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CHARACTERIZATION OF TYPE II TOXIN ANTI-TOXIN SYSTEMS IN  
*AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

By  
Blair W. Schneider

A dissertation submitted to the faculty of the School of Medicine of the University of  
Louisville in partial fulfillment of the Requirement for the degree of

Doctor of Philosophy in Microbiology and Immunology

Department of Microbiology and Immunology

University of Louisville

School of Medicine

May 2018



CHARACTERIZATION OF TYPE II TOXIN/ANTI-TOXIN SYSTEMS IN  
*AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

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Blair W. Schneider

A Dissertation approved on

03/30/2018

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## ACKNOWLEDGEMENTS

I would first like to thank my mentor, Dr. Donald Demuth, for all of his patience and guidance as I worked through my degree. Without his knowledge and experience, I do not think that I would have been able to complete this program. I am truly fortunate to have had the opportunity to work with him.

I would also like to thank my committee members: Drs. James Graham, Matthew Lawrenz, Donghoon Chung, David Scott, and Uldis Streips for the many suggestions and guidance along the way.

My work within the laboratory was made less stressful and much more entertaining with two of my colleagues: Whitney Weigel and Julie Tan. Julie is the nicest lab manager that has ever existed. She has always been willing to help when she could, in and out of the lab. I am truly thankful that I can call her a friend. Whitney and I learned the importance of magic in the laboratory together while we struggled through many of the same road blocks. She has been a wonderful friend throughout the years and I am very grateful that I was able to meet her.

Finally, I would not have considered even entering a doctorate program, let alone finish one, without the support and encouragement of my loving boyfriend, Donald Walker. I know it was a struggle some days, but he always encouraged me to move forward. I would also like to thank both of our families for their support and willingness to travel to visit us in a different state.

## ABSTRACT

### CHARACTERIZATION OF TYPE II TOXIN/ANTI-TOXIN SYSTEMS IN *AGGREGATIBACTER ACTINOMYTEMCOMITANS*

By

Blair W. Schneider

March 30, 2018

Microbes express many protective mechanisms in response to environmental stress. Toxin/anti-toxin systems encode a biologically active toxin and a labile anti-toxin that inhibits the toxin's activity. These systems are known to contribute to persister cell and biofilm formation. *A. actinomycetemcomitans* thrives in the complex oral microbial community and is subjected to continual environmental flux. Little is known regarding the presence and function of TA systems in this organism or their contribution survival in the oral environment. Using BLAST searches and other informatics tools, we identified 11 intact TA systems that are conserved across all seven serotypes of *A. actinomycetemcomitans* and represent the RelBE, MazEF and HipAB families of TA systems. The *A. actinomycetemcomitans* TA systems identified selectively responded to various environmental conditions that exist in the oral cavity. Transcription of two putative RelBE-like TA systems, D11S\_1194-1195 and D11S\_1718-1719, were induced in response to low pH, and were selected for further study. Deletion of D11S\_1718-1719 significantly reduced metabolic activity of stationary phase *A. actinomycetemcomitans* cells during prolonged exposure to acidic conditions. The mutant also exhibited reduced biofilm biomass when cultured under acidic conditions. The D11S\_1194 and

D11S\_1718 toxins inhibited *in vitro* translation of dihydrofolate reductase (DHFR), and degraded ribosome-associated, but not free mRNA. In contrast, the corresponding antitoxins or equimolar mixtures of toxin and antitoxin had no effect on DHFR production or RNA degradation. Preliminary results comparing the proteomes of acid stressed mutants to the acid stressed wild-type suggest that metabolism proteins are the most affected by these two TA systems. Among these, proteins involved in nucleotide metabolism are largely over-represented in the mutants. Other identified proteins are directly involved in quorum-sensing, iron transport and virulence (e.g. leukotoxin). Results of these studies indicate that the anti-toxin proteins inhibit the activity of the corresponding toxins and suggest that D11S\_1194-1195 and D11S\_1718-1719 are RelBE-like type II TA systems that are activated under acidic conditions. The toxins of both systems may function to cleave ribosome-associated mRNA to inhibit translation in *A. actinomycetemcomitans*.

## TABLE OF CONTENTS

Acknowledgments.....	iii
Abstract.....	iv
List of Tables.....	vii
List of Figures.....	ix
Chapter One: Introduction.....	1
Chapter Two: Materials and Methods.....	22
Chapter Three: Identification and Characterization of Putative Type II Toxin/Anti-toxin Systems in <i>Aggregatibacter actinomycetemcomitans</i> .....	42
Chapter Four: Generation and Characterization of Isogenic Deletion Mutants.....	66
Chapter Five: Putative Toxin/Anti-toxin Systems Inhibit Translation.....	73
Chapter Six: Evaluation of Proteome Under Stress.....	87
Chapter Seven: Discussion.....	116
Chapter Eight: Future Directions.....	125
References.....	130
Curriculum Vita.....	147



## LIST OF TABLES

Table 1: Type II toxin/anti-toxin system families.....	19
Table 2A: <i>A. actinomycetemcomitans</i> strains used in this study.....	24
Table 2B: <i>Escherichia coli</i> strains used in this study.....	24
Table 2C: Plasmids for <i>A. actinomycetemcomitans</i> .....	25
Table 2D: Plasmids for <i>E. coli</i> .....	25
Table 3: CDM Composition.....	26
Table 4: Protein sequences used for BLAST searches.....	28
Table 5: Strains of <i>A. actinomycetemcomitans</i> used for BLAST searches.....	30
Table 6A: Primers used for real-time PCR to monitor expression of putative type II TA systems.....	35
Table 6B: Primers used for pJT4 and pJT1 derived plasmids.....	36
Table 7: Peptide sequences for toxins and anti-toxins.....	41
Table 8: Type II TA systems in <i>A. actinomycetemcomitans</i> .....	50
Table 9A: BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype A.....	51
Table 9B: BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype B.....	52
Table 9C: BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype C.....	53
Table 9D: BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype D.....	54
Table 9E: BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype E-G.....	55
Table 10A: Conservation of TA systems in <i>A. actinomycetemcomitans</i> , serotype A.....	56
Table 10B: Conservation of TA systems in <i>A. actinomycetemcomitans</i> , serotype B.....	57

Table 10C: Conservation of TA systems in <i>A. actinomycetemcomitans</i> , serotype C.....	58
Table 10D: Conservation of TA systems in <i>A. actinomycetemcomitans</i> , serotype D.....	59
Table 10E: Conservation of TA systems in <i>A. actinomycetemcomitans</i> , serotype E-G...	60
Table 11A: Proteins over-represented in $\Delta$ 1194-1195 vs Wild-Type.....	90
Table 11B: Proteins underrepresented in $\Delta$ 1194-1195 vs Wild-Type.....	92
Table 12A: Proteins over-represented in $\Delta$ 1718-1719 vs Wild-Type.....	99
Table 12B: Proteins underrepresented in $\Delta$ 1718-1719 vs Wild-Type.....	101

## LIST OF FIGURES

Figure 1: Prevalence of severe chronic periodontitis world-wide.....	3
Figure 2: Oral biofilm formation.....	6
Figure 3: Characteristics of clinical isolates of <i>A. actinomycetemcomitans</i> .....	9
Figure 4: Effects of catecholamines and iron on the growth of <i>A. actinomycetemcomitans</i> .....	13
Figure 5: Effects of AI-2 and MqsR on biofilm formation.....	15
Figure 6: Types of Toxin/Anti-toxin Systems.....	17
Figure 7: Growth of bacteria under environmental stress.....	45
Figure 8: Growth of bacteria with lactate and environmental stress.....	46
Figure 9: Growth of bacteria in osmotic stress.....	47
Figure 10A-D: Induction of type II TA system expression in <i>A. actinomycetemcomitans</i> exposed to various environmental stress conditions.....	65
Figure 11A-B: PCR of isogenic mutants to show gene deletion.....	68
Figure 12A-B: Metabolic activity of stationary phase <i>A. actinomycetemcomitans</i> .....	70
Figure 13A-B: 72 hour biofilms are sensitive to pH stress.....	72
Figure 14A-B: Putative TA systems can be expressed in an inducible plasmid.....	76
Figure 15: Dot Blot after purification on agarose beads.....	77
Figure 16: Toxin proteins after refolding and concentration.....	78
Figure 17: Synthesized peptides are the correct size for the proteins of interest.....	81
Figure 18: <i>In vitro</i> translation of DHFR in the presence of the synthetic peptides.....	82

Figure 19A-B: MS2 RNA degradation occurs in the presence of ribosomes.....	84
Figure 20A-B: FPLC results for peptide mixtures.....	86
Figure 21A: Proteins over-represented in $\Delta$ 1194-1195 vs Wild-Type.....	93
Figure 21B: Breakdown of over-represented metabolism proteins in $\Delta$ 1194-1195.....	94
Figure 22A: Proteins underrepresented in $\Delta$ 1194-1195 vs Wild-Type.....	95
Figure 22B: Breakdown of underrepresented metabolism proteins in $\Delta$ 1194-1195.....	96
Figure 23A: Proteins over-represented in $\Delta$ 1718-1719 vs Wild-Type .....	103
Figure 23B: Breakdown of over-represented metabolism proteins in $\Delta$ 1718-1719.....	104
Figure 24A: Proteins underrepresented in $\Delta$ 1718-1719 vs Wild-Type.....	105
Figure 24B: Breakdown of underrepresented metabolism proteins in $\Delta$ 1718-1719.....	106
Figure 25: Comparison of proteomics data between isogenic mutants.....	109
Figure 26A: Identified proteins in common between two isogenic mutants.....	110
Figure 26B: Breakdown of metabolism proteins in common between two isogenic mutants.....	111
Figure 27A: Identified proteins unique to $\Delta$ 1718-1719.....	112
Figure 27B: Breakdown of metabolism proteins unique to $\Delta$ 1718-1719.....	113
Figure 28A: Identified proteins unique to $\Delta$ 1194-1195.....	114
Figure 28B: Breakdown of metabolism proteins unique to $\Delta$ 1194-1195.....	115

## CHAPTER ONE: INTRODUCTION

### **Periodontal Diseases**

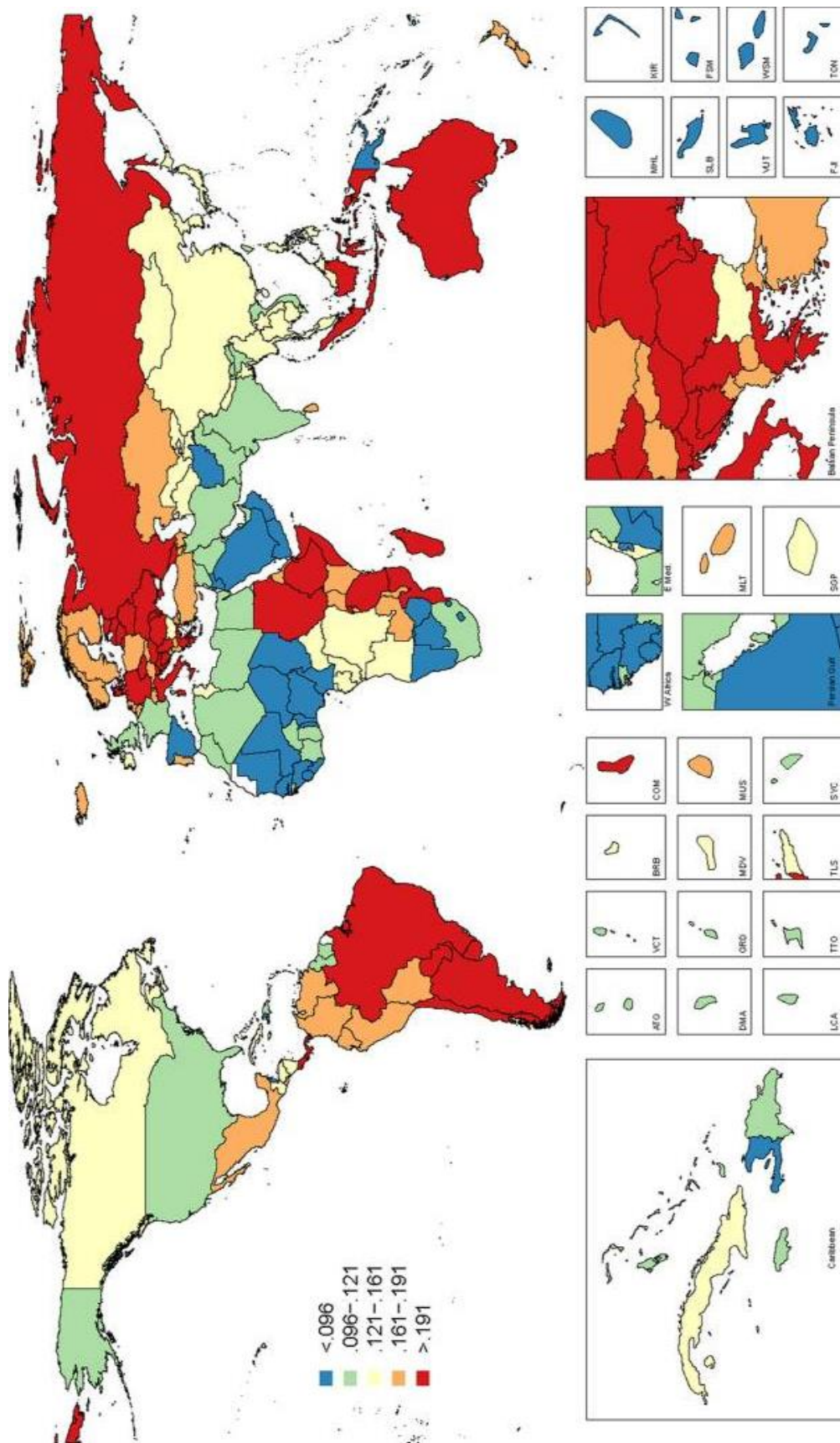
As far back 1500 BCE physicians were aware that infections within the mouth could lead to death if not treated properly (Olsen *et al* 2014). Though techniques and understanding have progressed over the centuries, dental care hit a setback in the late 19<sup>th</sup> century as dentists frequently removed all teeth, no matter how severe the infection or the number of teeth actually affected (Olsen *et al* 2014). In the late 1980s, the correlation between oral health and systemic health re-entered the minds of the general public as oral infections became associated with the conditions, such as cardiovascular disease, complications during pregnancy (including premature birth), and respiratory infections. During this time, we also started to see a rise in a class of immuno-compromised patients, for example, due to infection with HIV virus and chemotherapy treatments for cancer, which were at an increased risk for uncommon infections.

Periodontal diseases are a group of inflammatory diseases of the periodontium, the tissues surrounding the teeth (Albandar 2014). The initial stage of periodontal disease is gingivitis, which is characterized by swelling and bleeding of the gingival tissue (Armitage 1995). At this point, gingivitis is reversible with proper oral hygiene. If left untreated, the disease progresses into a chronic inflammatory condition called periodontitis. Periodontitis is characterized by persistent inflammation that leads to the

destruction of the supporting tissues surrounding teeth, resulting in recession of the gingiva and resorption of the alveolar bone. If still left untreated, the affected teeth will eventually be lost. At this point, proper treatment is necessary and it can involve antibiotic treatments, mechanical debridement of the area, and oral surgery (Dorfer 2003, Teughels *et al* 2014).

Periodontal diseases are prevalent world-wide in both children and adults (Figure 1). In the United States, approximately half of the adult population has some form of the disease and nearly 1% of children and young adults are affected by aggressive periodontitis (Susin *et al* 2014, Eke *et al* 2015). In the USA, approximately \$14.3 billion was spent on periodontal dental care in 1999 and expenditures increased to \$81 billion in 2006, not including the cost of oral hygiene products found in stores (Brown *et al* 2001, Beikler *et al* 2010). It is also estimated that dental patients pay approximately 45% of associated costs out of pocket, which is about four times more than the cost of seeing a physician (Mariotti *et al* 2015). The high cost of treatment, both preventive and reactive, could possibly explain why only 40% of American adults visit the dentist regularly (Mariotti *et al* 2015). In addition to the high cost, many other risk factors can increase an individual's likelihood of developing one of the diseases. Risk factors include genetics, smoking, other illicit drug use, nutrition, and socio-economic status.

Socio-economic status has significant impact on the overall progression of disease due to lack of access to care and/or poor education in oral healthcare procedures. It is estimated that more than 47 million Americans do not have regular access to dental treatment. These Americans are typically low-income, racial minorities, elderly, or from a rural community with a variety of reasons for not seeing a doctor (Mariotti *et al* 2015).



**Figure 1. Prevalence of severe chronic periodontitis world-wide. In 2010, age-standardized prevalence of severe**

periodontitis world-wide. Red countries have high incidence rates whereas blue countries have low incidence rates.

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For example, cultural differences can influence how people respond to illness, such as a religious opposition to modern medicine. Genetic backgrounds can also increase disposition to illness, e.g., people of African descent tend to have higher rates of aggressive periodontitis when compared to other ethnicities. Another important risk factor is tobacco and illicit drug use. Tobacco users are more susceptible to periodontal diseases, and they often have more severe disease (Shekarchizadeh *et al* 2013). With the growing opiate epidemic nationwide and marijuana use becoming main-stream, more evidence is becoming available about the effects of these drugs on the oral cavity. Heroin users experience a variety of oral problems, but usually in the form a unique caries pattern, in addition to generalized periodontal diseases, while marijuana users may experience early onset of periodontitis that is dependent on how often they smoke the substance (Shekarchizadeh *et al* 2013).

Periodontitis can be classified in a variety of ways, including chronic, necrotizing, or aggressive, as well as localized or generalized. Aggressive periodontitis, unlike the other forms, typically develops early in life and has historically been associated with children near puberty (Albandar 2014). First described more than 40 years ago, aggressive periodontitis is unique from chronic periodontitis for a number of reasons, including the age of onset (younger than 25 years old), the high rate of disease progression, and no systemic conditions that affect the host's immune response (Albandar 2014). Unlike some of the other periodontitis types, aggressive periodontitis is often associated with elevated counts of certain bacterial species, such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, as well as several of the herpesviruses (Kononen *et al* 2014). Prevalence of aggressive periodontitis varies around

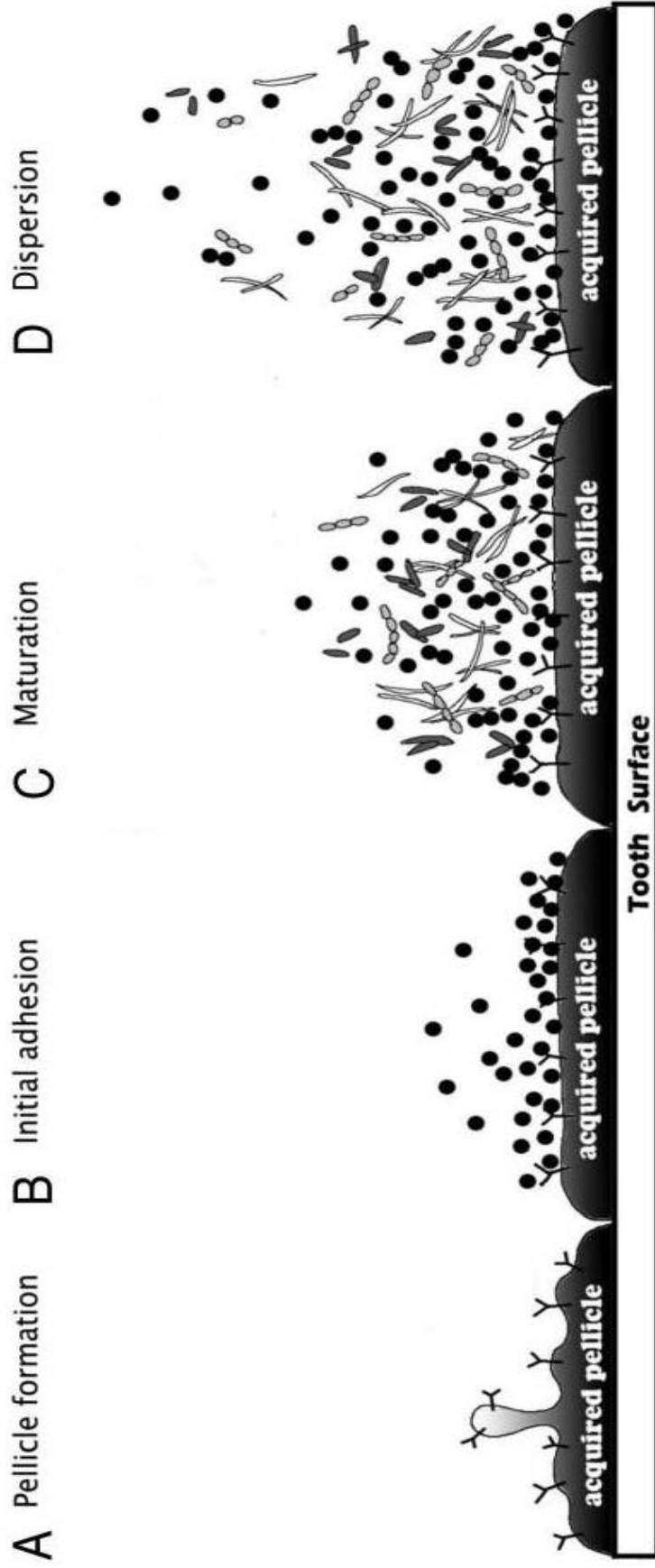


the world, with 0.1% in Swiss children to 3% Sudanese children being afflicted (Susin *et al* 2014).

