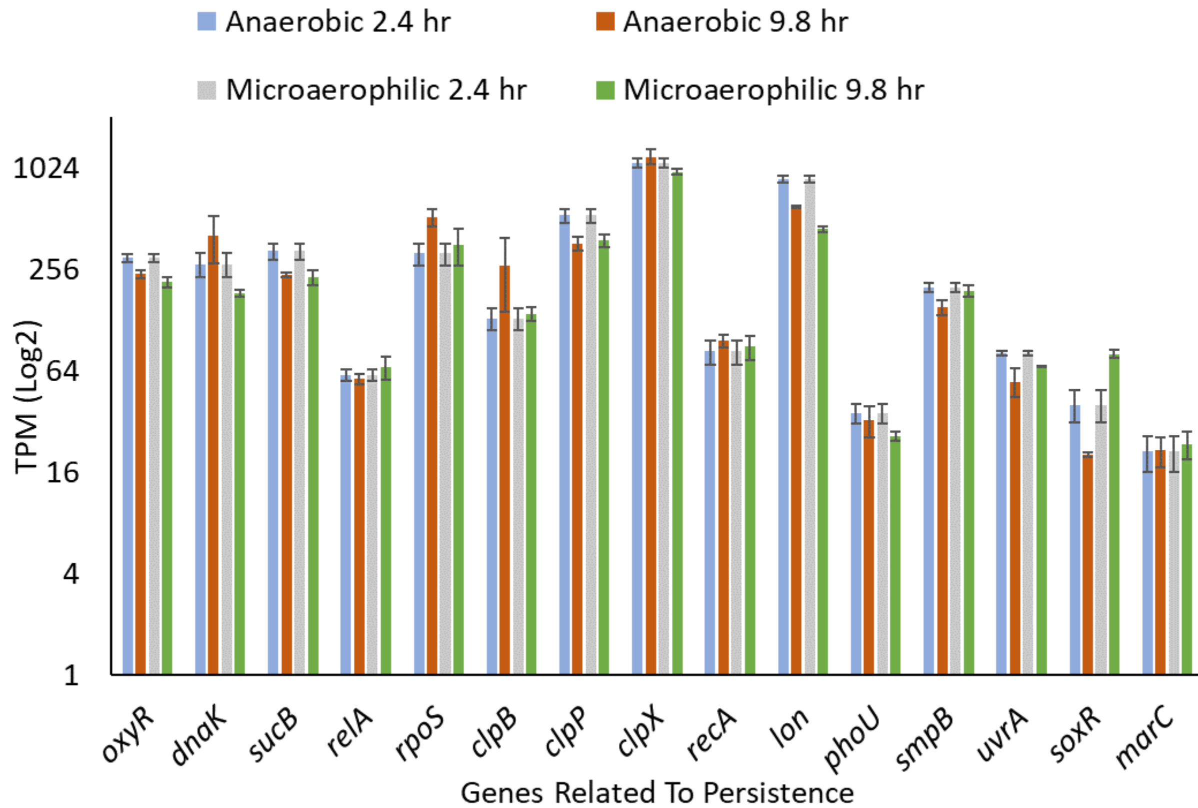

Figure 2.12. Differential expression of persister related genes in anaerobic and microaerophilic growth environment [GSE71880]. 2.4 hour doubling time indicates cells growing without stress and 9.8 hour indicates slowly growing cells in anaerobic and microaerophilic condition.

During the anaerobic slow growth, absence of oxygen increased the expression level of the *dnaK, rpoS, clpB, clpX, recA*, and *marC*. On the other hand, expression levels for *oxyR, sucB, relA, clpP*, *lon, phoU, smpB, uvrA* and *soxR* decreased after prolonged growth under oxygen limitation. A similar pattern was found during microaerophilic slow growth phase except *dnaK, relA, clpX, smpB, soxR*, and *marC* showed overexpression compared to the anaerobic growth condition.