Periodontal diseases are described as inflammatory diseases. Upon colonization of the oral cavity, the host innate immune system detects and responds to the microbes. Similar to the gastrointestinal tract, the biofilm within the oral cavity is necessary for health, which means that the periodontal tissues will constantly have a low-grade immune response in the area to maintain the biofilm (Kulkarni *et al* 2014). In the progression of periodontal disease, there is shift in the immune response from cell-mediated to humoral. In aggressive periodontitis, however, there is an increase in the number of T-cells and a decrease in the macrophage population, as well over-activated neutrophils in the blood and potentially defects in the neutrophil chemotaxis (Kulkarni *et al* 2014).

## **Biofilms**

The microbiota within in the oral cavity is typically found in a biofilm, and is commonly called dental plaque. The formation of this biofilm is dependent upon the presence of glycoproteins coating the tooth surface that are derived from the saliva of the host, referred to as a pellicle (Huang *et al* 2011, Mahajan *et al* 2013). Early colonizing bacteria adhere to this saliva-derived film. As these bacteria begin to grow, an extracellular matrix is produced that stabilizes the biofilm by aiding in further attachment and enhancing bacterial communication. During this time, both the extracellular matrix and the adherent bacteria provide additional sites for additional bacterial species to adhere and join the biofilm, culminating in a mature microbial community (Figure 2).



**Figure 2. Oral biofilm formation.** This diagram represents each step of oral biofilm formation. (A) Pellicle formation due to glycoproteins and contents of the saliva. (B) Initial adhesion. Early colonizing bacteria recognize the binding proteins on the pellicle. The adhesion is reversible at this point. (C) Maturation. As bacteria, late colonizing bacteria recognize additional binding sites and join the biofilm. (D) Dispersion. Bacteria detach from the biofilm due to death, or lack of nutrients or force from the saliva to spread a new site. Copyright permission to reproduce figure granted by Landes Bioscience and Huang *et al.* 2011.

During this maturation process, more than 700 different species of bacteria may colonize the oral biofilm. As cell density increases, the extracellular matrix changes (secreted proteins, compounds, and extracellular DNA accumulate), a gradient of oxygen and nutrients forms, and ultimately, biofilm cells are dispersed to the planktonic state (Huang *et al* 2011, Mahajan *et al* 2013). Dispersion occurs when cells, both alive and dead, are released from the biofilm, by both active and passive processes (i.e. due to lack of sufficient nutrients or by the shear force of the saliva flowing across the tooth surface) and these cells can colonize other tissue or tooth surfaces, or may be cleared from the oral cavity. Importantly, bacterial cells in a biofilm take on phenotypic traits that are markedly different from characteristics seen during planktonic growth. For example, *Pseudomonas aeruginosa* is a well-known opportunistic pathogen that is associated with biofilms on medical implants. Many early studies showed that *Pseudomonas aeruginosa* was resistant to antibiotics when grown in a biofilm, due to the inability of the antibiotic to disseminate into the extracellular matrix, resulting in an antibiotic gradient (Anwar *et al* 1989, Holmes *et al* 1989, Hoyle *et al* 1990, Hoyle *et al* 1991).

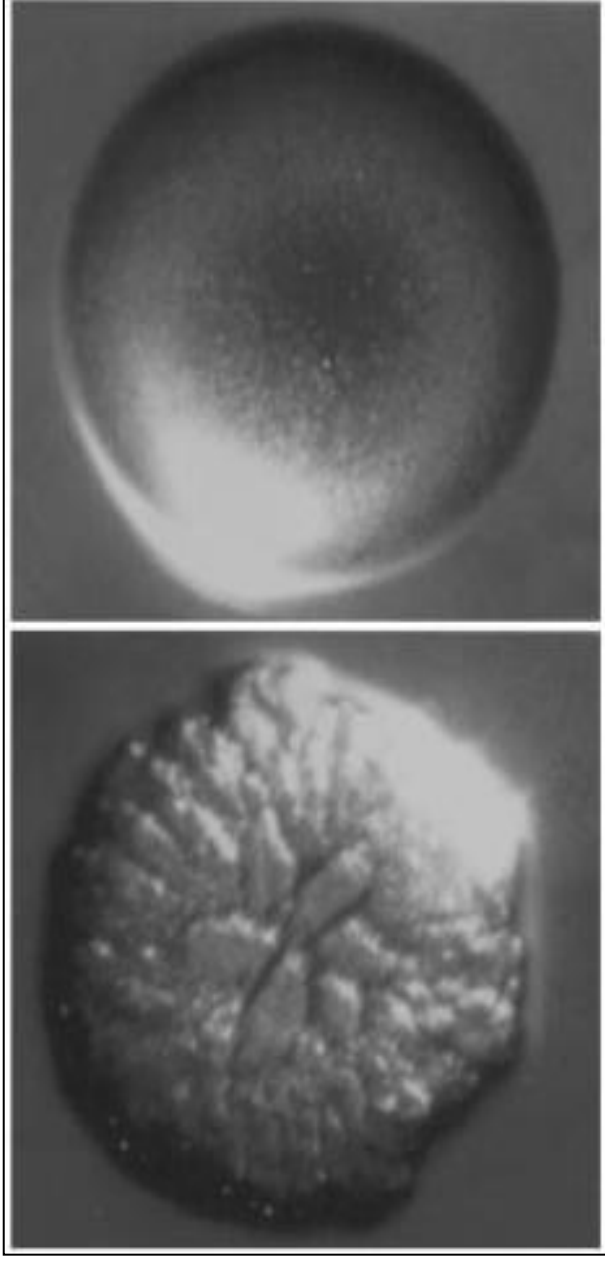
Since the extracellular matrix can act as a passive defense mechanism by limiting outside environmental influences on the biofilm, it also stands to reason that the extracellular matrix can form other gradients that will have influences on biofilm growth and which bacteria can adhere (Huang *et al* 2011, Mahajan *et al* 2013, Amarasinghe *et al* 2009). For example, the pH in crevicular fluid can vary depending on health of the sample site and food consumption, yet many species of bacteria within the oral cavity are sensitive to pH fluctuation (Bickel *et al* 1985, Eggert *et al* 1991, Kleinberg *et al* 1969). If a person frequently eats and drinks foods with high simple sugar content, the bacteria

within the oral cavity will produce massive amounts of lactic acid and other organic acids. This results in a shift of the pH in the biofilm, which will eventually lead to the elimination of acid sensitive bacteria. This shift in biofilm composition will allow the acid-tolerant bacteria, such as *Streptococcus mutans*, to survive and proliferate, which can result in the erosion of the tooth enamel, resulting in dental caries (Socransky *et al* 2005, Hajishengallis *et al* 2012).

General studies looking at the biofilm composition of healthy and diseased oral cavities showed that certain species of bacteria are found in increased numbers in the diseased mouth. This suggests that disease is caused by a shift in biofilm composition, and may result in loss of normal host-microbe homeostasis (i.e. dysbiosis). If left untreated, disease progresses and bacteria can disseminate to have systemic effects. Multiple studies have shown that there is correlation between oral health and many systemic diseases, such as diabetes, endocarditis, brain and lung abscesses, rheumatoid arthritis and osteomyelitis (Yew *et al* 2014, Pyysalo *et al* 2016, Hagiwara *et al* 2009, König *et al* 2016, Sharma *et al* 2017).

### ***Aggregatibacter actinomycetemcomitans***

*A. actinomycetemcomitans* is a gram-negative, non-spore forming, non-motile, facultative anaerobic coccobacillus commensal that is commonly located within the oral cavity (Figure 3). *A. actinomycetemcomitans* was first isolated in 1912 from an oral lesion, it was not considered a member of oral microbiota until 1975 (Klinger 1912, Kilian *et al* 1975). Although it is generally considered a commensal, it has the potential to be an opportunistic pathogen. *A. actinomycetemcomitans* has been associated with



**Figure 3. Characteristics of clinical isolates of *A. actinomycetemcomitans*.** When *A. actinomycetemcomitans* is initially isolated from the oral cavity and cultured on solid medium, it grows as a rough colony with a characteristic star-like structure. The bacteria can spontaneously mutate to lose the fimbriae and the colonies exhibit a smooth colony phenotype. Copyright permission to reproduce figure granted by Society for General Microbiology and Fine *et al* 1999.

many systemic conditions, such as aggressive periodontitis, endophthalmitis, endocarditis, urinary tract infections, rheumatoid arthritis, and brain abscesses (Binder *et al* 2003, Yew *et al* 2014, Townsend *et al* 1969, Konig *et al* 2016, Pyysalo *et al* 2016).

*A. actinomycetemcomitans*, like many pathogens, expresses several virulence factors that aid its ability to survive within the host. The leukotoxin is a protein that is directly associated with disease progression in some populations. Leukotoxin is a protein of the RTX family (repeats in toxin) that binds to leukocytes to induce apoptosis (Raja *et al* 2014, Taichman *et al* 1980, Yamaguchi *et al* 2004, Korostoff *et al* 2000). Studies have shown that a specific strain of *A. actinomycetemcomitans*, strain JP2, is highly associated with aggressive periodontitis. This strain has a deletion in the promoter region of the leukotoxin gene that results in high-level expression of this protein. Leukotoxin is unique among other RTX-family proteins due to the fact that the leukotoxin has a specific target (LFA-1), which allows it to target polymorphonuclear leukocytes, monocytes and T-cells (Spitznagel *et al* 1991, Brogan *et al* 1994, Hritz *et al* 1996, Taichmann *et al* 1986, Henderson *et al* 2002, Raja *et al* 2014). Strains of *A. actinomycetemcomitans* that express high levels of leukotoxin also tend to secrete more vesicles, which can contain the leukotoxin and actinobacillin, which is a bacteriocin produced by *A.*

*actinomycetemcomitans* that inhibits the growth of other bacterial species (Henderson *et al* 2002, Raja *et al* 2014, Stevens *et al* 1987, Hammond *et al* 1987, Oldak *et al* 2017).

Other virulence factors include a catalase, IgG protease, ompA-like proteins, cytolethal-distending toxin, a chemotaxis inhibitor, a capsular polysaccharide biosynthetic enzymes, and fimbriae (Raja *et al* 2014). The cytolethal distending toxin is believed to suppress the host immune response by interfering with the cell division cycle

(Henderson *et al* 2002). OmpA proteins in *E. coli* have been associated with virulence; in the case of *A. actinomycetemcomitans*, Omp34 binds to the IL-10 receptor to modulate monocyte function (Henderson *et al* 2002). Capsular polysaccharides are generally associated with the ability of a bacterium to evade the host immune response (i.e. by inhibiting the complement cascade, and mimicking host antigens) (Agarwal *et al* 2014, Cress *et al* 2014). There are currently seven recognized serotypes of *A. actinomycetemcomitans* and an unusual sugar, 6-deoxy-D-talose is found in many of the serotypes, but its function is currently not known (Henderson *et al* 2002, Nakano *et al* 2000, Suzuki *et al* 2000). The fimbriae are important for disease as well as general survival within the oral cavity because they aid in adherence to tissues and biofilm formation, however, the presence of fimbriae is not the sole determining factor for biofilm formation.

*A. actinomycetemcomitans* is also unique in that it prefers to utilize a carbon source that does not result in the highest growth rates. Instead, the organism preferentially utilizes lactate that is present in the biofilm extracellular matrix, but not utilized by many of the bacterial species found within the oral cavity (Brown *et al* 2007). It is not currently clear why *A. actinomycetemcomitans* prefers to use this growth limiting carbon source even in the presence of other carbon sources, such as glucose. As mentioned previously, many of the bacteria within the oral cavity can utilize simple sugars from the host's diet to produce lactic acid as a by-product. With an abundance of lactate within the extracellular matrix and high competition for the simple sugars, it is plausible that *A. actinomycetemcomitans* evolved to prefer the carbon sources that are readily available in the gingival crevice.

Competition for nutrients in the biofilm can affect the composition and health of the bacterial community. One common limiting growth factor for many bacteria within the human body is iron. Many bacteria utilize siderophores that can be secreted to scavenge iron from extracellular sources, but *A. actinomycetemcomitans* does not encode any of the genes commonly associated these iron-acquiring systems (Rhodes *et al* 2006).

Common sources of iron sources found in the human host include lactoferrin, hemoglobin, and transferrin, however, *A. actinomycetemcomitans* is not able to utilize these sources either (Rhodes *et al* 2006). Further experimental assays eventually revealed that *A. actinomycetemcomitans* is able to utilize hemin using a TonB-ExbB-ExbD-like system, as well as inorganic iron using a periplasmic-binding protein-dependent transport system (Rhodes *et al* 2006). A more recent study showed that *A. actinomycetemcomitans* may internalize host derived catecholamines, which can function as pseudo-siderophores by scavenging iron from host-derived chelators, such as lactoferrin (Figure 4; Weigel *et al* 2015, Freestone *et al* 2000, Sandrini *et al* 2010).

Living in a multi-species biofilm presents a multitude of stimuli to the bacteria. It has been suggested that the progression of periodontal diseases is linked to the formation of the dental plaque and a shift in the biofilm composition at the inflamed sites (Kononen *et al* 2014). For example, *A. actinomycetemcomitans* can be detected in healthy children at all ages in very low numbers. There are multiple studies that suggest this number increases in patients that exhibit localized aggressive periodontitis.

*A. actinomycetemcomitans* was one of the first oral pathogens to be examined for production of the quorum-sensing signal, autoinducer-2 (AI-2). Previous studies showed that *A. actinomycetemcomitans* contained the genes necessary to produce AI-2 (Blehert



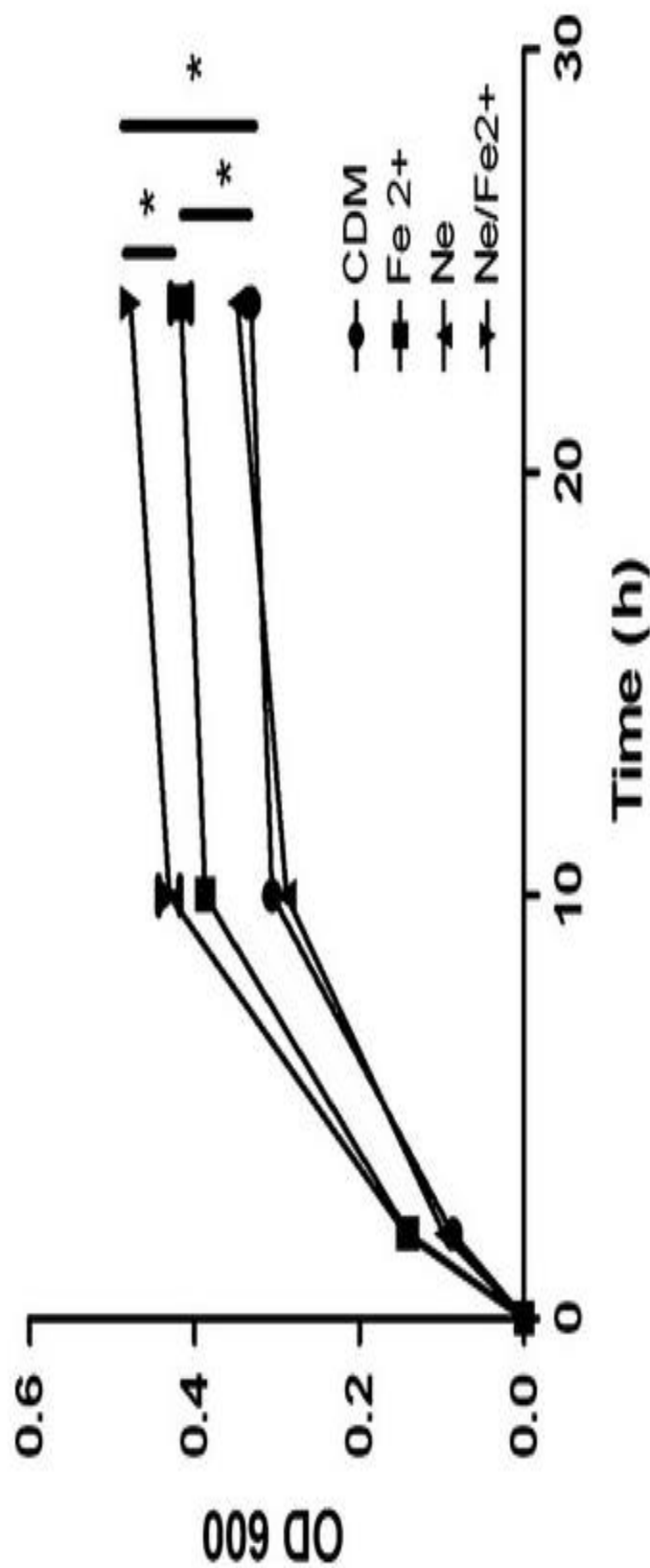


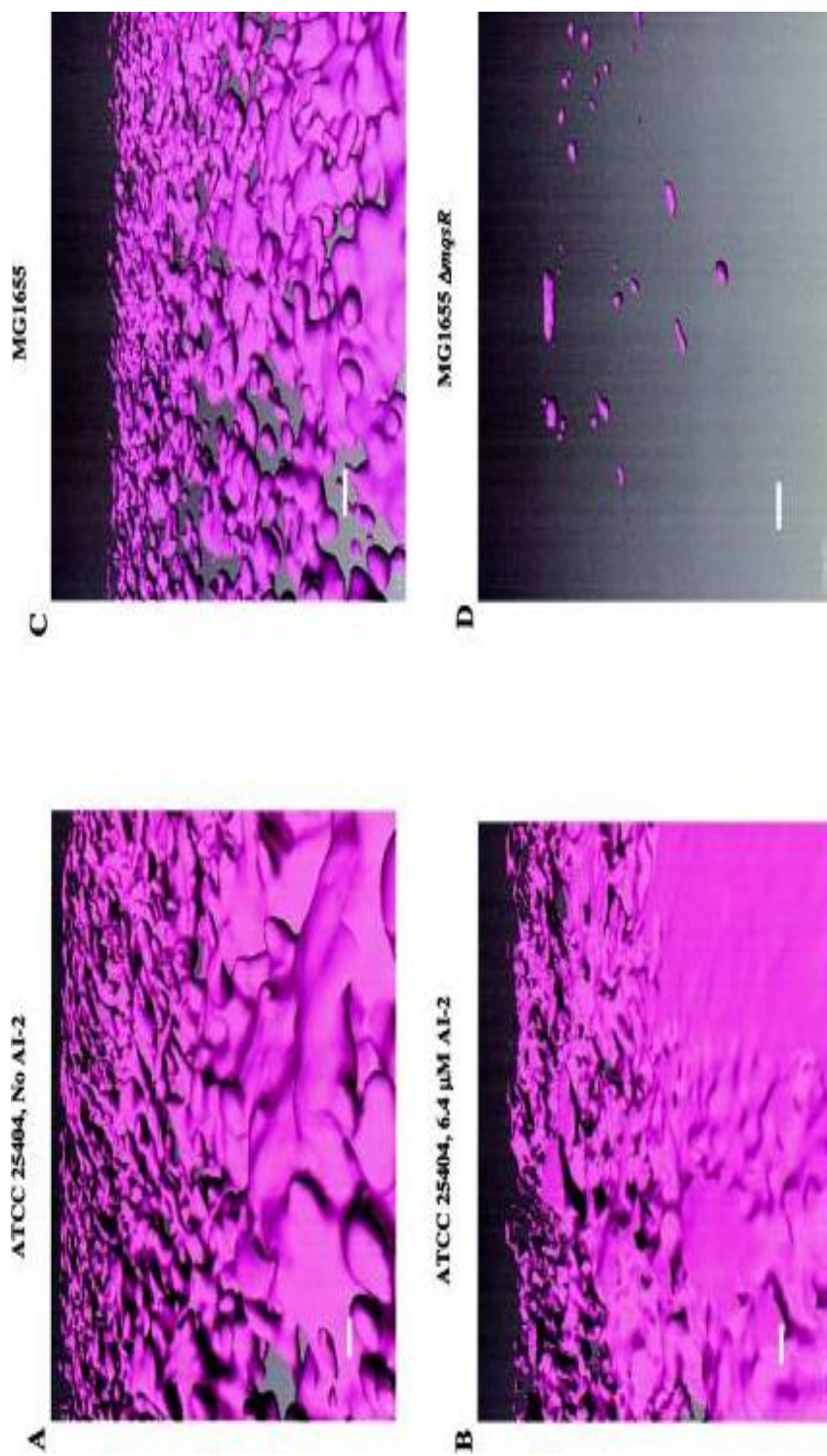
Figure 4. Effects of catecholamines and iron on the growth of *A. actinomycetemcomitans*. *A. actinomycetemcomitans*

was grown in a chemically defined medium either alone (CDM), with  $\text{FeCl}_2$ , (Fe2+), norepinephrine (Ne), or both iron and norepinephrine (Ne/Fe2+). Iron alone caused an increase in optical density, and iron with the catecholamine had a greater increase. Copyright permission to reproduce figure granted by Molecular Oral Microbiology and Weigel *et al*

2015

*et al* 2003, Fong *et al* 2001, Frias *et al* 2001, McNab *et al* 2003, Novak *et al* 2010, Wen *et al* 2004). The AI-2 pathway in *A. actinomycetemcomitans* has been shown to regulate biofilm formation, leukotoxin expression as well as iron acquisition genes, all of which affect the virulence of the bacteria (Fong *et al* 2003, Shao *et al* 2007). Previous research in our laboratory using *A. actinomycetemcomitans* showed that the AI-2 induces the expression of the QseBC two-component system (Novak *et al* 2010). In *E. coli*, QseBC is a quorum sensing regulator for the motility genes and deletion of *qseB* resulted in a large decrease in biofilm mass (Gonzalez *et al* 2006). Figure 5 shows results of an assay in *E. coli* that suggests that a functional MqsR protein is needed for biofilm formation and Gonzalez *et al* were also able to show that deletion of the *mqsR* also resulted in a reduction in the transcription of *qseB* as well as more than 40 other genes by acting as a global regulator of gene transcription (Gonzalez *et al* 2006, Yamuguchi *et al* 2009). A *mqsR*-like gene has also been identified in *A. actinomycetemcomitans* (Novak *et al* 2010).