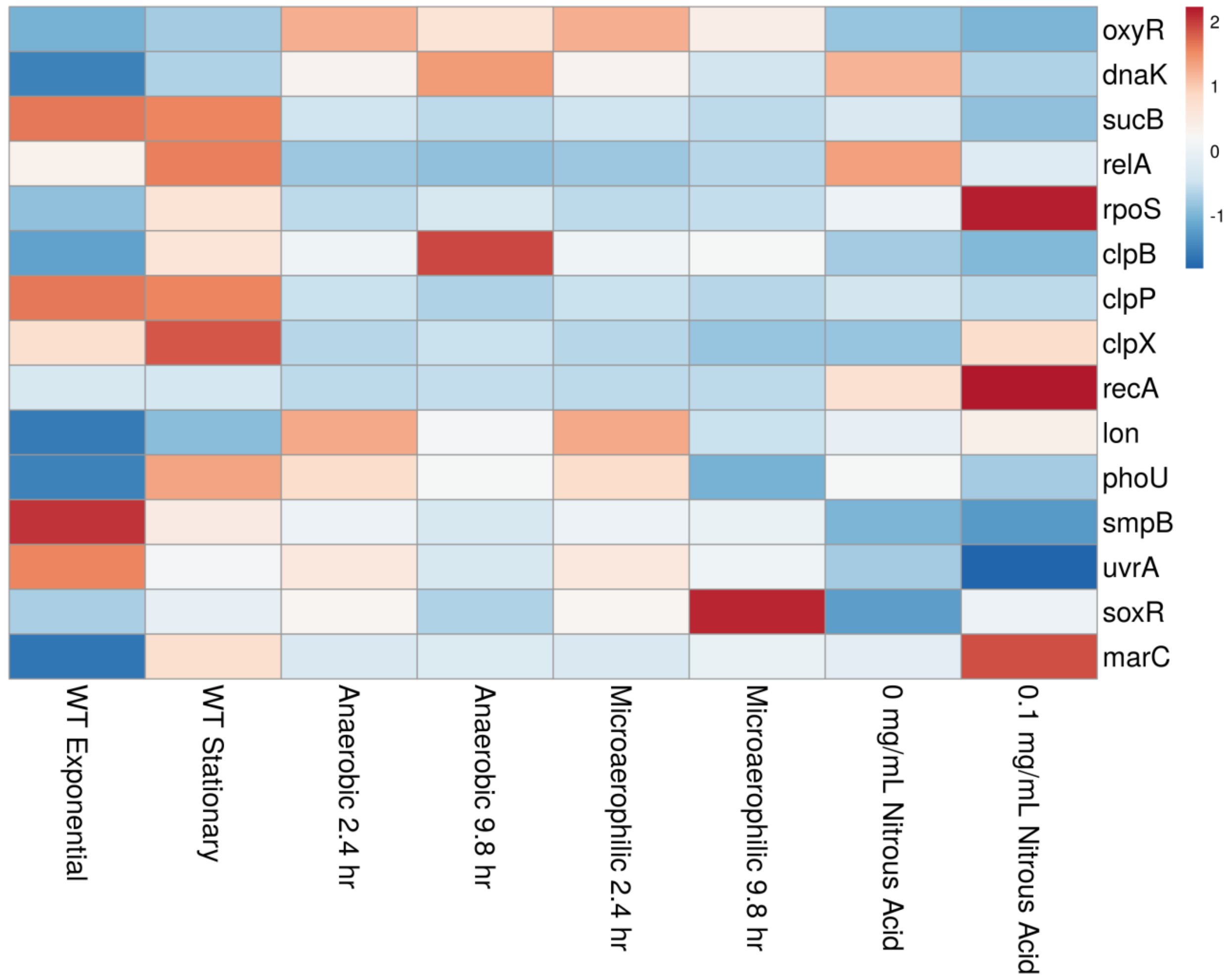

Figure 2.13. Heat map showing the relative expression level of persister related genes in different environmental conditions. Anaerobic 2.4 hour and Microaerophilic 2.4 hour are same samples used as control for the anaerobic and microaerophilic experiment.

In the heat map above, *recA, rpoS* and *marC* showed higher level of expression under Free Nitrous Acid stress. Highest levels of *smpB*, *uvrA*, *sucB* and *clpP* expression were found for the WT exponential growth condition. On the other hand, WT stationary phase showed highest level of *relA, clpX and phoU* expression. *soxR* was overexpressed under microaerophilic slow growth condition . *lon* and *phoU* were overexpressed under anaerobic and microaerophilic normal

growth conditions. The gene *dnaK* and *clpB* were overexpressed under anaerobic slow growth condition.

2.4. Conclusion

For the RNA-Seq datasets used in this experiment, the change of toxin to antitoxin ratio is not enormous. Here the change of the ratio is not only dependent on the expression of toxin molecules; it may also result from the changed expression of antitoxin molecules. The toxin and antitoxin pairs identified using the TADB databases are not biochemically characterized. Database searching using the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) showed different domains present in these molecules and their functions. The first TA pair PA0124:PA0125 belongs to the relBE TA gene family. The toxin RelE is a sequence specific, ribosome dependent mRNA endonuclease, which inhibits translation during amino acid scarcity in the cell. RelB antitoxin counter effect the toxin by direct protein-protein interaction. The ratio for RelE to RelB increased for all of the experiments except the microaerophilic slow (9.8 hour doubling time) growth environment. In the pair PA1878:PA1879 the toxin PA1878 is a metal dependent phosphohydrolases and the antitoxin is a DNA binding protein belonging to the xenobiotic response element (XRE) family of transcriptional regulators. For this pair, toxin to antitoxin ratio increases for all conditions except the free nitrous acid stress. Another pair PA3270:PA3269 consists of toxin PA3270 that contains Acetyltransferase (GNAT) domain. GNAT domain catalyzes N-acetyltransferase reactions. On the other hand, antitoxin PA3269 is an AraC family transcriptional regulator that controls the expression of genes with diverse biological functions including metabolism, stress response, and virulence. For this pair the ratio increases during the growth in the stationary and microaerophilic slow (9.8-hour doubling time) growth environment. The function of the TA pair PA5404:PA5403

is uncharacterized but the antitoxin PA5403 is characterized as helix-turn-helix (HTH) type transcriptional regulator. Toxin to antitoxin ratio only decreases for the stationary phase while increases for all other growth conditions for this pair. Another interesting TA pair is PA3056:PA3055 that consists of toxin PA3056. PA3056 may contain domains from antitoxin HipB. In addition, the antitoxin PA3055 contains HipB domains. HipB is an antitoxin, which neutralizes the toxic effect of toxin HipA or YjjJ. For this TA pair the ratio of toxin to antitoxin follows the same pattern as PA3056:PA3055. The last TA pair PA2781:PA2780 consists of toxin and antitoxins whose protein classification is still unknown. For this pair ratio of toxin to antitoxin decreased for all four experimental conditions. There is a strong correlation between the presences of excess toxin molecules and diminished metabolic capability of the cell. This diminished capability may result in dormant state of growth and increased resistance of bacteria to antimicrobial agents. Under normal physiological growth conditions, there is toxins are bound by the antitoxin in a similar fashion of molecular titration which does not interfere with cells biochemical activity. Also under stress, degradation of type II antitoxin molecules by ATP-dependent proteases changes the ratio of toxin to antitoxin results in the excess presence of toxins. In my study, I did not always find an increased ratio of toxin to antitoxin. Moreover, post-transcriptional regulation is not the only place where the toxin-antitoxin activity may be regulated. Since most of the toxin-antitoxin pairs in this study are not biochemically characterized, there is not enough insight how these TA module interact with cellular machinery. Recently, most focus shifted towards posttranslational mechanisms such as bacterial proteolytic degradation. Proteases are responsible for degrading the antitoxin proteins and shifting the balance toward more toxin rich conditions. The information found in this study is a first of kind to show how the dynamics of the toxin and antitoxin molecules changes with growth in *P. aeruginosa PAO1*.

CHAPTER 3

Discussion and Future Direction

Bacterial persistence is responsible for recurrent infection in patients when bacterial cells evade the immune system (PMID: 23563389). When stress in the growth environment poses a threat to bacterial survival, bacteria adjust the ratio of toxin and antitoxin to a point where toxin can interfere with essential cellular mechanisms. Altering the toxin to antitoxin ratio is not the only mechanism for the regulation of bacterial persistence; some post-translational regulation is evidenced to be involved, which is described below. The central mechanism of toxin-antitoxin interaction is the result of a dormant and slow metabolism that hinder the effectiveness of the antimicrobial drug. These interactions result in a vast array of metabolic perturbations in the cell. Though toxin affects multiple physiological functions, its prime target is the cellular translation machinery (Christensen-Dalsgaard, et al., 2010; Fu, et al., 2007). Toxins can affect cellular functions ranging from replication to growth. The most studied toxin, which interferes with replication, is the plasmid-encoded CcdB toxin. This CcdB toxin can interact with the replication enzyme DNA gyrase, which causes its stabilization (Maki, et al., 1992) . This results in the breakdown of the DNA double-strand causing the inhibition of replication, filamentous growth, and cell death. Another toxin, ParE from *Vibrio cholera,* is known to stabilize the DNA gyrase in a similar fashion to hinder replication (Yuan, et al., 2010).