MqsR is part of a two-gene operon that has been associated with biofilm formation, motility, and persister cell phenotype (Karimi *et al* 2014, Merfa *et al* 2016, Kim *et al* 2010). MqsRA has previously been shown to be a global gene regulator that can inhibit bacterial growth and protein translation *in vivo* (Kasari *et al* 2010). Expression of this protein does not lead to cell death immediately, but rather enables the cells to survive for a short period when exposed to environmental stress.



**Figure 5. Effects of AI-2 and *mqsR* on biofilm formation.** A) *E. coli* biofilm control with no AI-2

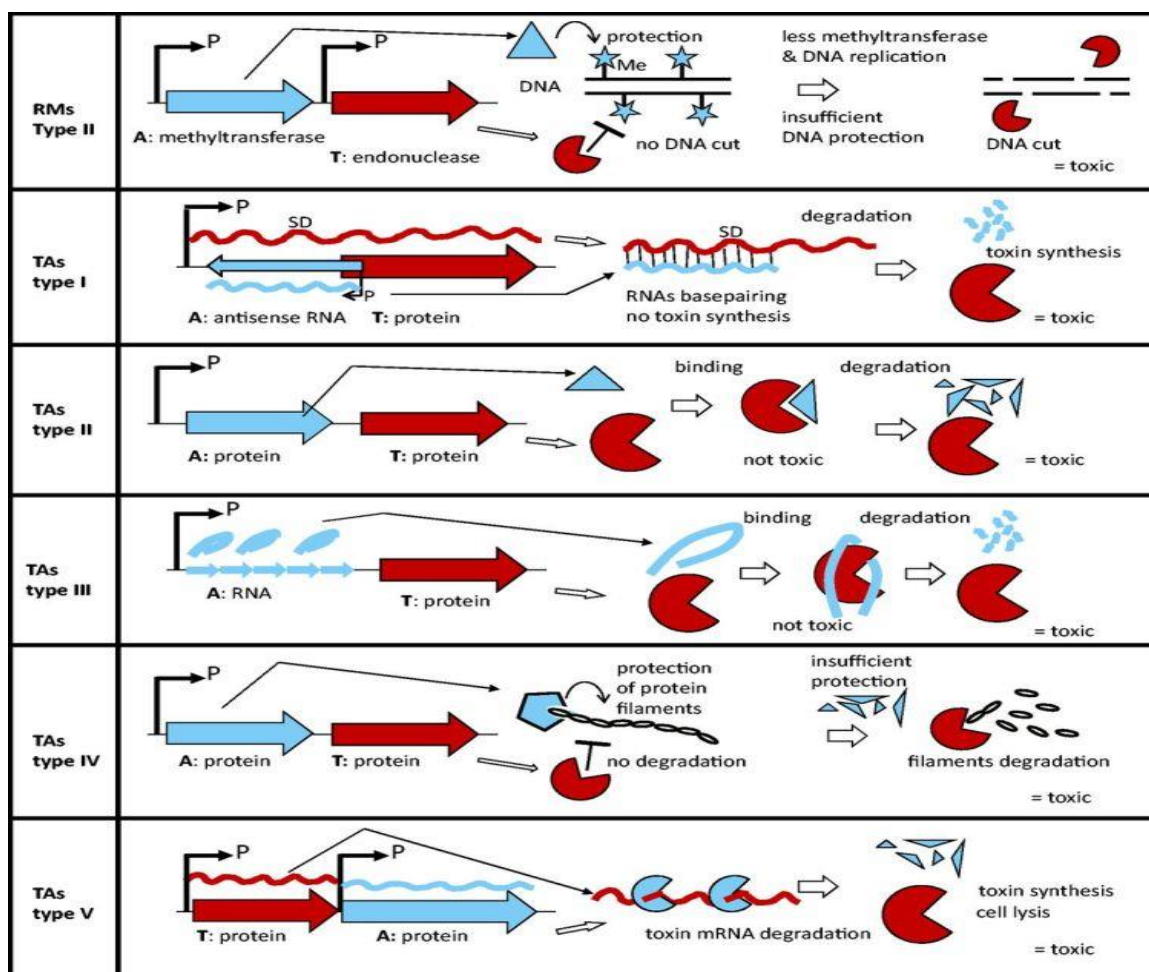
B) *E. coli* with AI-2 added C) *E. coli* strain MG1655 D) MG1655 with *mqsR* deleted. Copyright

permission to reuse granted by American Society for Microbiology. (Gonzalez *et al* 2006)

## **Toxin/Anti-Toxin Systems**

MqsR has since been determined to be a protein that functions as a sequence-specific mRNA interferase (Yamaguchi *et al* 2009). MqsR has also been shown to be part of a small, two gene operon called a toxin/anti- toxin (TA) system. Unlike many other proteins that are called toxins, the toxin is only toxic within the bacterial cell; it is not intended to kill other bacteria or host cells. Toxins are often co-expressed with their associated anti-toxin. As the name implies, the anti-toxin acts on the toxin to inhibit its function. There are currently six recognized classes of TA systems that are differentiated from each other based on how the anti-toxin interacts with the toxin (Kedzierska *et al* 2016, Mruk *et al* 2014).

In all classes of TA systems, the toxin is a protein that can act within the cell in a variety of modes to alter gene expression. The anti-toxin is usually labile and it does not need to be a protein to inhibit the toxin (Figure 6). In type I systems, the anti-toxin is an anti-sense RNA that binds directly to the toxin's mRNA preventing its translation. In type II systems, the anti-toxin is a protein that binds directly to the toxin's protein to inhibit function. In type III systems, the anti-toxin is an RNA that binds directly to the toxin protein to inhibit its function. In type IV systems, the anti-toxin is a protein that binds to the target of the toxin rather than the toxin itself. In type V systems, the anti-toxin is a protein that cleaves the mRNA of the toxin to inhibit its translation. In type VI systems, the anti-toxin acts as a protease adaptor to enable toxin protein degradation, which is not shown in the Figure 6 (Kedzierska *et al* 2016, Mruk *et al* 2014). There are also types of restriction-modification systems (Figure 6) that share many similarities to the TA systems.



**Figure 6. Types of TA systems.** In all cases, the toxin itself is a protein. Type II restriction-modification system anti-toxins methylate the genomic DNA to protect it from cleavage by the toxin. Type I TA system anti-toxin is an antisense RNA that binds directly to the toxin's mRNA. Type II TA system anti-toxin is also a protein that binds directly to the toxin protein. Type III TA system anti-toxin is RNA that binds to the protein toxin. Type IV TA system anti-toxin is a protein that binds to the target of the toxin. Type V TA system anti-toxin is a protein that cleaves the toxin mRNA. In the diagram above, A is anti-toxin; T is toxin. Copyright permission to reuse is granted by Creative Commons Attribution Non-Commercial License and the authors (Mruk *et al* 2014).

Type I and II systems are widely found among the entire prokaryotic world. In fact, many species contain multiple copies and many species contain no copies. There is growing evidence that TA systems respond to environmental cues to arrest cell growth. The advantage of these systems is that if environmental conditions are unfavorable to growth, such as lack of a carbon source, the toxins can temporarily stop cell growth without killing the cell until conditions become favorable. As some species contain multiple copies, it is theorized that different systems will respond to different stress conditions (Ghafourian *et al* 2014, Kedzierska *et al* 2016, Mruk *et al* 2014). With the type II TA systems, there are at least ten families of systems that are classified based on how the toxin functions (Table 1). Many of the systems, but not all, directly affect gene expression either by directly regulating translation, but also indirectly by acting as gene regulators.

Type II TA systems have been recently investigated as potential therapeutic targets. Possible applications include, artificial activation of the toxin since prolonged exposure to stress will lead to cell death, and the mass production of the toxin itself. Utilizing the bacteria's own defense systems could prove to be a low-cost, non-invasive method to treat certain infections (Lee *et al* 2016).

**Table 1. Type II TA system families.** There are currently ten recognized families of type II systems that all function to control cell growth and activity (Bukowski *et al* 2011, Ghafourian *et al* 2014, Mruk 2014, Van Melderren *et al* 2009).

<u>Family</u>	<u>Activity</u>	<u>Mechanism of Toxicity</u>
ccdAB	Gyrase inhibitor	Transcription inhibition
parDE	Gyrase inhibitor	Transcription inhibition
Phd/doc	Binds 30S ribosomal subunit	Translation inhibition
maxEF	Endoribonuclease	Translation inhibition
RelBE	Ribosome-binding endoribonuclease	Translation inhibition
higAB	Ribosome-binding endoribonuclease	Translation inhibition
vapBC	Endoribonuclease	Translation inhibition
$\zeta\epsilon$	Phosphotransferase	Peptidoglycan biosynthesis inhibition
hipAB	Serine/Threonine Kinase	Translation inhibition
hicAB	Endoribonuclease	Translation inhibition

## **Type II Toxin/Anti-Toxin Systems and *A. actinomycetemcomitans***

The genome for *A. actinomycetemcomitans* encodes approximately 1877 open-reading frames, with 32% having no known function. Among those unknown genes, 6% are unique to *A. actinomycetemcomitans* (Henderson *et al* 2003). Previous work in the lab suggests *A. actinomycetemcomitans* contains at least one type II TA system. More evidence that these systems may exist and function in *A. actinomycetemcomitans* is indicated by a number of studies.

One general study looked at the effects of temperature and pH on the protein expression of *A. actinomycetemcomitans* (Goulhen *et al* 2003). This study showed that each of the stress conditions showed different protein expression patterns, with some proteins being more abundant and others being less abundant. Since it has been suggested that bacteria contain multiple TA systems to adapt to changing environmental stress conditions, it is possible that multiple TA systems exist within *A. actinomycetemcomitans* that respond differentially to stress. Another study determined viability of *A. actinomycetemcomitans* across a pH range (Bharracharjee *et al* 2011). By storing and passaging stationary phase bacteria, Bharracharjee *et al* showed a decrease in viability over time. As *A. actinomycetemcomitans* grows to stationary phase, the pH of the medium becomes acidic. However, if the pH of spent medium was adjusted to neutral, then the cells would continue to grow without added nutrients. Since stationary phase is not necessarily a marker for bacterial cell death, it is possible that the bacteria are able to survive for a period of time in the unfavorable environmental conditions before dying.

Most strains of *A. actinomycetemcomitans* contain a gene that encodes for a catalase and certain strains are highly resistant to killing by hydrogen peroxide (Miyasaki



*et al* 1986). Miyasaki *et al* did not investigate gene expression changes as a result of the reactive oxygen species stress, however, a study in *C. crescentus* suggests that ParDE and RelBE TA systems can and do respond to general oxidative stress. Another study in *M. smegmatis* showed that a ParDE-like system also responded to oxidative stress (Fiebig *et al* 2010, Gupta *et al* 2016). As mentioned previously, *A. actinomycetemcomitans* prefers to use lactate as a carbon source when it is present. The utilization of lactate inhibits the transport of glucose into the bacterial cell, so it is possible that the gene expression changes responsible for this reaction are due to TA system activation (Brown *et al* 2007). Finally, certain isolates of *A. actinomycetemcomitans* have been shown to invade epithelial cells (Henderson *et al* 2003). This drastic change in environment could also activate TA systems to ensure survival of the bacteria within the host cell in a variety of ways, including increasing the likelihood of persister cell formation (Helaine *et al* 2014, Cardenas-Mondragon *et al* 2016).

At present, the presence of type II TA systems in *A. actinomycetemcomitans* has not been investigated. Based on previous work down within the laboratory, it is hypothesized that *A. actinomycetemcomitans* contains multiple type II TA systems and that these systems aid in the adaptation and survival of the organism with the oral cavity. Understanding how oral bacteria, especially the pathogens, survive within the oral cavity in the constantly fluctuating environment will help researchers develop novel therapies for pathogens, including *A. actinomycetemcomitans*, that utilize type II TA systems for survival that will be cost-effective and less invasive to the patient.

## CHAPTER TWO: MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 2A-D. Luria-Bertani (LB) broth and LB agar (LB broth plus 1.5% agar) were routinely used for the propagation and plating of *E. coli*. Bacteria were grown at 37°C in either a shaking incubator for planktonic growth or a microaerophilic (5% CO<sub>2</sub>) incubator for plates. Brain-heart infusion (BHI) broth and BHI agar (BHI plus 1.5% agar), Super Optimal Broth with Catabolite repression (SOC; 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, and 20 mM Glucose) and Tryptone-Yeast Extract Broth (TYE; 1% tryptone and 0.5% yeast extract) and TYE agar (TYE plus 2% agar) were routinely used for the propagation and plating *A. actinomycetemcomitans*. *A. actinomycetemcomitans* strain 652 is afimbriated and was grown at 37°C under microaerophilic conditions, either in a candle jar for plates or in an incubator with 5% CO<sub>2</sub> for broth cultures. In some assays, *A. actinomycetemcomitans* was grown in chemically defined medium (CDM) as described by Socransky et al. (1985) with some modifications (Table 3A-B). In some assays, the pH of the broth was adjusted to various points between 5.0 and 8.0. When necessary, medium was supplemented with 25 ug mL<sup>-1</sup> kanamycin, 50 ug mL<sup>-1</sup> spectinomycin, 12.5 ug mL<sup>-1</sup> tetracycline, 50 ug mL<sup>-1</sup> ampicillin, 40% sucrose or 1mM isopropyl B-D-1-thiogalactopyranoside (IPTG).

**Identification of putative TA systems.** Putative type II toxin/anti-toxin (TA) systems in *A. actinomycetemcomitans* were identified by two methods. First, the genome of *A. actinomycetemcomitans* strain D11S-1, a serotype c strain that is related to strain 652, (Chen *et al* 2009) was probed for sequence similarities to known *E. coli* TA systems using the protein basic local alignment search tool (pBLAST; NCBI). The sequences for known toxins that were used in these BLAST searches are listed in Table 4. Next, the entire genome of *A. actinomycetemcomitans* strain D11S-1 was examined using TAFinder (<http://202.120.12.133/TAFinder/TAFinder.php>) to identify operons composed of two small genes that were not previously identified in the BLAST searches. TA Finder detects type II TA loci based on sequence alignments and conserved domain searches against a database of known TA families. The genes that were identified as putative TA systems in strain D11S-1 were subsequently used to probe 33 other *A. actinomycetemcomitans* genome sequences, representing all seven serotypes that were present in the NCBI database (Table 5).

Table 2A. <i>A. actinomycetemcomitans</i> strains used in this study		
Bacterial strain	Description	Source
652	Wild-type, serotype C	Laboratory Strain
652-BWS1	652 $\Delta$ 1194-1195	<i>This study</i>
652-BWS2	652 $\Delta$ 1718-1719	<i>This study</i>
652-BWS1C	652 $\Delta$ 1194-1195::1194-1195	<i>This study</i>
652-BWS2C	652 $\Delta$ 1718-1719::1718-1719	<i>This study</i>

Table 2B. <i>Escherichia coli</i> strains used in this study		
Bacterial strains	Description	Source
XL1-Blue MRF <sup>1</sup>	$\Delta$ (mcrA)183 $\Delta$ (mcrCB-hsdmSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB. lacIqZ $\Delta$ M15 Tn10 (Tcr)]	Stratagene
One Shot Top10	F- mcrA $\Delta$ ( mrr-hsdRMS-mcrBC) $\Phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 recA1 araD139 $\Delta$ ( araleu)7697 galU galK rpsL (StrR) endA1 nupG	Thermo Fisher Scientific

<b>Table 2C. Plasmids for <i>A. actinomycetemcomitans</i></b>		
<b>Plasmid</b>	<b>Description</b>	<b>Source</b>
pJT1	Sp <sup>R</sup> suicide vector	Juárez-Rodríguez 2013b
pJT4	Km <sup>R</sup> promoterless expression vector	Juárez-Rodríguez 2013b
pBWS1	pJT1 derived, 1194-1195	<i>This study</i>
pBWS1	pJT1 derived, 1718-1719	<i>This study</i>
pBWS1C	pJT4 derived, 1194-1195	<i>This study</i>
pBWS2C	pJT4 derived, 1718-1719	<i>This study</i>

<b>Table 2D. Plasmids for <i>E. coli</i></b>		
<b>Plasmid</b>	<b>Description</b>	<b>Source</b>
pQE60	Amp <sup>R</sup> expression plasmid with C-terminal 6X His-tag, multiple cloning site and T5 promoter/lac operator, T5 transcription start	Qiagen
p1194	pQE60 derived, 1194	<i>This study</i>
p1195	pQE60 derived, 1195	<i>This study</i>
p11TA	pQE60 derived, 1195-1195	<i>This study</i>
p1718	pQE60 derived, 1718	<i>This study</i>
p1719	pQE60 derived, 1719	<i>This study</i>
p17TA	pQE60 derived, 1718-1719	<i>This study</i>

<b>Table 3. CDM composition</b>			
<b>Chemical/Compound</b>		<b>Concentration</b>	
		<b>(mg/L)</b>	
ZnSO <sub>4</sub>	0.7	L-Serine	100
KI	0.1	L-Lysine HCl	100
CuSO <sub>4</sub>	0.065	L-Histidine	135.1
Boric Acid	0.5	L-Glutamine	100
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	700	L-Asparagine	113.6
FeSO <sub>4</sub> · 7 H <sub>2</sub> O	5	L-Methionine	100
MnSO <sub>4</sub>	5	L-Isoleucine	100
NaCl	100	L-Proline	100
K <sub>2</sub> PO <sub>4</sub>	200	L-Aspartic Acid	100
CaCl <sub>2</sub>	100	L-Phenylalanine	100
KH <sub>2</sub> PO <sub>4</sub>	1000	L-Tyrosine	20
NaMo <sub>4</sub>	0.5	L-Cystine	5
KNO <sub>3</sub>	100	L-Ornithine HCl	20
L-Glutamic Acid	249.6	L-Hydroxyproline	20
DL-Alanine	200	Adenine	12.5
L-Leucine	100	Guanine	10
Glycine	100	Cytosine	13.5
L-Valine	100	Thymine	10
L-Tryptophan	100	Xanthine	10
L-Threonine	100	Hypoxanthine	10

Table 3 (continued). CDM Composition			
Chemical/Compound		Chemical/Compound	
	Concentration (mg/L)		Concentration (mg/L)
Uracil	10	D-Biotin	0.1
Choline Chloride	50	DL-6,8-Thioctic Acid amide	0.1
B-Alanine	10	Vitamin B12	0.01
Pyridoxal	1	NaHCO <sub>3</sub>	1000
Pyridoxine HCl	1	L-Cysteine	650
Pyridoxamine · 2HCl	1	Glucose	3600
Spermidine · 3 HCl	1		
Nicotinic Acid	1	Magnesium L-Lactate*	4050
Nicotinamide	1		
Calcium Pantothenate	1		
Spermine · 4 HCl	1		
Thiamine HCl	1		
Myo-inositol	10		
Nicotinamide adenine dinucleotide	1		
p-Aminobenzoic Acid	0.1		
Pimelic Acid	0.1		
Folic Acid	1		
Riboflavin	1		
		<i>*MgSO<sub>4</sub> was not added with lactate</i>	

**Table 4. Protein sequences used for BLAST searches**

<b>Toxin</b>	<b>Toxin Sequence (protein)</b>
ccdB	mqfkvytykr esryrlfvdv qsdidtpgr rmviplasar llsdkvsrel ypvvhigdes wrmmttqmvs vpvsvigeev adlshrendi knainlmfwg i
parE	mlpvlwlesa dtdldditsy iarfdidaae rlwqrlgcv lplsehpyly ppsdrvpglr eivahpnyii lyrvttssve vvnviharrq fp
phD	mqsinfrtar gnlsevlennv eageeveitr rgrepavivs katfeaykka aldaefaslf dtdlstdnkel vnr
mazF	mvsryvpdmg dliwvdfdpd kgseqaghrp avvlspfmyn nktgmclcvp cttqskgypf evvlsqgerd gvaladqvks iawrargatk kgtvapeelq likakinvli g
chpK	mtrgeiwwvd lgipfgsepg fqrpvliqn nafnhsnint iivvplttnl hlatapgnsm lkkedtnlsk dsivnvsqiv tidrerfikk vteiknkhmk kveegmklvl sles
kid	xergeiwlvs ldptagheqq gtrpvliivp aafnrvtlrp vvpvtsqgn fartagfavs ldvgirttg vvrcdqpti dmkarggkrl ervpetimne vlgrlstilt
pemK	mlkyqlknen gwmhrrlvrr ksdmergeiw lvsldptagh eqqgtrpvi vtpaafnrvt rlpvvpvts ggnfartagf avslldgvgir ttgvvrcdp rtidmkargg rlervpeti mnevlgrlst ilt
chpBK	mvkkseferg divlvqfpa sgheqqagr palvsvqaf nqlgmtlvap itqggnfary agfsvplhce egdvhgvvvl nqvrmmldha rlakriglaa devveallr lqavve
relE	mayfldfder alkewrkls tvreqlkkkl vevlesprie anklrgmpdc ykiklrssgy rlvyyvidek vvvfvisvgk rersevysea vkril
yoeB	mkliwseesw ddylywqetd krivkkinel ikdtrtrtpe gkgkpeplkh nlsqfwsrri teehrlvyav tddsliaac ryhy
ygiN	mhlitqkalk daaekypqhk telvalgnti akgyfkkpes lkavfplsldn fkyldkhyvf nvgnelrvv amvffesqkc yirevmthke ydfftavhrt kgkk
yafO	mrvfktklir lqltaeelda ltadfisyr dgvlpdifgr dalyddsftw plikervah ihlanennpf ppqlrqfsrt ndeahlvycq gafdeqawll iailkpephk lardnnqmhk igkmaeafm rf
ygiU	mekrtphtrl sqvklvnag qvrttrsall nadelglldfd gmcnviigs esdfyksmtt ysdhtiwdv yrprlvgtqv ylkitvihdv livsfkek