The effect of any TA modules in cellular metabolism is also strongly controlled by degradation of the antitoxin by ATP-dependent bacterial proteases. When toxin and antitoxins are in the same operon, antitoxins are responsible for the self-repression of its own TA modules under normal physiological conditions. Antitoxin degradation (mostly under stress) releases this repressing effect and results in the change of toxin to antitoxin ratio in the cell since the antitoxin is degraded faster than the toxins under stress. (Muthuramalingam, et al., 2016). Bacterial proteases play a major role in maintaining the intricate network of toxin and antitoxin modules. The most important

protease for the degradation of the antitoxin is Lon, which is responsible for the degradation of almost half of the antitoxins in all bacterial species except some of the Gram-positive bacteria where the Lon protease is absent. The second most important protease for the degradation of the antitoxin is the ClpXP, which counts as one-third of the total protease pool in the cell.

Bacterial proteases perform multiple functions ranging from the degradation of mistranslated proteins to stress response. Therefore, the proteases provide a hub for crosstalk among different cellular regulatory mechanisms. This relation is similar to the server-client relationship in customer care centers, where different types of customers come to the same service provider for a different purpose (Cookson, et al., 2011). In bacteria, the limited number of proteases handles multiple types of clients such as sigma factors, misfolded proteins, and antitoxins. Therefore, they have to prioritize which function to prioritize under certain physiological conditions. Change of this prioritized processing (e.g. degradation of antitoxin under stressful condition) occurs during the stress which results in the degradation of antitoxins.

Due to this vast array of functions, proteases sometimes bottleneck their functional capability. Since the resource (protease) available for the proteolytic capability is limited, there is always a queue in the degradation process. However, most of the bacteria have multiple proteases, but only little is known about the dynamics of the multi-protease network. One model sheds light on the qualitative behavior of these networks depending on the absolute affinity of rather than the cross affinity of the substrate to protease (Ogle and Mather, 2016). This model prioritizes that the proteolytic order in a queue is based on relative affinity for proteases. To this end, Synthetic Biology approaches are used to validate this affinity-based degradation of bacterial proteases. One of these studies (Butzin and Mather, 2018) used a synthetic construct to show the selective overloading of the bacterial protease, which in turn slows bacterial growth. This selective

overloading of bacterial proteases may serve as the mechanism of drug discovery to eradicate chronic infection.

Pseudomonas aeruginosa is the ubiquitous microorganism and present everywhere in the environment. This microorganism considered as nonpathogenic in healthy individuals, but turns pathogenic in patients with a diminished immune system. Computational predictions found some putative toxin and antitoxin pairs but no biochemical study has been performed to date to show the characteristics of those TA modules and their cross interaction in biofilm formation.

TA modules are auto regulated at the transcriptional level by the method known as the conditional cooperativity. The principle mechanism of this method is that transcription factors can bind to the operator region of the TA operon in a certain stoichiometric ratio of toxin to antitoxin. Conditional cooperativity works at different ratio of the toxin and antitoxin and solely depends on the toxin to antitoxin complex. Therefore, without proper biochemical and structural studies, I cannot determine the optimum ratio of toxin to antitoxin where antitoxin repress the toxin or their complex represses their transcriptions.

Bacterial persistence may be the model for the detailed study of bacterial antibiotic resistance. Elucidating the detailed mechanism of bacterial persistence will help us to understand the underlying mechanism of bacterial antibiotic resistance. Therefore, comprehensive system biology studies are requires further development of novel antimicrobial strategies. With the improvement of microfabrication techniques, it is now convenient to use microfluidics in the field of single cell analysis (Weibel, et al., 2007). Microfluidics enable the analysis of physiological responses of single cells to stress such as antibiotic, oxidative and chemical stress. For this reason, it is an interesting tool for the study of microbial physiology and pathogenesis. Combined with higher

resolution microscopy techniques, microfluidics can yield more reproducible results without missing any minute details in bacterial growth dynamics. Therefore, combined classic microbiology, next generation sequencing, and modern microfabrication techniques will make the study of persister behavior more advanced and will reveal the detailed mechanisms underlying bacterial persistence.

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5. Appendix

Table A. Ratio of Toxin to Antitoxins (WT vs Stationary Phase).

Toxin	PA0124 (T)	PA1878 (T)	PA3270 (T)	PA5404 (T)	PA3056 (T)	PA2781 (T)
Log Phase 1	28.15	4.53	36.41	20.7	35.83	8.22
Log Phase 2	28.16	5.22	37.46	18.83	34.62	13.46
Log Phase 3	33.2	4.59	33.21	21.84	27.62	9.37
Stationary Phase 1	31.58	13.41	41.13	8.35	88.72	37.39
Stationary Phase 2	57.21	24.57	46.43	4.34	92.89	59.44
Stationary Phase 3	67.57	20.79	59.31	10.27	105.13	76.92
Antitoxin	PA0125 (A)	PA1879 (A)	PA3269 (A)	PA5403 (A)	PA3055 (A)	PA2780 (A)
Log Phase 1	8.95	2.93	15.86	206.79	7.37	23.45
Log Phase 2	11.23	5.32	13.8	358.58	11.36	27.22
Log Phase 3	6.7	4.79	13.85	373.38	10.02	27.7
Stationary Phase 1	17.83	9.05	28.93	347.85	39.79	77.58
Stationary Phase 2	15.16	7.11	32.28	271.5	32.92	100.21
Stationary Phase 3	17.19	8.7	35.82	308.94	35.9	103.22

Table B. Ratio of Toxin to Antitoxins (No vs Free Nitrous Acid Stress).