**Table 4 (continued). Protein sequences used for BLAST searches**

<b>Toxin</b>	<b>Toxin Sequence (protein)</b>
mqsR	mekrtphtrl sqvkklnag qvrttrsall nadelgldfd gmcenviigls esdfyksmtt ysdhtiwdqv yrprlvgtqv ylkitvihdv livsfkek
yafQ	miqrddieysg qyskdvklaq krhkdmnklk ylmtilinnt lplpavykdh plqgswkgyr dahvepdwil iykldklr fertgthaal fg
higB	mhlitqkalk daaekypqhk telvalgnti akgyfkpes lkavfplsln fkyldkhyvf nvggnelrvv amvffesqkc yirevmthke ydfftavhrt kgkk
vapC	mldtnicsfi mreqpealk hleqsvlrg h rivvsaitys emrfgatgpk asprhvqlvd afcerldavl pwdraavdat teikvalrla gtpigpnda iaghaiaaca ilvtnnvref ervpglvled wvr
Zeta	manivnftdk qfenrlndnl eelvqgkkav esptaflgg qpqsgktslr saifeetqgn vvvidndtfk qqhpnfdelv klyekdvvk h atpysnrmte alisrlsdqg ynlviegtgr ttdvpigtat mlqakgyetk tyamavpkie sylgtierye tmyaddpmta ratpkqahdi vvknlptnle tlhktglfsd irlynregvk lyssletpsi spketlerel nrkvsgkeiq ptlerieqkm vqnqhgetpe fkaiqqkmes lqpptppipk tpklpgi
hipA	mpklvtwmnn qrvgeltkla ngahtfkyap ewlasryarp lsllplqrg nitsdavfnf fdnlldspi vrdrivkryh aksrqpfdll seigrdsvga vtlipedetv thpimawekl tearleevlt aykadiplgm ireendfris vazaqektal lrigndwcip kgitptthii klpigeirqp natldlsqsv dneyycllla kelglnvpa eiikagnvra laverfdrw naertvllrl pqedmcqtfg lpssvkyesd ggpgiarima flmgssealk drydfmkfv fqwligatdg haknfsvfiq aggsyrltpf ydiisafpvl ggtgihisd klamglnask gkktaidkiy prhflatakv lrfpevqmhe ilsdfarmip aaldnvktsl ptdfpennvt avesnvrlrh grlsreygsk
hicA	mgktdkllak flnskktfew delvvlfs sl gyvkkemqgs rvrffnaein htilmhrphp esyikggtlk aikqnlkeag ll
yncN	mkqsefrwl esqgvdivang snhlklrfhg rrsvmprhpc deikeplrka ilkqlgls

<b>Table 5. Strains of <i>A. actinomycetemcomitans</i> used for BLAST searches</b>		
<b>Strain</b>	<b>Serotype</b>	<b>GenBank Accession Number</b>
624	A	CP012959.1
D7S-1	A	CP003496.2
H5P1	A	AEJK00000000.2
D17P-3	A	ADOA00000000.2
A160	A	AJME00000000.2
ANH9381	B	CP003099.1
HK1651	B	CP007502.1
Y4	B	AMEN00000000.1
RhAA1	B	AHGR00000000.1, JPZI00000000.1
SCC1398	B	AEJP00000000.2
I23C	B	AEJQ00000000.2
SCC4092	B	AJMF00000000.2
S23A	B	AJMH00000000.2
D11S-1	C	CP001733.2
SC38S	C	AZTR00000000.1
SCC2302	C	AEJR00000000.2
D17P-2	C	ADOB00000000.2
AAS4A	C	AJMG00000000.2
SA2200	D	AZTY00000000.1
SA269	D	AZTX00000000.1
SA3033	D	AZTW00000000.1

<b>Table 5 (continued). Strains of <i>A. actinomycetemcomitans</i> used for BLAST searches</b>		
<b>Strain</b>	<b>Serotype</b>	<b>GenBank Accession Number</b>
SA3733	D	AZTV000000000.1
SC1083	E	AEJM000000000.1
SA2149	E	AZTT000000000.1
SC936	E	AZTP000000000.1
SA2876	E	AZTS000000000.1
ANH9776	E	AZTZ000000000.1
SA3096	E	AZTQ000000000.1
SCC393	E	AEJN000000000.2
SC29R	F	AZTO000000000.1
D18P1	F	AEJO000000000.2
NUM 4039	G	AP014520.1

**Expression of putative TA systems under environmental stress.** The expression of the putative TA systems was determined under various environmental stress conditions using real-time PCR. Cells were grown in CDM supplemented with 20 mM glucose or 20 mM lactate (Brown *et al* 2007) at 37°C in an aerobic incubator to mid-log phase and were subsequently exposed to various environmental stress conditions for 20 minutes. Environmental conditions used were: acidic pH (pH 5.0), oxidative stress (0.1% hydrogen peroxide), microaerophilic conditions (5% carbon dioxide), elevated temperature (39°C), anaerobic conditions, iron starvation (250  $\mu$ M bipyridyl), and reduced temperature (30°C). Bacteria were then harvested for RNA extraction using the cesium chloride step-gradient method as described by Reddy *et al.* (2001). RNA was reverse transcribed to cDNA using random primers provided in the cDNA synthesis kit (Quanta Bio), and the resulting cDNA was used in SYBR Green real-time PCR using primers for the TA system (Table 6A) as recommended by the manufacturer (Quanta Bio). Data was analyzed using the  $\Delta\Delta C_t$  method and fold change expression was determined by normalizing the results to the levels of an unstressed control (5S rRNA was used for normalization).

**Generation of expression plasmids.** DNA manipulations were carried out as described by Juárez-Rodríguez *et al.* (2013). PCR products were amplified using high fidelity PCR supermix as recommended by manufacturer (Invitrogen and Alkali Science). All primers (Table 6B) used in this study were flanked with restriction enzyme recognition sites. Primers were designed using the genome sequence of *A. actinomycetemcomitans* D11S-1 strain available in the NCBI database. Restriction

enzymes and ligation reactions were used as recommended by the manufacturer (New England Biolabs). Transformation of both *E. coli* and *A. actinomycetemcomitans* was done by electroporation (2 mm cuvette with 1800 V, 25 uF, 200  $\Omega$ ). For transformation of *E. coli*, ~3 ug plasmid was used for electroporation. Plasmids that were transformed into *A. actinomycetemcomitans* were first produced from 25 mL of *E. coli* harvested for plasmid extraction using a miniprep kit as recommended by the manufacturer (Qiagen and Zymo Research) and 20 ug of plasmid were used for each electroporation. Transformant colonies were recovered in SOC broth for 45 minutes or 5 hours, for *E. coli* and *A. actinomycetemcomitans*, respectively, and then plated on either LB agar or BHI agar supplemented with appropriate antibiotics. Samples were sent to the DNA sequencing facility for further confirmation of gene insertion.

**Generation of isogenic deletion mutants.** The generation of markerless deletion mutations was carried out as described by Juárez-Rodríguez *et al.* (2013) with some modifications. PCR fragments of the upstream and downstream flanking regions for the genes of interest were amplified by PCR and cloned into the pJT1 suicide vector (Table 6B). pJT1 was first cloned into *E. coli* cells to ensure propagation of the plasmid. Colonies showing spectinomycin resistance were analyzed for gene insertion. Plasmids that were positive for gene insertion were analyzed by restriction digestion for appropriate size and sent to the DNA sequencing facility for further confirmation. Once confirmed, plasmid was harvested for use in *A. actinomycetemcomitans*. Electroporated cells were recovered in SOC broth for 5 hours before being plated. Colonies that underwent a single recombination event were selected on the agar using spectinomycin

resistance. Multiple colonies were selected and sub-cultured in BHI broth with antibiotics and then passaged into BHI broth lacking antibiotics for a minimum of two days. A final passage was performed into TYE broth supplemented with IPTG to induce the expression of levansucrase, encoded by *sacB* carried on the plasmid vector. Levansucrase is an enzyme that forms long levan chains from sucrose and is lethal to gram negative bacteria in the presence of sucrose. The cultures were then serially diluted and plated on TYE agar supplemented with IPTG and sucrose. A minimum of 100 colonies were replica plated from the TYE agar plate on to TYE agar plates with IPTG and sucrose and BHI with antibiotics. Colonies that were sucrose resistant and antibiotic sensitive were selected for PCR confirmation that the genes of interest were deleted. Colonies that were positive for deletion were sent to DNA sequencing facility for further confirmation.

Table 6A. Primers used for real-time PCR to monitor expression of putative type II TA systems.			
Primer	Primer Sequence (5' – 3')	Target	Product Size
D11S_0150-0151 Fwd Rev	CTT GCT GAA GCG GAA ACG TTT TTT G CAC CCA ATA CGC CAC AAG GCA A	D11S_0150-0151	157 bp
D11S_0499-0500 Fwd Rev	ACG GGC GGG TCA TTC TGA CAG AA CGG TTA AGT CGC AGT TTT TGT GAT GG	D11S_0499-0500	248 bp
D11S_0905-0906 Fwd Rev	TAA TCC TGT TAC TGG GTG AAC GTC TAA CCG TAC GGC TTG ATT GCG TCC G	D11S_0905-0906	175 bp
D11S_0919-0920 Fwd Rev	CAG CCC AAA GAA ACG ATT AAC CTT AGA TG CCT TGC GTG ATC GGG CAG ACT AAA A	D11S_0919-0920	257 bp
D11S_1023-1024 Fwd Rev	GAA GGT CAG GGG TAT CGA GTT TAT CTG CGC CTA ACG CAG ACA AAA TAA GCT C	D11S_1023-1024	255 bp
D11S_1069-1070 Fwd Rev	CAG CGA ACC AAC ACC TGC ATT TTC CG TCC ATG CCT AAC GGA AAA GTG CGG TAG	D11S_1069-1070	465 bp
D11S_1194-1195 Fwd Rev	ACG CCC GCT CAG GTG TTA AAT ATG TTT CAG GGA TTT CGA GAT TGC CAC ATC AAA	D11S_1194-1195	386 bp
D11S_1417-1418 Fwd Rev	TTA AAC CGC AAT GAA CCC GC CCG AAA ATC CCC GAG TGT CA	D11S_1417-1418	269 bp
D11S_1718-1719 Fwd Rev	CCG TGC AGA ATT AGC AAC AAC ACT TGA TC GAC GAT GCT CTT TAT CTA TTC TTC TAG ACC	D11S_1718-1719	418 bp
D11S_1798-1799 Fwd Rev	CGC AAG CCA AGA TGG TGC CGT A GAT AAC CTT TTA ATT TGT CGC CGG GTA CTC	D11S_1798-1799	309 bp
D11S_2133-2134 Fwd Rev	AAC GCG TGA TTC AAG CGG CGG TAA AA CCC ATC AGA GGC ATA TAG GCA AGC ATT T	D11S_2133-2134	200 bp

Table 6B. Primers used for pJT4 and pJT1 derived plasmids. Sequences in bold indicate restriction enzyme sites for cloning.			
Primer	Primer Sequence (5' – 3')	Target	Product Size
17TA-500	CTC CGC AAA GGT ACC GAC ATT GAA GCC AGT	1718-1719 plus 500 bp	1022 bp
17TA - rev	ATT AAA CGT TTT GGA TCC TTA GTA GTG ATA GCG GCA TTG	upstream for pJT4	
11TA- 500	TTA ACT ATG ATG GGT ACC TTA GTA CTC GCC GTC ATC	1194-1195 plus 500 bp	1143 bp
11TA-rev	ATG TAA TGG ATA TGA ATT GGA TCC TTA TTT AAA CAC TTC CGA GTG	upstream for pJT4	
BWS1-115	GTG GCA GCA GCT TAA CTA TGA TGG CTA GCT TAG TAC TCG CCG TCA TCA CCA TTT TAG CCA CCA TG	Upstream for 1194-1195	616 bp
BWS1-113	CAC TGC GCT ATC TAA CAT AAT TTT TTC CTC CAT GGG CCC GGT GAA ATA	for pJT1	
BWS2-115	GGA AGT GTT TAA ATA AGG GCC CAA TTC ATA TCC ATT ACA TAT TAG	Downstream 1194-1195	575 bp
BWS2-113	CAC GCC GTA GAA ACG ACG ATT TTC ATC AGA CTG CAG CAT GGC GGC AAT CGG	for pJT1	
BWS3-175	GAA AAG AGT GAG AAA ATG GCT AGC AAT CAA CAA CTT ATT GAT TTA AAA CAT AAG CTG GCG	Upstream 1718-1719 for pJT1	1120 bp
BWS3-173	CGC AGA ATA ACT TAT TAC ATT GGG CCC CAT GTT ATT CTC C	pJT1	
BWS4-175	CGC TAT CAC TAC TAA GGG CCC AAA ACG TTT AAT ATG TTC GAA CTC	Downstream 1718-1719	937 bp
BWS4-173	GGA GCA TAG GTA AAG GCG CGC TGC AGC TAT AGA TAA ACA ATG CCT GTG	for pJT1	



**Measurement of metabolic activity.** To assay for metabolic activity of isogenic mutants over time, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed as described by Wang *et al.* (2010) with some modifications. Cells were grown to stationary phase, removed from the incubator ( $t=0$ ), and stored at room temperature for up to seven days. At each time point, 3 mL of culture was pelleted, re-suspended in fresh BHI, diluted 1:10 into fresh BHI broth containing MTT. Cultures were then incubated for two hours (approximately one doubling period) at 37°C under microaerophilic conditions before being harvested. When MTT is dissolved, the initial solution is yellow. Cells with metabolic activity will reduce the MTT via NADH<sup>+</sup>/NADPH<sup>+</sup>-dependent oxidoreductase enzymes in the electron transport chain into water-insoluble formazan (Stepaneko *et al* 2015). The resulting formazan crystals were harvested by centrifugation and allowed to air-dry for 30 minutes before being dissolved in dimethyl sulfoxide (DMSO). Absorbance was read at 550 nm.

**Growth and analysis of static biofilms.** Static biofilms were grown in multi-well tissue culture plates (Merritt *et al* 2005, Haase *et al* 2006, Izano *et al* 2008). *A. actinomycetemcomitans* cultures were grown in BHI broth overnight and OD<sub>600</sub> was measured. To form mature *A. actinomycetemcomitans* biofilms, cells were diluted into fresh BHI broth to a final OD<sub>600</sub> of 0.005 and this dilution was used to inoculate the plate wells. Cultures were incubated at 37°C for 72 hours and the resulting biofilms were supplied with fresh BHI broth that was pH adjusted from pH 5.0 – pH 8.0 to represent the entire range of conditions that might exist in the gingival pocket (Kleinberg *et al* 1969; Bickel *et al* 1985; Eggert *et al* 1991). Biofilms were incubated for an additional 24 hours,

then rinsed gently with sterile water before staining with 0.1% crystal violet. After staining, cells were rinsed with sterile water until the water was clear. After drying, crystal violet was solubilized with 30% acetic acid and the absorbance at OD<sub>570</sub> was measured for each biofilm.

**Statistical analysis.** All assays were carried out in at least triplicate and data were analyzed using the unpaired *t*-test with statistical significance defined as  $P \leq 0.05$ .

**Functional analysis of putative TA systems.** Individual toxin and anti-toxin proteins were cloned into pQE60 (Qiagen) for expression and isolation. Recombinant bacteria were grown in LB broth that was supplemented with ampicillin, tetracycline and IPTG in a shaking incubator at 37°C until late log phase. Cells were harvested and protein was extracted as follows: The cell pellet was suspended in a denaturing protein buffer (50 mM phosphate, pH 7.8, 300 mM NaCl, 0.1% SDS) and sonicated on ice with 5 second bursts until clear. Samples were centrifuged and the supernatant was removed into a clean tube. The pellet was suspended in denaturing protein buffer and the sonication process was repeated. After centrifugation for a second time, the supernatant was transferred to a clean tube. Samples were analyzed via NuPAGE Bis-Tris SDS-PAGE gels (Thermo Fisher) according to manufacturer instructions. Samples were then transferred to a PVDF membrane (GE) for a western blot analysis. Membranes were probed with a primary antibody to the hexa-histidine tag (Takara) according to manufacturer instructions. A secondary antibody, a horseradish peroxidase conjugate (Sigma) and a 3,3',5,5'-tetramethylbenzidine (TMB) substrate (VWR) was used to visualize samples. As needed,

samples were affinity purified using cobalt resin (Takara and Goldbio) according to manufacturer instructions using buffers that were adjusted to a final concentration of 0.1% SDS. Samples were then refolded according to Roussel et al (2013) using 2-methyl-2,4-pentanediol (MPD). Samples were concentrated using Vivaspin-20 (Sartorius).

To eliminate the possibility of RNase contamination from protein expression and production in *E. coli*, peptides (Table 7) representing the toxin and anti-toxin proteins of each TA system were chemically synthesized (Biosynthesis Inc.). Synthetic peptides were dissolved in a protein buffer (0.05M Phosphate buffer, pH 7.8, 300mM NaCl, 0.01% TFA) and peptide purity and size was confirmed using a NuPAGE Bis-Tris SDS-PAGE gel (Thermo Fisher). Samples were prepared in NuPAGE LDS Sample Buffer, Reducing Agent and Antioxidant (Thermo Fisher) and gels were electrophoresed using NuPAGE MES Buffer (Thermo Fisher) in the XCell SureLock MiniCell as recommended by manufacturer. To test the ability of the synthetic toxins and anti-toxins to interfere with protein translation, a cell-free protein synthesis system was used. Toxin (200 ng), anti-toxin (200 ng), and equal concentrations of both were added to the PURExpress In Vitro Protein Synthesis Kit (New England BioLabs) using the control plasmid DNA that expresses dihydrofolate reductase (DHFR). Reactions were incubated in a thermocycler at 37°C for 2 hours. 5 uL of the reaction was used for analysis on a Tris-Glycine SDS-PAGE gel (Thermo Fisher) according to manufacturer's instructions. All protein gels were stained with AcquaStain 1-Step Protein Gel Stain (Bulldog Bio) overnight and destained with sterile water for 20 minutes before imaging. To determine if the toxin and anti-toxin proteins function to degrade mRNA in the presence of ribosomes, MS2 bacteriophage genomic RNA (Roche) was mixed with toxin (200 ng), anti-toxin

(200 ng) or both together in the presence and absence of ribosomes. Reactions were incubated at 37°C for 30 minutes before the reaction was stopped with RNA loading dye containing 47.5% formamide (New England BioLabs). Reactions were denatured at 85°C for 15 minutes before analysis on TBE-Urea gels (Thermo Fisher) according to manufacturer's recommendation. Gels were rinsed in DEPC-treated water for 10 minutes and were stained for 20 minutes with ethidium bromide and rinsed twice with DEPC-treated water before imaging.

**Protein-Protein Interactions.** To determine if the toxin and associated anti-toxin bind together, samples were analyzed on a Superdex 75 Increase, 10/300 GL column according to the manufacturer instructions using a AKTApurifier UPC10 FPLC (flow rate = 0.5 mL/min, Pressure = 1.25 MPa, Temperature = room temperature). 1 mg of each peptide was analyzed separately as well as mixed together in HPLC-grade PBS with 0.5% TFA.

**Proteomic analysis.** Cultures of *A. actinomycetemcomitans* wild-type and both isogenic mutants were grown to mid-log phase in CDM. Cells were gently pelleted and the media decanted. The pellet was suspended in fresh CDM at pH 5.0, and incubated for 4 hours (approximately two doubling periods). The cultures were harvested with gentle centrifugation and the pellet was suspended in a denaturing buffer (50 mM phosphate buffer, pH 7.8, 300 mM NaCl, 8M Urea). Samples were sent to the Proteomics Core at the University of Louisville for analysis. Results were analyzed using the Scaffold Software (Proteome Software) by comparing the number of hits for a given protein

sequence between a mutant and wild-type as well as between the both mutants. Limits were set to only examine sequences that were present at least twice within a given sample. The resulting ratio (mutant vs wild-type) indicates a fold-change in abundance in the mutant as it relates to the wild-type. A number greater 1.0 indicates that the protein was found more frequently in the mutant than in the wild-type whereas a number below 1.0 indicates that the protein was found less frequently in the mutant

Proteins that were differentially represented were analyzed using the Pfam (Finn *et al* 2016) and String Protein-Protein Interaction Networks Database (ELIXIR infrastructure) to determine the function, when possible, of each protein and what other pathways may be affected by the gene of interest.