Toxin	PA0124 (T)	PA1878 (T)	PA3270 (T)	PA5404 (T)	PA3056 (T)	PA2781 (T)
No HNO2_1	86.2	26.25	98.3	6.14	11.02	88.46
No HNO2_2	74.84	28.74	89.7	8.11	12.98	72.3
0.1 mg/ml_HNO2_1	59.13	9.02	88.52	23.27	72.67	318.89
0.1 mg/ml_HNO2_2	74.71	10.92	76.06	16.85	54.19	255.28
0.1 mg/ml_HNO2_3	67.41	8.43	99.15	25.35	80.03	308.11
Antitoxin	PA0125 (A)	PA1879 (A)	PA3269 (A)	PA5403 (A)	PA3055 (A)	PA2780 (A)
No HNO2_1	78.5	13.24	33.99	25.92	29.14	120.67
No HNO2_2	95.52	15.26	32.52	32.76	18.99	111.24
0.1 mg/ml_HNO2_1	85.13	13.42	22.94	24.97	102.21	269.7
0.1 mg/ml_HNO2_2	77.11	11.22	31.57	29.11	103.47	268.08
0.1 mg/ml_HNO2_3	85.61	11.54	20.15	27.26	96.01	225.28

Table C. Ratio of Toxin to Antitoxins (Anaerobic slow growth).

Toxin (T)	PA0124 (T)	PA1878 (T)	PA3270 (T)	PA5404 (T)	PA3056 (T)	PA2781 (T)
2.4_hr_1	108.02	1.5	56.25	4.3	104.92	71.26
2.4_hr_2	118.69	8.26	54.6	6.75	95.95	65.91
9.8_hr_1	106.37	23.78	68.58	7.29	136.08	84.83
9.8_hr_2	87.09	11.2	53.59	11.44	258.42	124.66
Antitoxin (A)	PA0125 (A)	PA1879 (A)	PA3269 (A)	PA5403 (A)	PA3055 (A)	PA2780 (A)
2.4_hr_1	305.39	7.84	24.43	52.56	95.79	176.59
2.4_hr_2	269.63	14.77	27.96	74.25	91.65	211.85
9.8_hr_1	273.91	17.72	21.86	92.65	88.66	192.42
9.8_hr_2	400.4	15.03	31.44	94.02	89.38	257.89

Table D. Ratio of Toxin to Antitoxins (Microaerophilic slow growth).

Toxin (T)	PA0124 (T)	PA1878 (T)	PA3270 (T)	PA5404 (T)	PA3056 (T)	PA2781 (T)
2.4_hr_1	108.02	1.5	56.25	4.3	104.92	71.26
2.4_hr_2	118.69	8.26	54.6	6.75	95.95	65.91
9.8_hr_1	100.39	19.75	101.85	2.69	195.75	144.86
9.8_hr_2	104.28	16.2	131.94	3.12	211.43	150.87
Antitoxin (A)	PA0125 (A)	PA1879 (A)	PA3269 (A)	PA5403 (A)	PA3055 (A)	PA2780 (A)
2.4_hr_1	305.39	7.84	24.43	52.56	95.79	176.59
2.4_hr_2	269.63	14.77	27.96	74.25	91.65	211.85
9.8_hr_1	124.17	16.68	54.76	77.59	121.56	326.66
9.8_hr_2	146.39	18.2	59.93	47.07	115.36	325.27

Code A. Code used for filtering data.

```
import os

import pandas as pd

os.chdir('C:\\Users\\User\\Desktop')

df = pd.read_csv('.csv')

TA_List = df.loc[df['locus_tag'].isin(['PA0124', 'PA0125', 'PA1030', 'PA1029', 'PA1878', 
'PA1879',

            'PA3270', 'PA3269','PA5404', 'PA5403', 'PA3056', 'PA3055', 'PA2781', 'PA2780'])]

# Change the name of the output file

TA_List.to_csv('TA_List_SRX245813.csv', sep=',', encoding='utf-8')
```