<b>Table 7. Peptide sequences for toxins and anti-toxins.</b>		
<b>Protein</b>	<b>Sequence</b>	<b>M.W.</b>
D11S_1718	MNVISYSAFRAELATTLQVADHSPVMITRQNGKHAVVMSLEDFAA YEETAYLLRSPKNRERLLASIDQLNSGKIIERELQE	9394.6
D11S_1719	MILAWTETAWEDYLYWQQVDKKTLLRINKLIQNITRAPFEGLGNPPEL KHQLSGFWSRRIDKEHRLVYQVSDSHLTIIQCRYHY	10201.6
D11S_1194	MDYVLSKEYKRDLLKLPVEIQSGPEYAEVLYCLFNQKSLPERYKDHALQ GNWQGFQDCHIKNDLILYKIEADTLYFARLNSHSEVFK	10515.9
D11S_1195	MLDSAVNFRTQADIEQAFNVIKSYGLTPAQVLNMFQIAKTNTIPLS LDYQPNTKTANAINELMSGKGERFSVDSFDEFQQKMRDLSK	10165.5

CHAPTER THREE: IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE  
TYPE II TOXIN/ANTI-TOXINS IN *AGGREGATIBACTER*  
*ACTINOMYCETEMCOMITANS*

**Introduction**

Microbes have evolved many mechanisms to detect and respond to environmental cues. Type II toxin/anti-toxin (TA) systems are small gene systems that encode a biologically active protein, the toxin, and a labile protein, the anti-toxin, that prevents toxin activity by binding to it. Type II TA systems have been found in both bacteria and archaea and many species contain more than one TA system. The toxins function in a variety of ways, including controlling gene expression by inhibiting transcription or translation, and controlling cell growth by inhibiting peptidoglycan synthesis (Syed *et al* 2012). These systems typically respond quickly because of the labile nature of the anti-toxin.

Under normal conditions, the anti-toxin and the toxin form a stable complex that inhibits the toxin function, but many TA complexes also auto-regulate their own expression. When a bacterial cell encounters environmental stress, general proteases, such as Lon and Clp, become active and the anti-toxin is quickly degraded. The toxin is more stable and is not as readily degraded. Once the toxin is active, gene expression of the TA system increases and the toxin is free to perform its task.

The oral cavity is an environment that experiences constant change due to the intake of food, beverages, and the constant flow of saliva. Normal consumption of food and drink can result in changes in temperature, pH, osmotic conditions and nutrients. It is feasible that bacteria within the oral cavity utilize TA systems to temporarily alter cell growth to adapt to environmental stress. The presence of TA systems in *A. actinomycetemcomitans* has not been fully investigated as of yet.

## Results

### ***A. actinomycetemcomitans* growth is affected by environmental stress:**

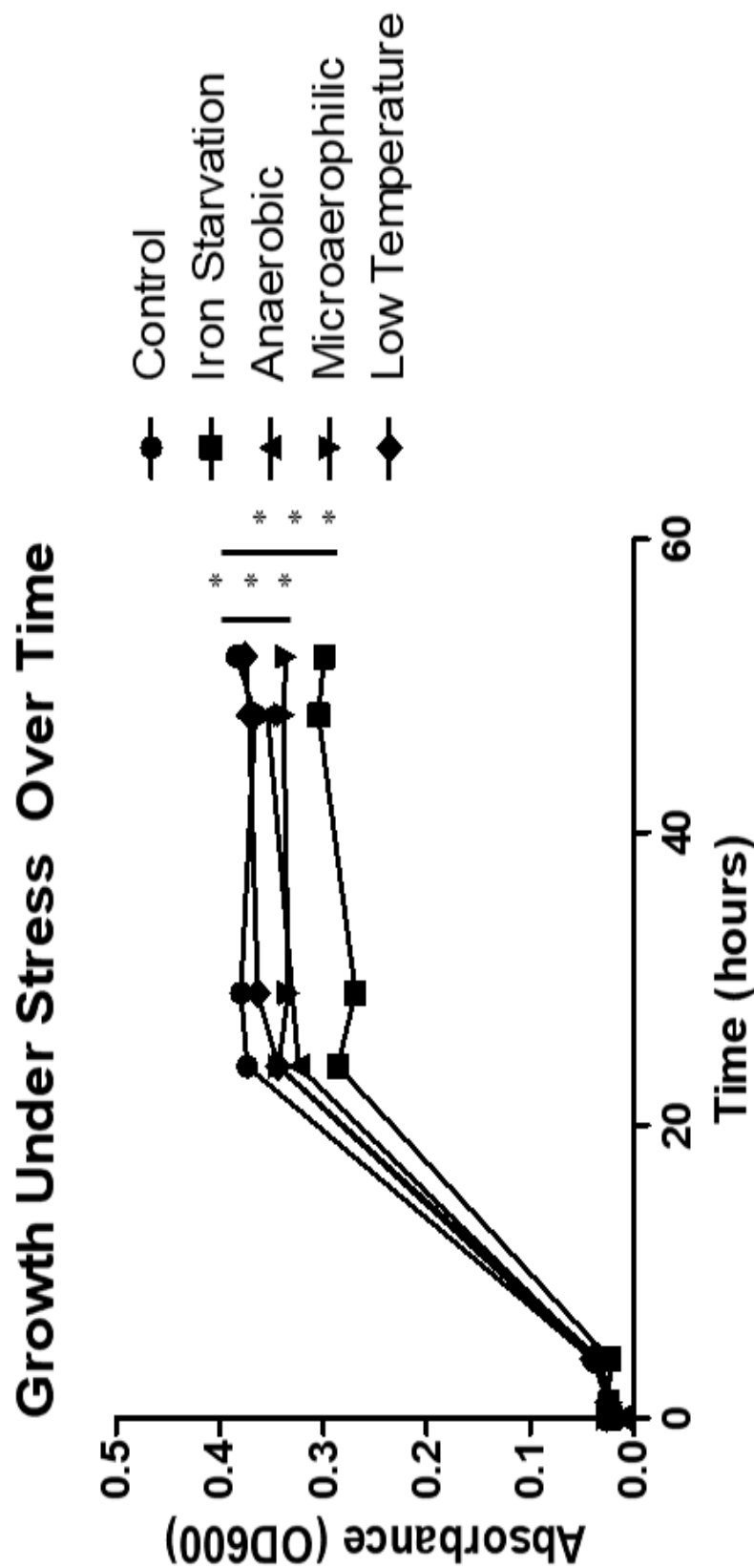
To determine the effects of environmental stress on wild-type bacteria, *A. actinomycetemcomitans* was grown in CDM under one of five stress conditions for 48 hours. The five test conditions were: iron limitation (250  $\mu$ M bipyridyl), aerobic (no added carbon dioxide), anaerobic, and microaerophilic (5% carbon dioxide) conditions, and reduced temperature (30°C). In Figure 7, bacteria growing in aerobic conditions exhibit a doubling time of approximately two hours. Doubling time has previously been calculated within the laboratory and these growth curves are consistent with those assays (data not shown). Iron limitation reduced the OD<sub>600</sub> at stationary phase but had little effect on doubling time. One possible explanation for this is that *A. actinomycetemcomitans* is able to store iron for use when it cannot acquire iron from its environment (Fong *et al* 2003). Reduced temperature and anaerobic conditions did not significantly affect the growth of the bacteria when supplemented with glucose, suggesting that bacteria adapt rapidly to these conditions. Growth under microaerophilic conditions also reduces the OD<sub>600</sub> at stationary phase, but doubling time is also

unaffected . Taken together, these results suggest that *A. actinomycetemcomitans* is capable of efficiently responding to environmental stress.

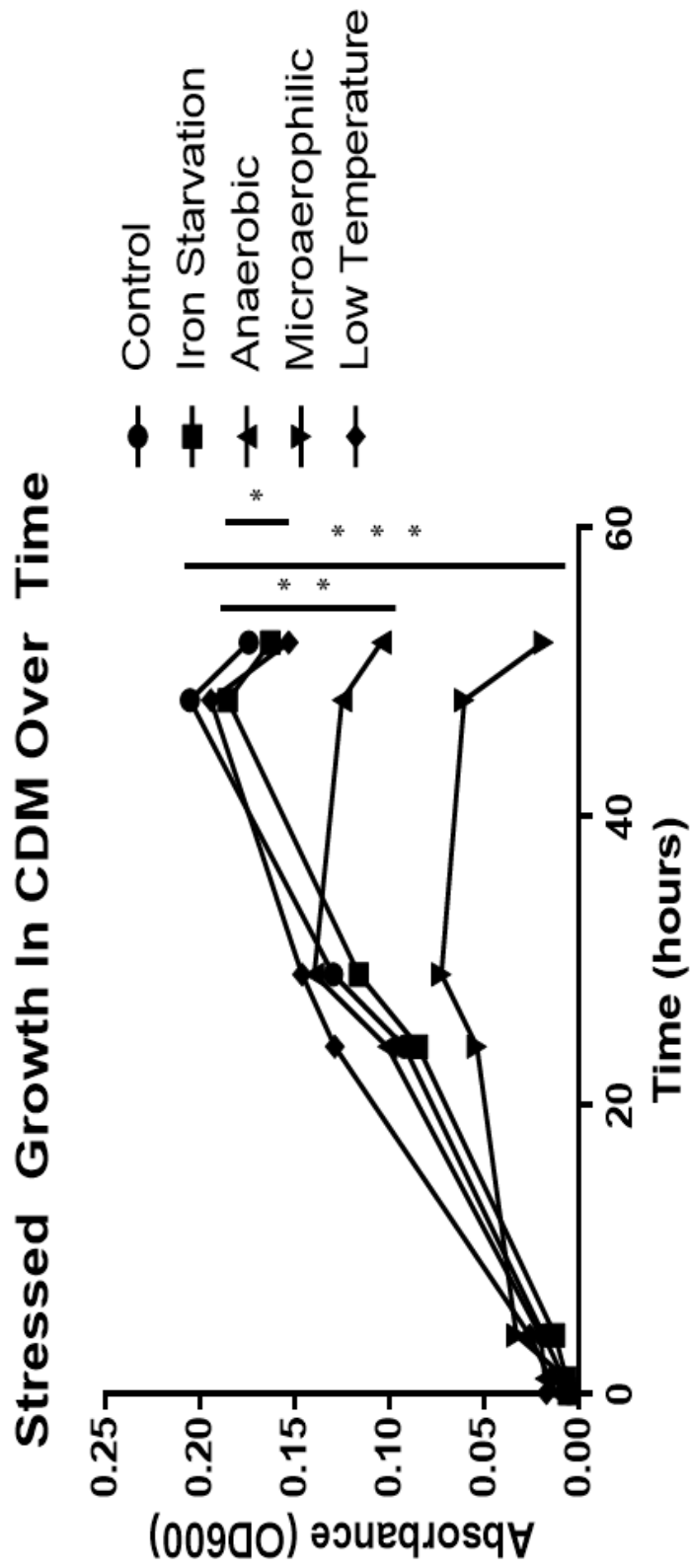
Since previous studies show that *A. actinomycetemcomitans* prefers to utilize lactate as a carbon source, cells were also cultured in CDM that was supplemented with lactate instead of glucose. As shown in Figure 8, doubling time in CDM/lactate increased to four hours and cell density began to drop significantly after 48 hrs. Utilization of lactate under anaerobic or microaerophilic conditions resulted in a loss of cell density after 24 hours.

CDM itself is hypotonic and contains only 100 mg/L (1.7 mM) of sodium chloride, whereas BHI contains 5.0 g/L (85mM). To determine if salt concentration influences the growth of *A. actinomycetemcomitans*, cultures were grown in CDM with increasing concentrations of sodium chloride. As shown in Figure 9, sodium chloride concentration in CDM has little effect on growth. However, the growth of bacteria in BHI when compared to the growth in CDM with additional sodium chloride is still greater, indicating that the growth difference is not due to osmotic stress.



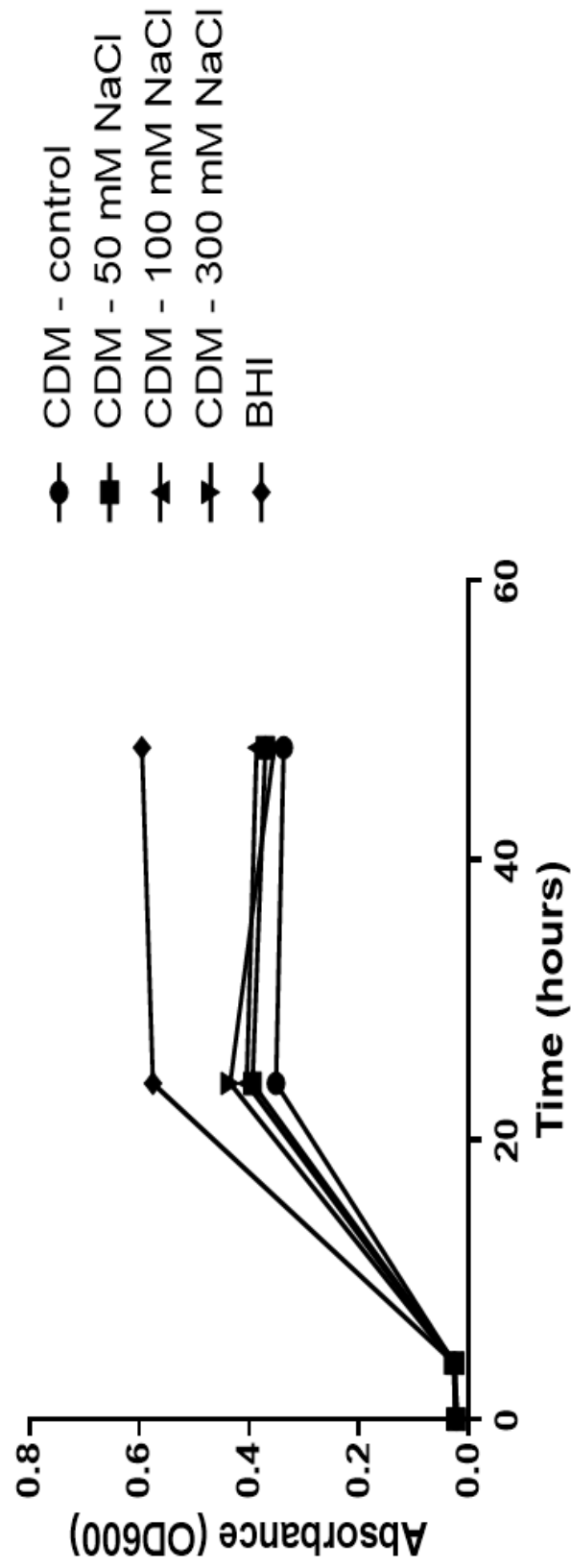


**Figure 7. Growth of bacteria under environmental stress.** Bacteria were grown in CDM with glucose for at least 48 hours under different stress conditions in triplicate. Growth was measured by reading the absorbance of the culture at OD<sub>600</sub>. Final OD<sub>600</sub> values were analyzed using a two-tailed t-test. \*\*\*  $p < 0.001$ . Error bars were omitted to ensure all data points can be seen.



**Figure 8. Growth of bacteria with lactate and environmental stress.** Bacteria were grown in CDM with lactate under different environmental stress conditions in triplicate. Growth was measured by absorbance at OD<sub>600</sub>. Final OD<sub>600</sub> were analyzed using an unpaired t-test \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Error bars were omitted to ensure all data points can be seen.

## Osmotic Stressed Growth in CDM Over Time



**Figure 9. Growth of bacteria in osmotic stress.** Bacteria were grown in CDM with glucose and added salt to determine if the growth differences between CDM and BHI was due to osmotic stress in triplicate. Growth was measured by reading the absorbance of the culture at OD<sub>600</sub>. There were no statistically differences between the control CDM and any of the test CDMs. Error bars were omitted to ensure all data points can be seen.

### **BLAST results for type II toxin/anti-toxin systems in *A. actinomycetemcomitans*:**

Type II TA systems encode two small proteins (each between 41-206 amino acids) that can regulate bacterial cell function. Previous studies have shown that TA systems are involved with biofilms, antibiotic stress, and starvation (Horak *et al* 2017). To determine if the *A. actinomycetemcomitans* genome encodes type II TA systems, protein sequences of known type II TA systems found in *E. coli* were used to perform BLAST searches of the *A. actinomycetemcomitans* D11S-1 genome (Chen *et al* 2009). Table 8 shows the results of these BLAST searches. The BLAST searches identified nine putative TA systems. Two have homology to the MazEF-family, which inhibit translation through endoribonuclease activity that cleaves mRNA independently of ribosome binding. One TA system has homology to HipAB-family, which inhibits translation through a serine/threonine kinase that targets tRNA. The remaining six putative TA systems exhibit homology to the RelBE-family, which cleave mRNA, but endoribonuclease activity of these TA systems is ribosome dependent.

Since approximately 32% of the genes in *A. actinomycetemcomitans* do not have a known function, it is possible that unique TA systems may exist that were not identified in the BLAST searches. To address this possibility, the entire genome was examined using TAFinder to identify two gene operons that encode small proteins in the size range of known toxin and anti-toxin proteins. Five additional putative TA systems were identified from this search. Using the protein sequence of these genes, a BLAST search was performed and no significant results for known TA systems were found, suggesting that these five putative TA systems may be unique to *A. actinomycetemcomitans*. In addition, D11S\_0469-0470 and D11S\_2094-2095 were unique in that each putative TA

system contains a third open reading frame, which encodes a gene unrelated to the toxins or antitoxins, with homology to diadenosine tetraphosphatase and *O*-succinylbenzoate synthase, respectively. Finally, sequence analysis suggested that three of the putative TA systems may contain pseudogenes (indicated by an asterisk in Table 8). Hence, the *A. actinomycetemcomitans* D11S-1 genome encodes at least 14 putative type II TA loci, three of which may contain pseudogenes and may be non-functional.

There are currently seven recognized serotypes of *A. actinomycetemcomitans* and there are marked differences in their ability to cause disease (Minquez *et al* 2014, Brigido *et al* 2014). Using the sequences from *E. coli* toxin and anti-toxin protein sequences described above, BLAST searches were performed across 33 *A. actinomycetemcomitans* genome sequences present in the NCBI database. Table 9A-G summarizes the results of these searches. Each 'X' in the chart indicates a BLAST match that showed sequence similarity across the entire probe peptide sequence. A majority of the *A. actinomycetemcomitans* strains examined possess only the TA systems that were identified in the initial BLAST search of the D11S-1 genome. None of the other *E. coli* TA systems were found in the *A. actinomycetemcomitans* genome sequences tested, with the exception of the presence of a TA system related to *E. coli yncN* in two serotype c strains of *A. actinomycetemcomitans* (SCC2302 and AAS4A). In addition, several serotype d and e strains lack homology to several *E. coli* systems, e.g., strain SA2200 serotype d lacks homologs of PemK, ChpBK and YafO (see Table 7D). Finally, the HipA toxin is a large protein compared to many other toxins classified as type II TA systems. Many of the homologs found in *A. actinomycetemcomitans* are annotated as

**Table 8. Type II TA systems identified in *A. actinomycetemcomitans* D11S-1 using BLAST and TAFinder.**

<b>Toxin</b>	<b>Anti-toxin</b>	<b>Gene Identity</b>	<b>Family</b>	<b>Total Score</b>	<b>Query Cover</b>	<b>E Value</b>	<b>Max Identity</b>
mazF	mazE	D11S_0905-0906	mazEF	101	92%	2e-28	33%
chpBK	chpBI	D11S_0919-0920	mazEF	88.6	93%	2e-23	42%
hipA	hipB	D11S_1069-1070	hipAB	108	21%	1e-28	45%
yafQ	dinJ	D11S_1194-1195	relBE	89.7	95%	2e-24	36%
yoeB	yafM	D11S-1718-1719	relBE	125	100%	1e-38	63%
relE	relB	D11S_1798-1799	relBE	112	94%	6e-33	57%
relE	relB	D11S_2133-2134	relBE *	44.7	88%	2e-7	12%
relE	relB	D11S_1417-1418	relBE	102	98%	3e-30	54%
relE	relB	D11S_1144-1145	relBE	149	97%	2e-46	59%
		D11S_1023-1024					
		D11S_0150-0151	*				
		D11S_0499-0500	*				
		D11S_0469-0470	**				
		D11S_2094-2095	**				

\* - sequence analysis indicated this operon contained a toxin and/or antitoxin pseudogene

\*\* - identified by TAFinder but present in a 3 gene operon with a non-TA related gene (see text)

**Table 9A. BLAST results for all *A. actinomycetemcomitans* strains, serotype A**

[illegible]

O - only the anti-toxin gene is present 1 - partial sequence match 2 - internal deletion in toxin open reading frame 3 - frameshift mutation in toxin open reading frame 4- toxin only

Strain	CcDB	ParE	Phd	MazF	ChpK	Kid	PemK	ChpBK	RelE	YoeB	YafO	YglN	YglU	MqsR	YafQ	HigB	VapC	Zeta	HipA	HicA	YncN
ANH9381		X <sup>2</sup>		X <sup>1</sup>		X	X <sup>1</sup>	X	XX	X					X				X <sup>1</sup>		
HK1651		X		X <sup>1</sup>					X <sup>1</sup> X <sup>1</sup>	X					X <sup>1</sup>				X <sup>1</sup> X <sup>1</sup> X <sup>1</sup>		
Y4		X <sup>2</sup>		X <sup>1</sup>		X	X <sup>1</sup>	X	X <sup>1</sup> X	X					X <sup>1</sup>				X <sup>1</sup> X <sup>1</sup> X <sup>3</sup>		
RbAA1		X <sup>2</sup>		X <sup>1</sup>		X	X <sup>1</sup>	X	XX X	X											
SCC1398		X <sup>2</sup>							X	X					X <sup>1</sup>				X <sup>1</sup>		
I23C		X <sup>2</sup>		X <sup>1</sup>		X	X <sup>1</sup>	X	X	X					X				X <sup>1</sup>		
SCC4092		X <sup>2</sup>							X	X					X <sup>1</sup>				X <sup>1</sup>		
S23A		X <sup>2</sup>		X <sup>1</sup>		X	X <sup>1</sup>	X	X	X					X				X <sup>1</sup>		

O - only the anti-toxin gene is present 1 - partial sequence match 2 - internal deletion in toxin open reading frame 3 - frameshift mutation in toxin open reading frame 4- toxin only





Table 9D. BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype D																					
Strain	CcdB	ParE	Phd	MazF	ChpK	Kid	PemK	ChpBK	RelE	YoeB	VarO	YglN	YglU	MqsR	VarQ	HgbB	VapC	Zeta	HtpA	HicA	YncN
SA3733		X				X	X	X	X										X		
I63B		X <sup>4</sup>		X					X	X					X				X		
SA3033		X		X		X	X	X	X	X					X				X		
SA269		X		X		X	X	X	X	X					X				X		
SA2200		X		X					X	X									X		
SA508		X		X		X	X	X	X	X					X				X		

O - only the anti-toxin gene is present 1 - partial sequence match 2 - internal deletion in toxin open reading frame 3 - frameshift mutation in toxin open reading frame 4- toxin only

Table 9E. BLAST results for all <i>A. actinomycetemcomitans</i> strains, serotype E-G																					
Strain	CcDB	ParE	PhD	MaxF	ChpK	Kid	PemK	ChpBK	RolE	YoeB	YafO	YgjN	YgtU	MgsR	YafQ	HigB	VapC	Zeta	HipA	HicA	YucN
SC1083 (E)		X		X		X			XX	X					X				X <sup>12</sup>		
SA2149 (E)		X		X		X	X	X	X <sup>1</sup> X <sup>1</sup>	X					X				X <sup>12</sup>		
SC936 (E)		X		X		X	X	X	X <sup>1</sup> XX	X					X				X		
SA2376 (E)		X					X	X	X <sup>1</sup> XX	X					X				X <sup>1</sup> X <sup>1</sup>		
ANB9776 (E)		X							X	X					X				X		
SA3096 (E)		X				X	X	X	XX	X					X				X		
SCC393 (E)		X		X		X	X	X	X	X					X				X		
SC29R(F)		X		X		X	X	X	X <sup>1</sup> XX	X					X				X		
D18P1(F)		X		X		X	X	X	X	X					X				X		
NUM 4029 (G)		X		X		X	X	X	X <sup>1</sup> XX	X					X				X		

O - only the anti-toxin gene is present 1 - partial sequence match 2 - internal deletion in toxin open reading frame 3 - frameshift mutation in toxin open reading frame 4- toxin only

Table 10A. Conservation of type II TA systems in <i>A. actinomycetemcomitans</i> , serotype A											
Strain	D11S_0150	D11S_0499	D11S_0906	D11S_0920	D11S_1023	D11S_1069	D11S_1194	D11S_1418	D11S_1719	D11S_1799	D11S_2133
A160	X	X	X	X	XX	O	X	X	X	X	X
HSP1	X	X	X	X	XX	O	X	X	X	X	X
D7S-1	X	X	X	X	XX	O	X	X	X	X	X
D17P-3	X	X	X	X	XX	O	X	X	X	X	X

O - only the anti-toxin gene is present 1 - putative pseudogene Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider *et al* 2018).

Table 10B. Conservation of type II TA systems in <i>A. actinomycetemcomitans</i> , serotype B											
Strain	D11S_0150	D11S_0499	D11S_0906	D11S_0920	D11S_1023	D11S_1069	D11S_1194	D11S_1418	D11S_1719	D11S_1799	D11S_2133
ANH931	X	XX	X	X	X	X	X	XX	X	XX	X
HK1651	X	XX	X	X	X	<sup>1</sup> X	X	<sup>1</sup> X	X	<sup>1</sup> X	X
Y4	X	X	X	X	XX	<sup>1</sup> X	X	XX	X	XX <sup>1</sup>	X
RhAA1	X	X	X	XX	XXX			XX	X	XXX	XX
SCC1398	X	XX			X	<sup>1</sup> X	X	X	X	X	X
I23C	X	XX	X	X	X	O	X	X	X	X	X
SCC4092	X	XX			X	O	X	X	X	X	X
S23A	X	XX	X	X	X	O	X	X	X	X	X

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Table 10C. Conservation of type II TA systems in <i>A. actinomycetemcomitans</i> , serotype C											
Strain	D11S_0150	D11S_0499	D11S_0906	D11S_0920	D11S_1023	D11S_1069	D11S_1194	D11S_1418	D11S_1719	D11S_1799	D11S_2133
D11S-1	X <sup>1</sup>	XX <sup>1</sup>	X	X	X	X	X	X	X	X	X
SC383		XX	X	X	X	X	X	XX <sup>1</sup>	X	XX <sup>1</sup>	X
SCC2302		XX	X	X	X	O	X	X	X	X	X
D17P-2		XX	X	X	X	O	X	X	X	XX	X
AAS4A		XX	X	X	X	O	X	X	X	X	X

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Table 10D. Conservation of type II TA systems in <i>A. actinomycetemcomitans</i> , serotype D											
Strain	D11S_0150	D11S_0499	D11S_0906	D11S_0920	D11S_1023	D11S_1069	D11S_1194	D11S_1418	D11S_1719	D11S_1799	D11S_2133
SA508	X	X	X	X	XX	O	X	XX <sup>1</sup>	X	XX <sup>1</sup>	X
SA2200	X		X	XX	XX	O		XXX	X	XX <sup>1</sup>	X
SA269	X	X	X	X	XX	O	X	XX <sup>1</sup>	X	XX <sup>1</sup>	X
SA3033	X	X	X	X	XX	O	X	XX <sup>1</sup>	X	XX <sup>1</sup>	X
I63B	X	X	XX	X	XX		X	X	X	X	X
SA3733	X	X		X	X	O		X <sup>1</sup>	X	X <sup>1</sup>	X

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Table 10E. Conservation of type II TA systems in <i>A. actinomycetemcomitans</i> , serotype E-G											
Strain	D11S_0150	D11S_0499	D11S_0906	D11S_0920	D11S_1023	D11S_1069	D11S_1194	D11S_1418	D11S_1719	D11S_1799	D11S_2133
SC1083 (E)	X <sup>2</sup>	XX		X	XXX	X <sup>1</sup>	X	XX	X	XX	X
SA2149 (E)		XX	X	X	X		X	XX <sup>1</sup>	X	X <sup>1</sup>	X
SC936 (E)	X	XX		X	XXX	O	X	XX	X	XXX <sup>1</sup>	X
SA2876 (E)	X <sup>2</sup>	X	X	X	XX	O	X	XX <sup>1</sup>	X	X	
ANH9776 (E)	X <sup>2</sup>	XX			X		X	X	X	X	X <sup>2</sup>
SA3096 (E)	X	XX		X	XXX	O	X	XX	X	XX	X
SCC393 (E)	X <sup>2</sup>	X	X	X	XX	O	X	X	X	X	X
SC29R (F)	X	X	X		XX	O	X	XX <sup>1</sup>	X	XX <sup>1</sup>	X
D18P1 (F)	X	X	X	X	XX	O	X	X	X	X	X
NUM 4029 (G)	X	X	X	X	XX	O	X	XX <sup>1</sup>	X	XX <sup>1</sup>	X

O - only the anti-toxin gene is present 1 - putative pseudogene Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider *et al* 2018).



smaller proteins and are usually present as multiple open reading frames that are identified as *hipA*-like. It is likely that these sequences in *A. actinomycetemcomitans* represent a *hipA* gene that has undergone deletion or rearrangement during its evolution.

A second BLAST search was performed using the sequences identified in D11S-1 to determine if the type II TA systems identified in the D11S\_1 strain are conserved in other strains within the C serotype, as well as across all known serotypes. As shown in Tables 10A – E, most of the D11S-1 TA systems are highly conserved across all of the other *A. actinomycetemcomitans* serotypes. Two exceptions were D11S\_150-151 and D11S\_1069-1070. A complete D11S\_1069-1070 operon was present in only three of the 33 strains examined (D11S-1, Sc383s and ANH9381); the other strains either lacked the toxin open reading frame or possessed a truncated gene, but interestingly most contained the anti-toxin gene. D11S\_150-151 was present in all serotype a, b, d, f and g strains but was absent in the serotype c organisms except for D11S-1 which possessed a truncated pseudogene. For many of the strains across all of the serotypes, some TA systems were present in multiple copies. For example, serotype b strain RhAA1 possesses at least 19 TA systems including two copies of D11S\_0469-0470, D11S\_0905-0906, D11S\_1418-1419 and D11S\_2133-2134, and three copies of D11S\_1023-1024 and D11S\_1798-1799, yet this strain also lacked D11S\_1069-1070, D11S\_1194-1195, and D11S\_2094-2095. Finally, PCR reactions using *A. actinomycetemcomitans* 652 (serotype c) DNA as a template indicated that all of the D11S-1 TA systems were present (not shown). Although a complete genome sequence is not yet available, strain 652 has been extensively characterized in our laboratory and was used for the functional characterization of the *A. actinomycetemcomitans* TA systems described below. Overall,

these results suggest that the TA systems identified in the D11S-1 genome are highly conserved in all *A. actinomycetemcomitans* serotypes suggesting that they may play an important role in *A. actinomycetemcomitans* physiology. However, D11S\_150-151 and D11S\_1069-1070 were more limited in distribution than the other TA systems.

#### **Putative TA systems in *A. actinomycetemcomitans* respond to environmental stress.**

Previous work done within the laboratory and elsewhere has shown that *A. actinomycetemcomitans* growth is influenced by environmental stress, such as iron starvation or pH and temperature fluctuations (data not shown). Since it is possible that TA systems are responsible for controlling bacterial growth when activated, mRNA expression of the putative TA systems was examined under different stress conditions. During normal cell activity, the anti-toxin is bound to the toxin. This inhibits toxin activity and in many cases, the complex of the two proteins has been shown to auto-regulate its own operon expression by binding to the promoter. When cells experience environmental stress, general proteases such as the Lon and Clp proteases are activated and the labile anti-toxin is rapidly degraded which de-represses operon expression.

To assess the effect of environmental stress on TA expression, wild-type bacteria were grown to mid-exponential phase and cells were exposed to various stress conditions for 20 minutes and then harvested for RNA extraction. RNA was converted into cDNA and the cDNA was analyzed via real-time PCR to determine changes in TA gene expression change.

Figure 10A displays the results obtained for the three of putative TA systems that were unique to *A. actinomycetemcomitans*. D11S\_0150-0151 was upregulated under

anaerobic (no oxygen) and under iron limiting (250 $\mu$ M bipyridyl) conditions, and slightly under acidic stress (pH 5.0). The other two systems showed very little change from the control, however, D11S\_0499-0500 was down-regulated under acidic (pH 5.0) and in the presence of hydrogen peroxide (0.1% hydrogen peroxide).

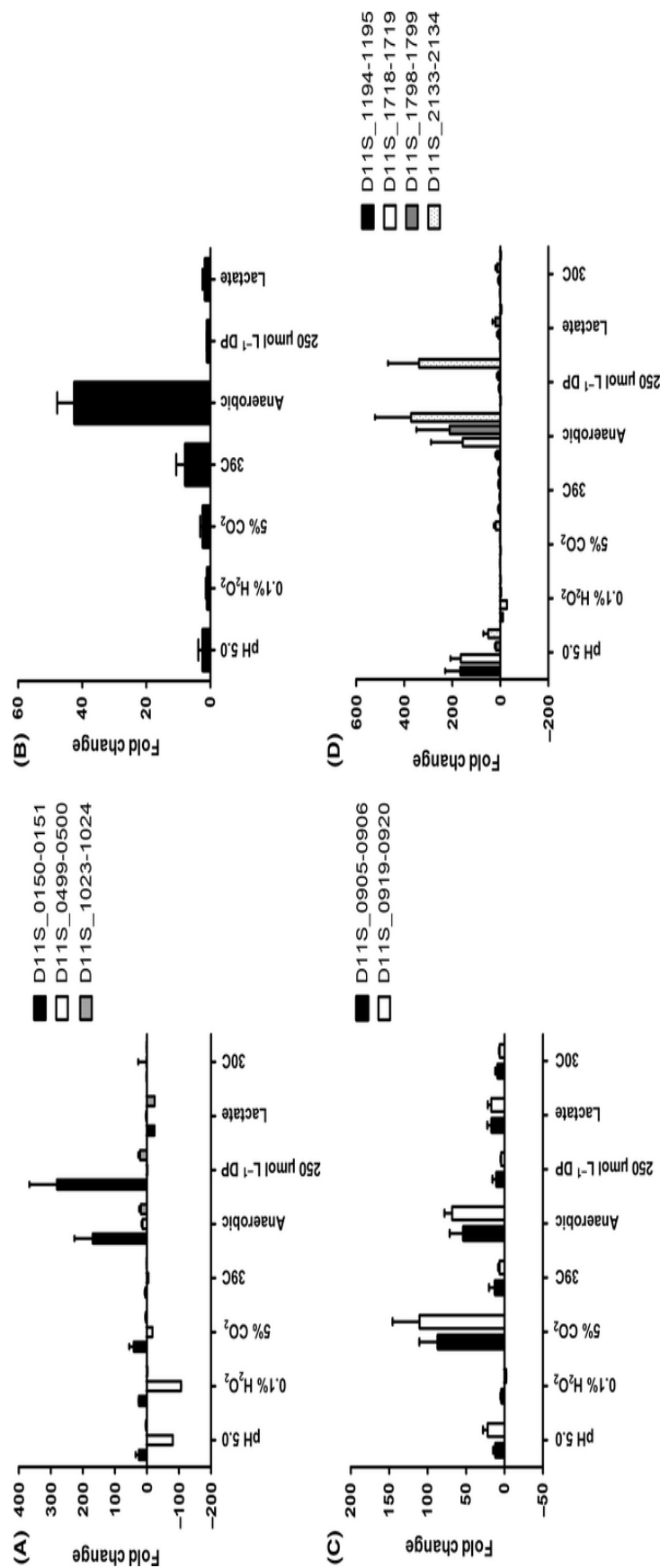
Figure 10B shows the HipAB homolog was largely unresponsive to most stress conditions tested. However, there was an induction in expression of this TA operon when the cells were cultured under anaerobic conditions and when the cells were exposed to elevated temperature (39°C).

Figure 10C focuses on the two putative systems with homology to MazEF-family systems. Interestingly, both systems exhibit a similar pattern of expression. Both systems also show large up-regulation under microaerophilic (5% carbon dioxide) and anaerobic conditions, and to a lesser extent under acidic stress (pH 5.0) and when lactate is the carbon source. Since these systems were activated in almost all stress conditions tested, with the exception of oxidative stress (0.1% hydrogen peroxide), these systems may aid in the adaption of the bacteria to a variety of environmental stimuli. These results also suggest functional redundancy may exist in the MazEF-like systems since both respond at similar levels to environmental stress.

Figure 10D focuses on four of the five RelBE-family homologs. There is a significant up-regulation of two of the systems under acidic conditions (pH 5.0), but the other two systems were not as responsive. One system was induced under iron starvation (250 $\mu$ M bipyridyl) but the other three systems were not responsive under this condition. Three of the systems experienced a large up-regulation under anaerobic conditions, but one was not responsive. For oxidative stress (0.1% hydrogen peroxide), microaerophilic

(5% carbon dioxide), elevated temperature (39°C), lactate for a carbon source, and reduced temperature (30°C), all of the systems were not responsive.

The results taken together suggest that the putative TA systems differentially respond to environmental stress and clearly, not all systems respond to every stress. Seven of the 11 systems comprising members of each of the TA families were induced under anaerobic conditions, suggesting that a high degree of functional redundancy may exist in the TA systems contributing to adaption to anaerobic growth. In contrast, only two systems were significantly induced when cultures were either grown under microaerophilic conditions, acidic conditions, or under iron limiting conditions, which suggests that some TA systems are functionally more specific. Only one of the TA systems, D11S\_1023-1024, did not respond to any of the environmental stress conditions that were tested.



**Figure 10A-D. Induction of type II TA system expression in *A. actinomycetemcomitans* exposed to various environmental conditions.** A. TA systems with no known homology. B. TA system related to the HipAB family. C. TA systems related to the MazEF family. D. TA systems related to the RelBE family. Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider *et al* 2018).

## CHAPTER FOUR: GENERATION AND CHARACTERIZATION OF ISOGENIC DELETION MUTANTS

### **Introduction**

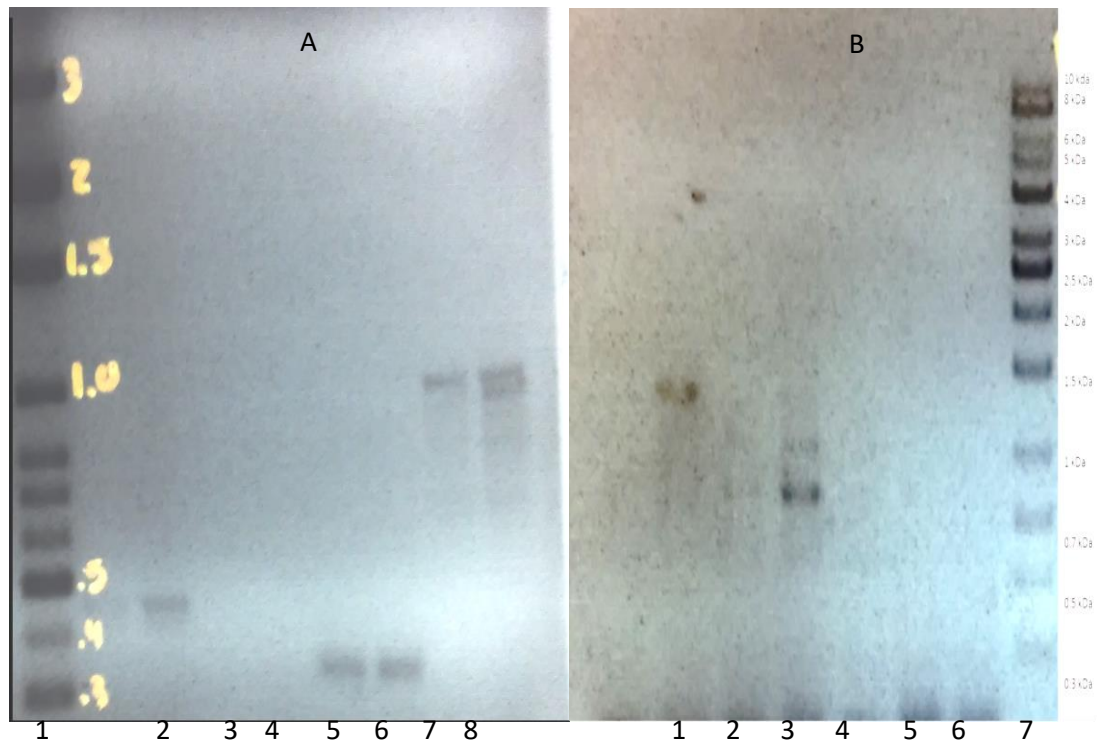
As *A. actinomycetemcomitans* enters stationary phase, the pH of medium drops from an initial pH of 7.5 to approximately a pH of 5.5 - 6.0 in stationary phase, depending on the medium used for culture. Previous work showed that when the pH of spent medium was adjusted back to neutral, *A. actinomycetemcomitans* continued to grow without any other additives (Bhattacharjee 2011), suggesting that *A. actinomycetemcomitans* entrance into stationary phase may be dependent on environmental pH rather than the lack of nutrients.

Two of the putative TA systems were highly responsive to acidic stress, which suggests that these systems may be important for overall fitness of the bacteria under acidic conditions. Since the pH of the gingival pocket can vary significantly, it is possible that these two systems are important for colonization and maintenance of biofilms in the oral cavity. Some studies have shown that removal of TA systems in bacteria has a noticeable effect on biofilm formation and stability (Yamaguchi *et al* 2009). Since the presence of a biofilm in the gingival crevice is crucial for progression of disease, these two systems in *A. actinomycetemcomitans* were chosen for further studies.

## **Results**

### **Generation of isogenic deletion mutants:**

Using a suicide vector that was previously designed in the laboratory, two isogenic mutants were created. The suicide vector contains two separate selection mechanisms: the first is to permit the identification of single recombination events that incorporate the plasmid into *A. actinomycetemcomitans* genome (Campbell 1957) and subsequently to isolate mutants that have undergone a second recombination that results in deletion of the target gene and the loss of the plasmid sequences. This results in a markerless and scarless deletion mutant. Colonies that appeared to have experienced two recombination events were analyzed with PCR to determine if the gene of interest was deleted (Figure 11A-B).



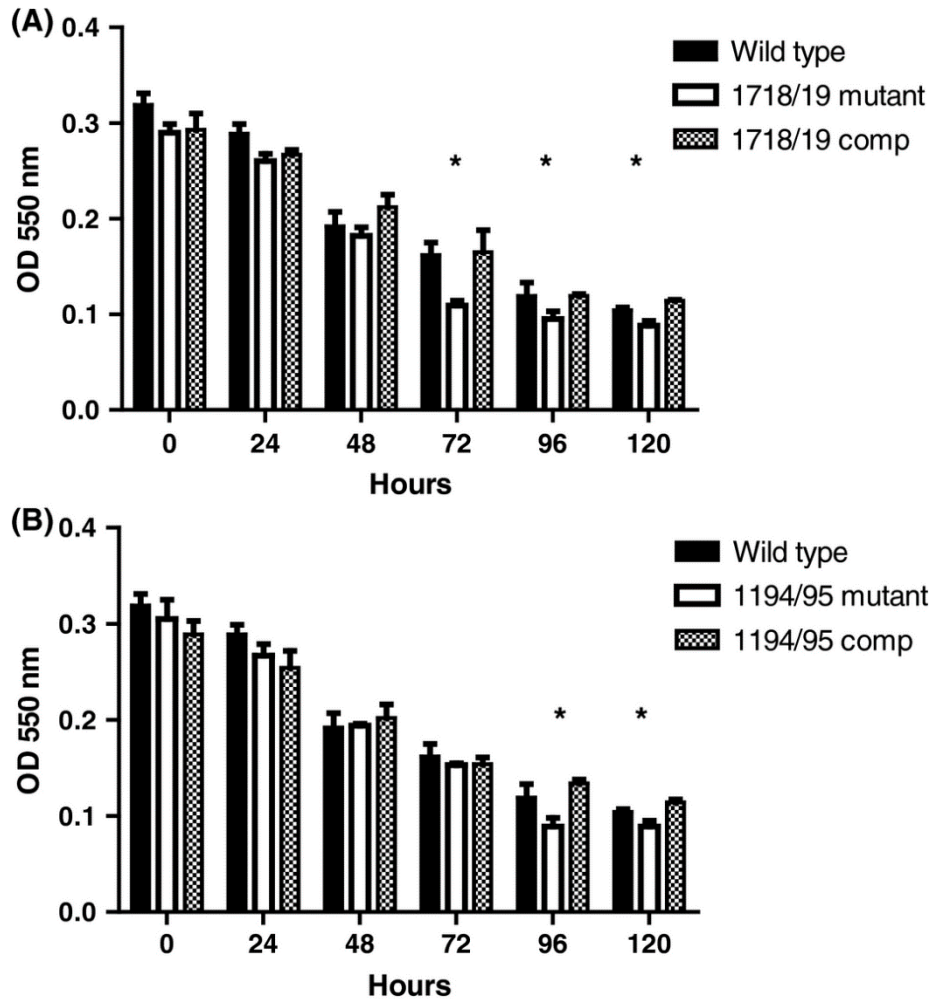
**Figure 11A-B. PCR of isogenic mutants to show gene deletion.** A) Lane 1 is the ladder, labeled in kDa. Lane 5 and 6 are the  $\Delta 1194-1195$  mutants and Lanes 7 and 8 are the wild-type. B) Lane 1 is wild-type, lane 3 is the  $\Delta 1718-1719$  mutant, and lane 7 is the ladder.



### **Metabolic activity at stationary phase decreases over time.**

Previous work showed that viable wild-type *A. actinomycetemcomitans* could be recovered from broth cultures for up to a week after entering stationary phase, when the cultures were maintained at room temperature (Bhattacharjee 2011). As *A. actinomycetemcomitans* grows into stationary phase in BHI broth, the pH of the medium drops from approximately 7.5 to near 5.0 in 24 hours. The pH of the medium is maintained over time and there is no difference between the wild-type strain and two isogenic mutants (data not shown). Since the two putative TA systems chosen for further analysis were responsive to acidic conditions, cell viability in stationary phase was determined using a MTT assay.

As shown in Figure 12A, the wild-type strain maintains a steady loss of metabolic activity over 120 hours. The  $\Delta 1718/1719$  is the more sensitive to sustained exposure to acidic conditions in stationary phase and lost metabolic activity to a greater extent than the wild-type, especially between 72 and 120 hours. Complementation of the deletion mutant with a functional copy of D11S\_1718-1719 restored the metabolic activity to wild-type levels. The  $\Delta 1194-1195$  mutant phenotype was similar to the wild-type strain through 72 hours, but exhibited a significant decrease in metabolic activity relative to the wild-type strain at 96 and 120-hour time points (Figure 12B). Complementation of the mutant restored metabolic activity to the wild-type level. Interestingly, the OD<sub>600</sub> of the cultures did not significantly decrease over the incubation period for any of the strains (data not shown), suggesting that cell lysis may not be occurring as metabolic activity decreases.



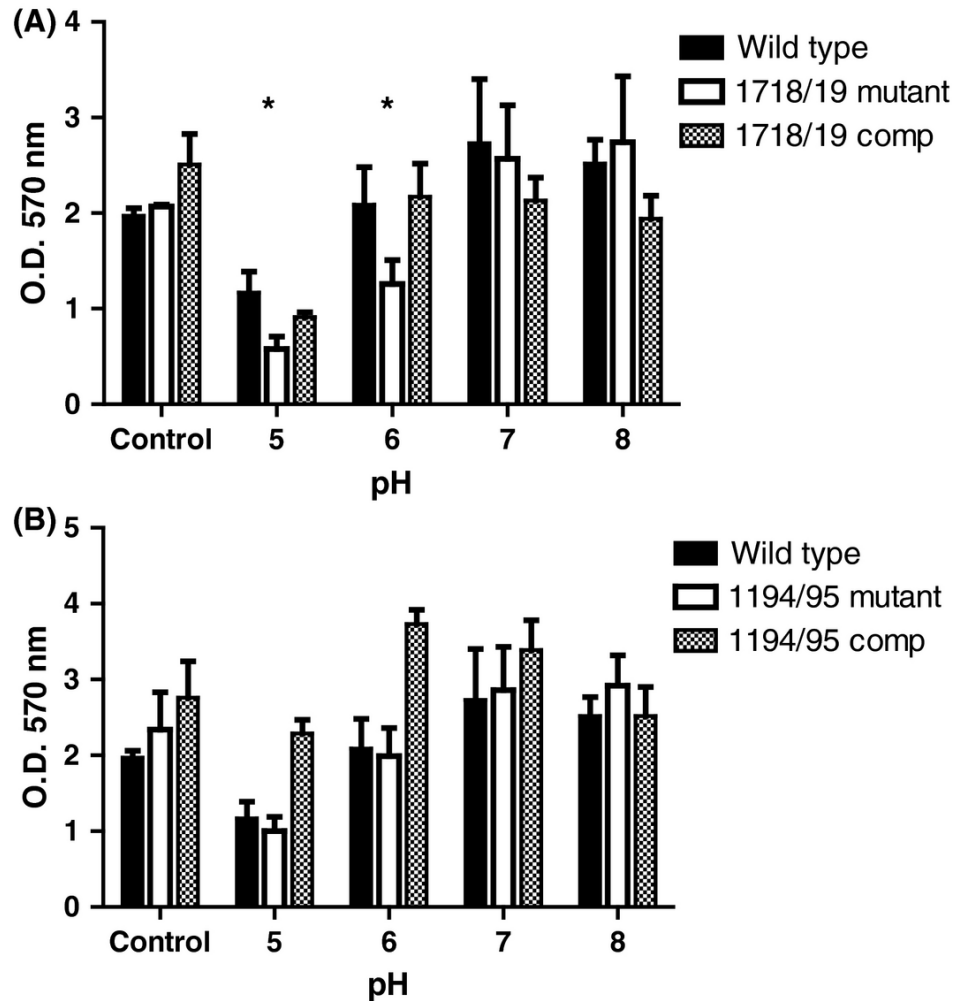
**Figure 12A-B. Metabolic activity of stationary phase *A. actinomycetemcomitans*.**

Metabolic activity was measured using a MTT assay. Asterisks indicate the time points where metabolic activity of the mutant strain was significantly reduced relative to the wild-type ( $P \leq 0.05$ ) and restored to wild-type levels when complemented with a functional copy of the TA system. Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider *et al* 2018).

### **Isogenic mutants exhibit reduced biofilm growth**

Type II TA systems have been implicated in biofilm formation in various bacterial species (Cardenas-Mondragon *et al* 2016, Wood *et al* 2016, Sun *et al* 2017, Schneider *et al* 2018). *A. actinomycetemcomitans* is an oral commensal that forms biofilms within the gingival pocket. Biofilms are important for survival of the bacteria within the oral cavity and necessary for disease progression.

To determine if the type II TA systems of interest are involved in biofilm formation, biofilms were grown in multi-well cell culture plates using BHI. Static biofilms were allowed to grow untouched for 72 hours and then provided with fresh medium at pH 5.0 to pH 8.0. After incubation for an additional 24 hours, biofilms were quantified by crystal violet staining. Figure 13 shows that biofilm biomass of the wild-type strain increased after the addition of fresh medium at pH 6.0, 7.0, or 8.0, consistent with previous findings of Bhattacharjee *et al*. However, a significant decrease in biomass occurred when the wild-type biofilm was incubated in fresh medium at pH 5.0. Biomass of the  $\Delta 1718-1719$  strain was significantly less than that of the wild-type at pH 5.0 and 6.0, but was restored to wild-type levels when the deletion strain was complemented with a function copy of the TA system. Biomass of the  $\Delta 1194-1195$  strain did not differ significantly from wild-type; however, complementation of the strain resulted in a significant increase ( $P \leq 0.01$ ) in biofilm biomass relative to the wild-type at pH 5.0 and pH 6.0, possibly arising from the presence of the TA system in multiple copy in the complemented strain. Together, these results suggest that the putative TA systems may contribute to the persistence of *A. actinomycetemcomitans* in mature biofilms exposed to acidic conditions.



**Figure 13A-B. 72 hour biofilms are sensitive to pH stress.** Biofilms were allowed to grow for 72 hours before media was refed with fresh BHI at pH 5.0 to pH 8.0. Biomass was quantified after staining with crystal violet. Control is a biofilm that was not refed with fresh medium. Asterisks indicate conditions where biofilm biomass of the mutant strain was significantly reduced ( $P \leq 0.05$ ) relative to the wild-type strain and restored to near wild-type levels after complementation. Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider *et al* 2018).

## CHAPTER FIVE: PUTATIVE TOXIN/ANTI-TOXIN SYSTEMS INHIBIT TRANSLATION AND FUNCTION AS AN ENDORIBONUCLEASE

### Introduction

Type II TA systems encode two proteins, the toxin and the anti-toxin. The toxin has a biologically relevant function that is necessary for the survival of the cell under environmental stress. The two systems that have been chosen for further analysis, D11S\_1194-1195 and D11S\_1718-1719 both belong to the RelBE-family of TA systems. Goltfredsen *et al* first identified *relEB* has a toxin/anti-toxin gene family where the induction of the RelE protein was resulted in an inhibition of growth that was prevented in the presence of the RelB (Goltfredsen *et al* 1998). Since then, the *relEB* has been shown to be a type II toxin/anti-toxin system where the RelE protein is a toxin that binds to the bacterial ribosomes to cleave mRNA in A-site of the ribosome in a sequence specific manner (Galvani *et al* 2001, Pedersen *et al* 2003). RelEB has specifically been implicated in apoptosis in human cells, adaption of pathogens to macrophages, antibiotic resistance, and persister cell formation (Yamamoto *et al* 2002, Korch *et al* 2009, Singh *et al* 2010, Tashiro *et al* 2012). Interestingly, the anti-toxin, RelB, has been shown to form a dimer is able to bind to the operator region of its own operon to repress its own expression and this autorepression is enhanced in the presence of the toxin protein (Li *et al* 2008, Overgaard *et al* 2009).

RelEB-like systems have been shown to differentially respond to environmental stressors, such as amino acid starvation, glucose starvation, and oxidative stress (Averina *et al* 2015, Christensen-Dalsgaard *et al* 2010, Yang *et al* 2012, Marsan *et al* 2017). Based on the real-time PCR data shown previously, two of the RelEB-like homologs were able to respond acidic pH stress. Deletion of D11S\_1718-1719 results in a more rapid loss of cell metabolic activity after cells enter stationary phase, suggesting that this TA system plays an important role in maintaining cellular fitness during stationary phase; deletion of D11S\_1718-1719 also resulted in a loss of biomass of mature biofilms when exposed to acidic stress conditions. To determine if these putative type II TA systems act at the level of translation, as many other RelBE TA systems do, and function as endoribonucleases, the functional properties of purified proteins representing full-length toxin and anti-proteins were examined.

## **Results**

### **Inducible expression of putative TA system**

To determine if the genes of interest truly encode TA systems, expression of the proteins by an inducible plasmid was carried out. The toxin only and anti-toxin only were cloned separately into pQE60, an inducible vector for expression of proteins in *E.coli* that produces proteins containing a C-terminal hexa-histidine fusion tag.

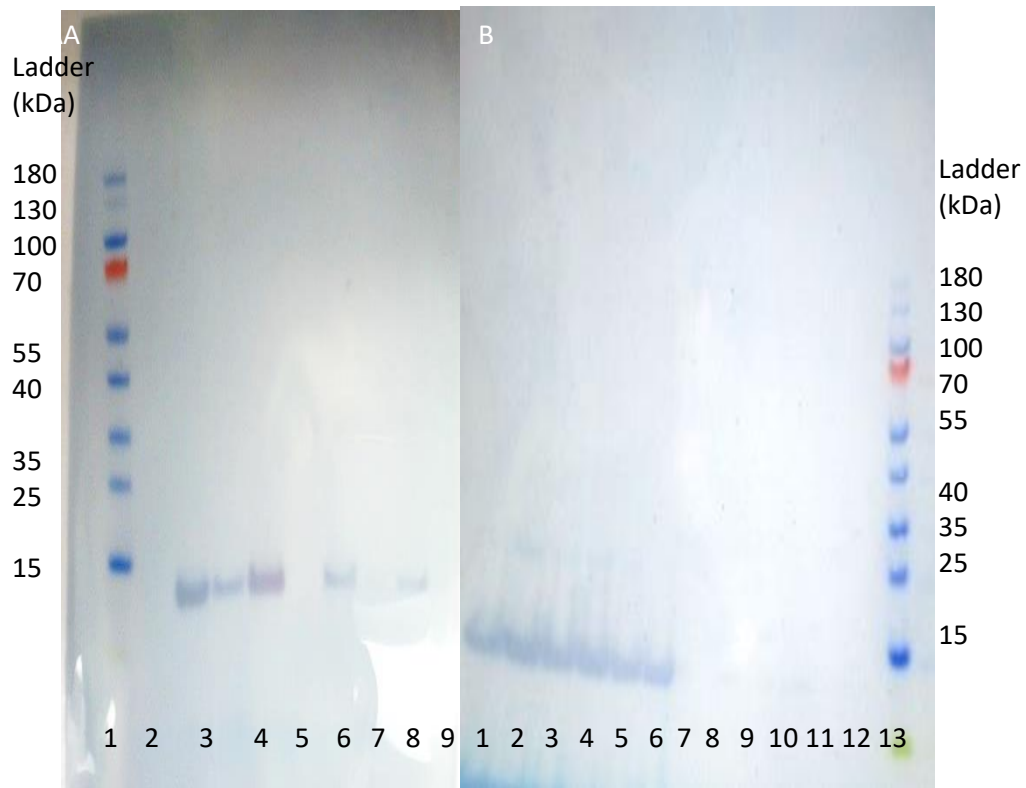
Initial experiments to express the TA system proteins showed that the tagged proteins were present in the insoluble fraction after cell lysis, but not likely in an inclusion body since it could be made partially soluble in low molarity solutions of urea (Figure 14A). There are many protocols suggesting a variety of methods to increase

solubility without the need to use a denaturant, including using a lower temperature for induction of expression, using more sonication buffer during lysis, and altering the buffer composition (i.e. increase salt concentration). Dialysis can be a long, difficult process to refold protein. While many companies offer membranes with an appropriate molecular weight cut off (MWCO), small proteins may be susceptible to degradation during this process, so other non-denaturing methods were considered and tested.

The most successful method to drive protein into the soluble fraction was to use a two-step sonication. Using a standard sonication buffer (50 mM phosphate, pH 7.8, 300 mM NaCl), the samples were sonicated and then pelleted. The supernatant was removed into a clean tube for further use and the pellet was suspended in fresh sonication buffer. The pellet was sonicated for a second time and pelleted again. Most of the tagged protein was present in the soluble fraction (Figure 14B) using this method.

The soluble protein obtained through this method did not bind to the metal affinity agarose beads commonly used to purify histidine tagged proteins. All of the His-tagged protein eluted in the initial flow through after allowing time for the protein to bind to the beads (data not shown). One explanation for this is that the His-tag is sterically shielded from interacting with the affinity resin. Since sonication and purification worked under denaturing conditions, samples were sonicated in protein buffer containing 0.1% SDS. The SDS denatures the proteins and this percentage is compatible with the affinity purification procedure. To verify this was effective, cultures of *E. coli* were induced at 37°C until late exponential phase. Cells were harvested and the pellet was suspended in the denaturing sonication buffer for lysis. The lysate was centrifuged to remove insoluble debris and the supernatant was purified using the cobalt agarose resin. Figure 15 shows

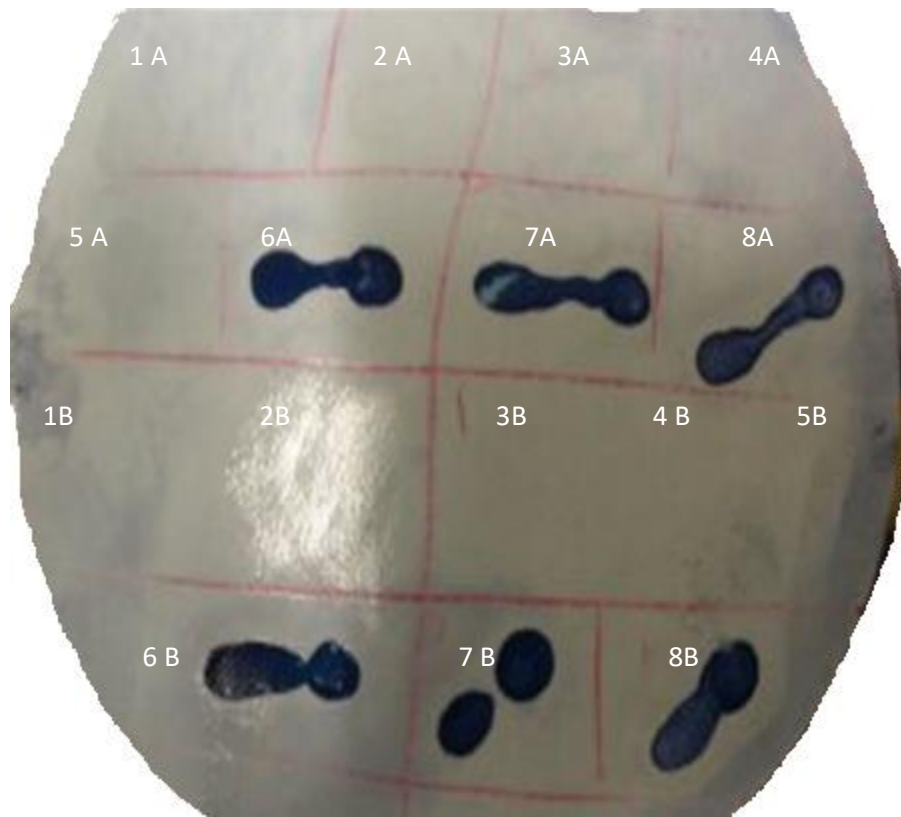
two representative dot blots of the purification process. Samples were refolded as described using 2-MPD and then samples were concentrated. Elution samples were analyzed on Bis-Tris SDS-PAGE gels (Figure 16).



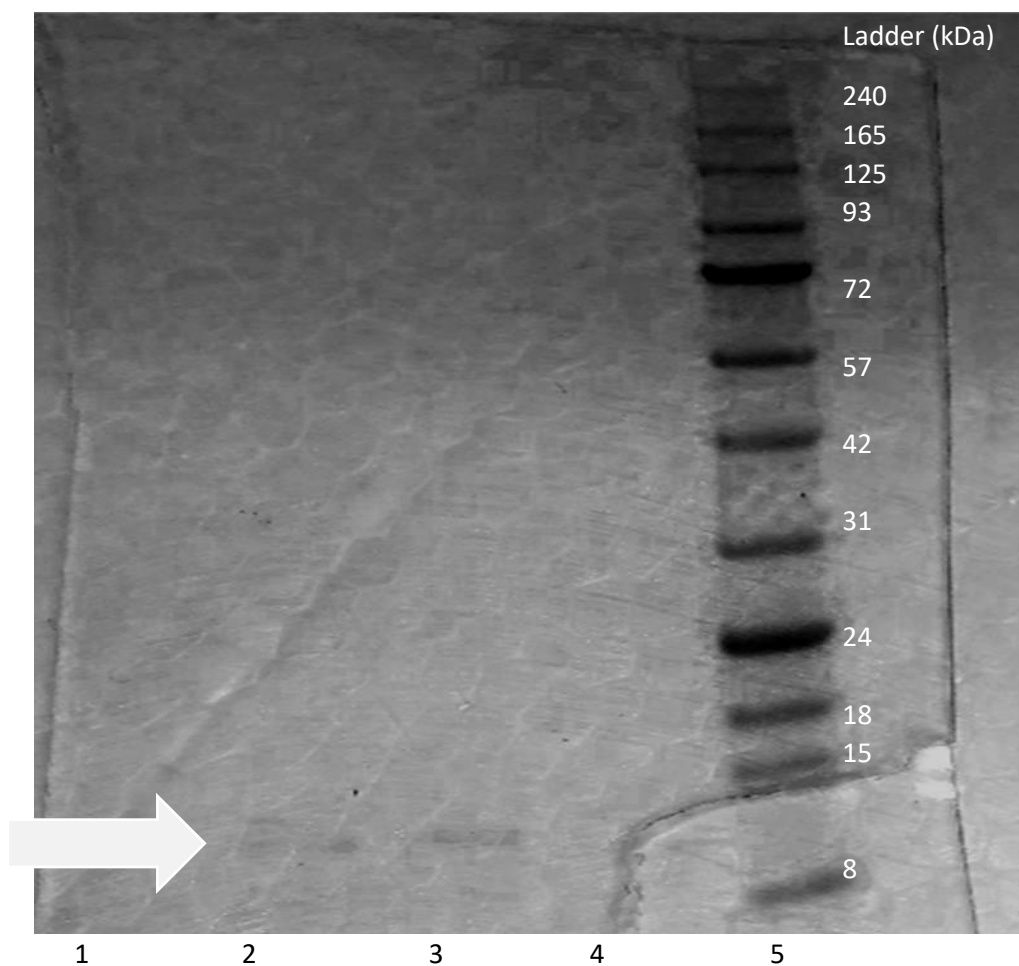
**Figure 14A-B. Putative TA systems can be expressed in an inducible plasmid.**

Western blot of crude protein extract following Bis-Tris SDS-PAGE. A) Crude protein extract following lysis with sonication. Lanes 1 – Ladder, 2-1194 soluble, 3- 1194 insoluble, 4-1194 4M Urea soluble, 5-1194 4M Urea insoluble, 6-1719 soluble, 7-1719 insoluble, 8-1719 4M urea soluble, 9-1719 4M Urea insoluble B) Crude protein extract following lysis with two separate sonication steps. 1-3: 1194 soluble, 4-6: 1719 soluble, 7-9: 1194 insoluble, 10-12: 1719 insoluble, 13- Ladder





**Figure 15. Dot Blot after purification on agarose beads.** Representative dot blot of 1194 (A) and 1195 (B) after SDS denaturation. SDS denatured proteins were purified using cobalt agarose beads. Each square represents a sample of fluid collected during the purification process. 1: Flow-through after initial binding period; 2: Wash Step #1 – 0 mM imidazole; 3: Wash Step #2 – 0 mM imidazole; 4: Wash Step #3 – 0 mM imidazole; 5: Elution #1 – 10 mM imidazole; 6: Elution #2 – 100 mM imidazole; 7: Elution #3 – 200 mM imidazole; 8: Elution #4 – 400 mM imidazole



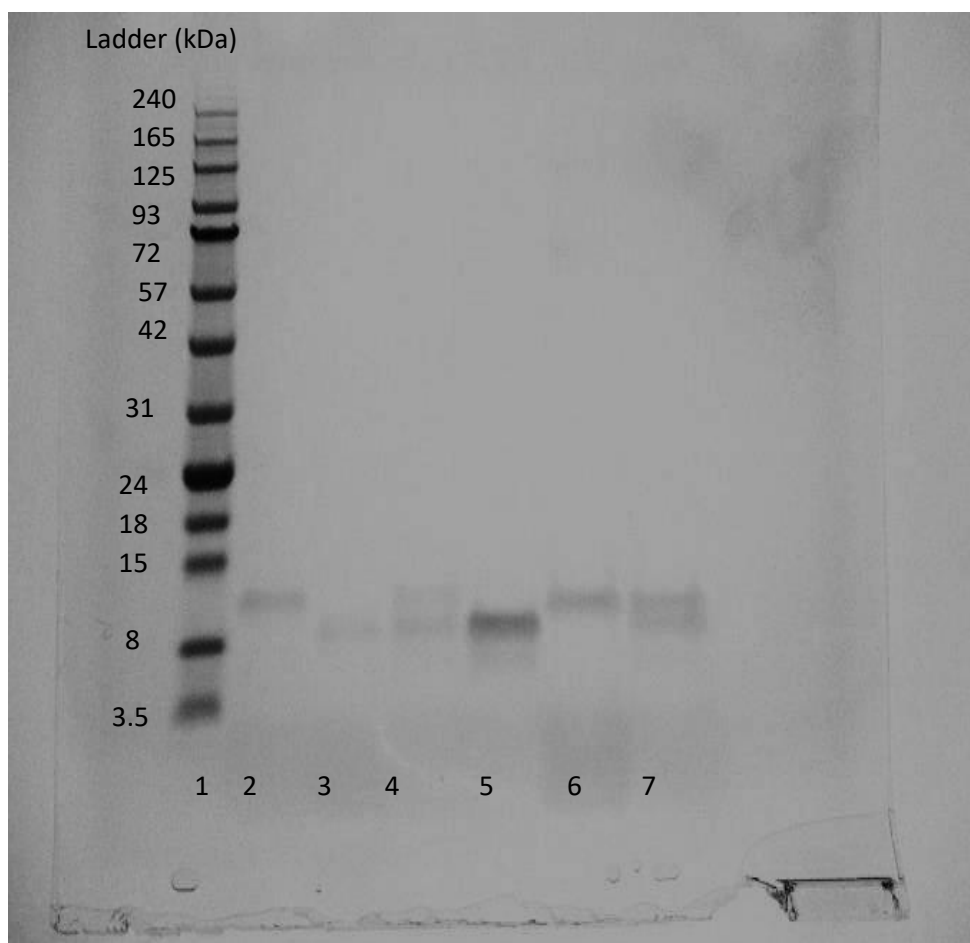
**Figure 16. Toxin proteins after refolding and concentration.** Lane 1 is empty, Lane 2 is 1194, Lane 3 is 1719, Lane 4 is empty and Lane 5 is the Ladder. Representative SDS-PAGE gel to show that soluble protein remained after purification, refolding, and desalting. Arrow indicates where the protein of interest is located.

**Putative toxins inhibit protein synthesis and degrade mRNA:**

RelE-like proteins are able to inhibit translation by cleaving mRNA. To determine if the putative toxins of interest act in a similar manner, the ability of the toxins to degrade mRNA was characterized. Initial tests (data not shown) showed that the purified protein obtained from expression in *E. coli* had RNase contamination that was not fully inhibited by traditional inhibitors. To ensure RNase free protein, synthetic peptides were produced by Biosynthesis, Inc. and were analyzed for purity by gel electrophoresis (Figure 17).

These peptides were tested for inhibition of translation using the PURExpress In Vitro Protein Synthesis system and a control plasmid that expresses mRNA encoding dihydrofolate reductase (DHFR). Synthetic peptide was added at 200 ng to the translation reaction (or 100 ng of each when mixtures were analyzed). The reaction was allowed to incubate for two hours before being analyzed on a Tris-Glycine SDS-PAGE gel (Figure 18). As shown in Figure 18, DHFR (indicated by a white arrow in Lanes 1 and 8) is readily produced when the control translation reaction was carried out in the absence of synthetic peptide. In contrast, no DHFR is produced when the reaction is conducted in the presence of the synthetic 1194 toxin (Lane 2). DHFR production is unaffected by the synthetic 1195 anti-toxin peptide (Lane 3) or by the presence of equal amounts of both the synthetic 1194 toxin and 1195 anti-toxin (Lane 4). This result indicates that the synthetic 1194 peptide functions as a toxin that inhibits DHFR translation and that the 1195 protein represents its corresponding anti-toxin that is capable of preventing toxin activity when applied together.

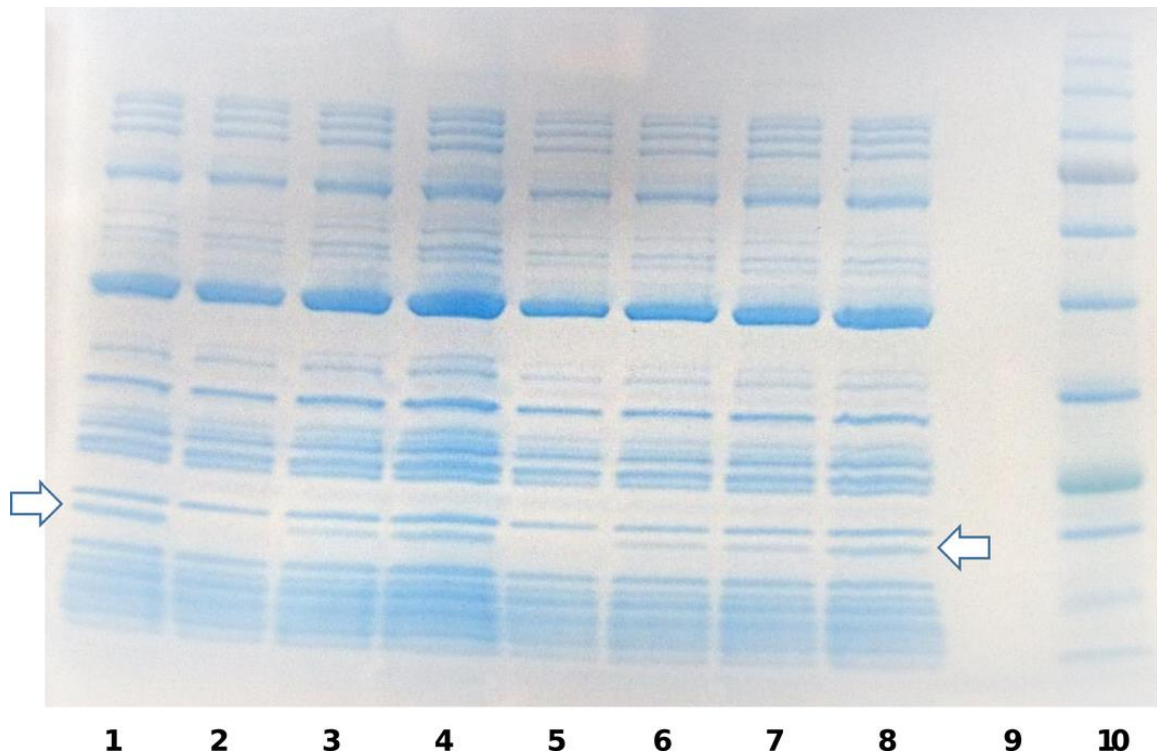
Similarly, synthetic 1718 protein prevents translation of DHFR (Lane 5) whereas the 1719 peptide or an equal mixture of 1718 and 1719 proteins have no effect on DHFR production (Lanes 6 and 7, respectively). This result was unexpected since from the structural organization of many *RelBE* TA operons, the open reading frame encoding the 1719 protein would be predicted to encode the toxin component. Our results clearly show that 1719 does not inhibit DHFR translation and thus functions as an anti-toxin and that the 1718 peptide represents the toxin in this TA system.



**Figure 17. Synthesized peptides are the correct size for the proteins of interest.**

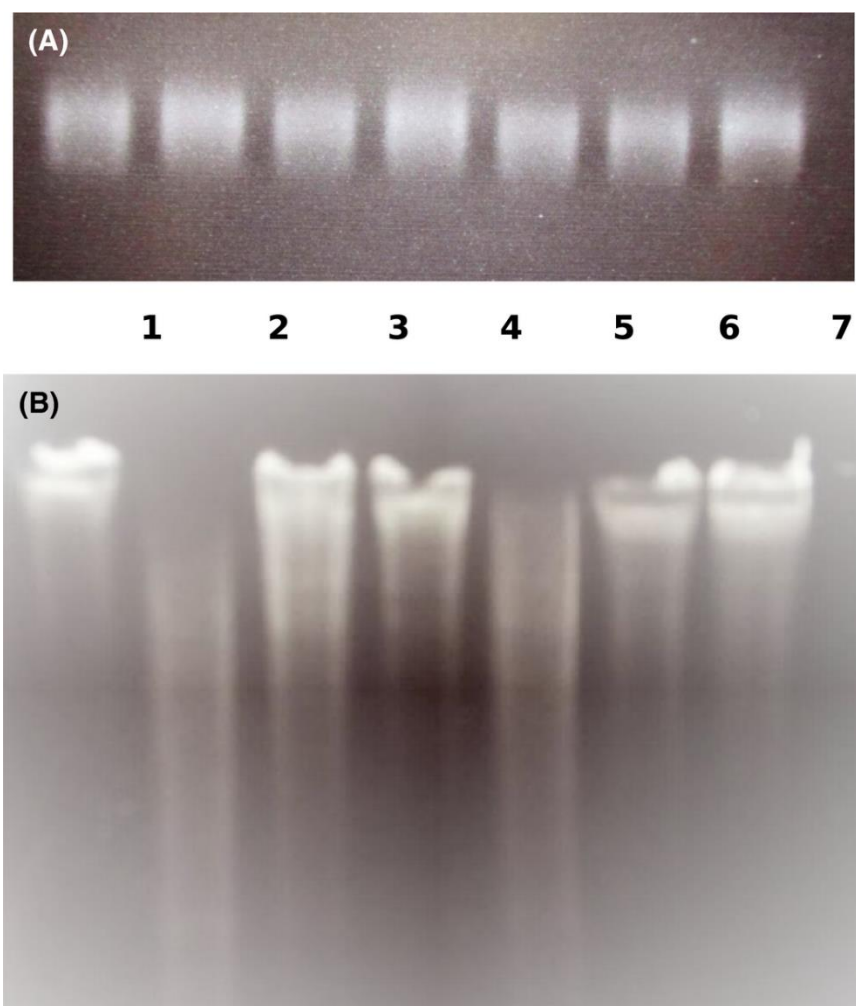
Peptides were dissolved in protein buffer containing 0.01% TFA. Samples need the TFA to go completely into solution. Samples were analyzed with a Bis-Tris SDS-PAGE gel.

Lane 1 – Ladder, Lane 2- 1194, Lane 3-1195, Lane 4 – 1194/1195 together, Lane 5- 1718, Lane 6- 1719, Lane 7 – 1718/1719 together



**Figure 18. *In vitro* translation of DHFR in the presence of synthetic peptides.** Tris-Glycine SDS-PAGE gel of *in vitro* translation samples that were incubated with the toxin or anti-toxin proteins of the TA system, either alone or in combination. Lane 1 – positive control (no inhibition), Lane 2 – 1194 only, Lane 3 – 1195 only, Lane 4- 1194/1195 together, Lane 5 – 1718 only, Lane 6 – 1719 only, Lane 7 – 1718/1719 together, Lane 8 – Positive Control, Lane 9 – empty, Lane 10 – ladder. Arrow marks the positive control protein. Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider et al 2018).

To examine the mechanism of translation inhibition, we next determined if the toxin proteins function as a ribosome dependent ribonuclease. To accomplish this, purified ribosomes were mixed with bacteriophage MS2 RNA (MS2 contains a positive-sense, single stranded RNA genome), and synthetic toxin and/or anti-toxin proteins, incubated at 37<sup>0</sup>C for 25 minutes and electrophoresed in a TBE-Urea gel. As shown in Lane 1 of Figure 19A, MS2 RNA exhibits only minor degradation in the absence of ribosomes and synthetic protein. In contrast, Figure 19B shows that MS2 RNA is significantly degraded in the presence of ribosomes and synthetic 1194 peptide (Lane 2) but not by synthetic 1195 or an equimolar mixture of 1194 and 1195 peptides (Lanes 3 and 4, respectively). Similarly, addition of ribosomes and synthetic 1718 results in MS2 degradation (Lane 5) whereas little degradation is observed in the presence of 1719 or a mixture of 1718 and 1719 proteins (Lanes 6 and 7, respectively). These results are consistent with the translation inhibition results and strongly suggest that the 1194 and 1718 toxins function as ribosome dependent endoribonucleases, which are inhibited by the 1195 and 1719 anti-toxin proteins.



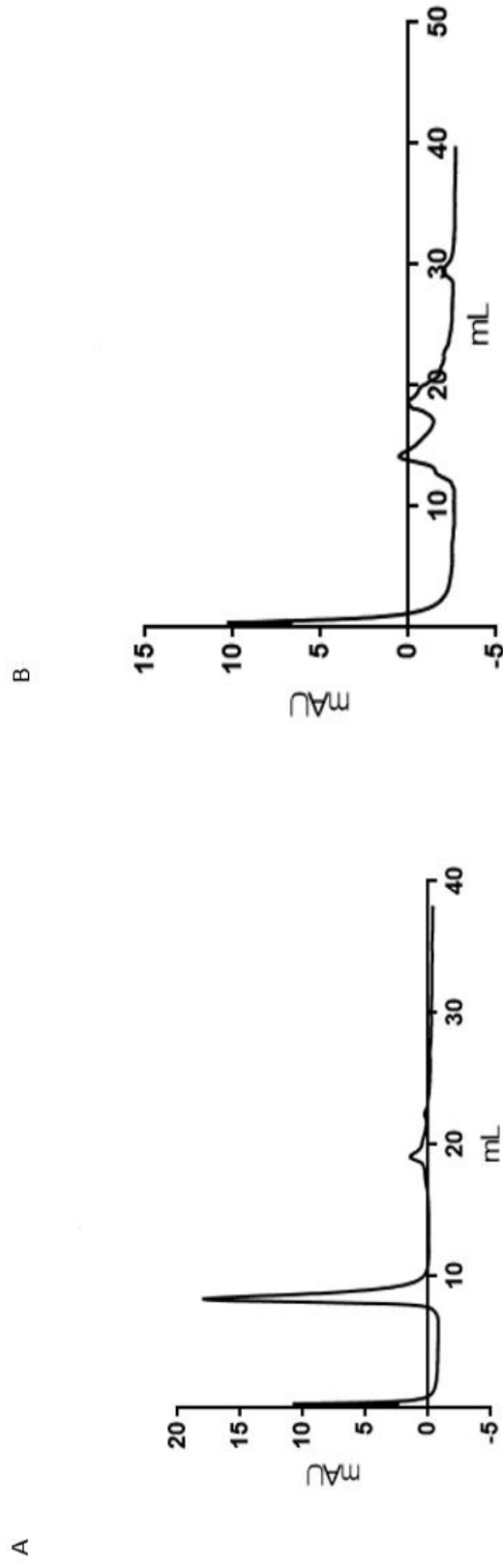
**Figure 19A-B. MS2 RNA degradation occurs in the presence of ribosomes.** RNA samples were incubated in the presence or absence of ribosomes and were analyzed on a TBE-Urea gel stained with ethidium bromide. Lane 1: Control – no toxin or anti-toxin proteins; Lane 2: D11S\_1194 toxin only; Lane 3: D11S\_1195 anti-toxin only; Lane 4: equal mixture of D11S\_1194 and 1195; Lane 5: D11S\_1718 toxin only; Lane 6: D11S\_1719 anti-toxin only; Lane 7: equal mixture of D11S\_1718 and 1719. Copyright permission to reuse granted by the Creative Commons Attribution License (Schneider et al 2018).



**Putative toxins and associated anti-toxins interact and bind to each other:**

An important aspect of the TA system is that the type II TA systems are composed of two small proteins in which the anti-toxin inhibits the activity of the toxin by directly binding to the toxin. To further demonstrate this interaction, the proteins were analyzed by FPLC. The peptides were solubilized the day of the experiments by suspending the peptide in HPLC grade PBS with 1% TFA and mixed before loading. The column was standardized using a variety of markers before the protein samples were loaded on to the column (data not shown). There is a large peak at 10 mL, which corresponds to the size of the individual peptides. However, there is a second peak that is observed at 20mL, which represents the size if protein or protein complex with a molecular weight 2-fold greater than the monomer (Figure 20A). Since both proteins in the TA system are approximately the same size, this peak likely represents a dimer of toxin and anti-toxin. The size of this second peak is small, suggesting that under these conditions, dimer formation may not be favored.

The D11S\_1718-1719 mix shows three peaks: one near where each monomer peptides elute, and a third peak that represents the proteins binding (Figure 20B). As seen with the D11S-1194-1195 system, the peak that corresponds to a complex appears to indicate a weak interaction between the two proteins under these conditions. The presence of three peaks and the low mAU readings in the D11S\_1718-1719 are both likely to do incomplete solubility of the peptides in the solutions needed for the FPLC.



**Figure 20A-B. FPLC results for peptide mixtures.** Purified, synthesized peptides were analyzed for interactions using FPLC.

Peptides were dissolved in HPLC grade PBS buffer containing 1% TFA. A: equal concentrations of 1194 and 1195 mixed together for analysis. B: equal concentrations of 1718 and 1719 mixed together for analysis. The smaller peak for each pair indicates where a protein complex would appear.

## CHAPTER SIX: EVALUATION OF PROTEOME UNDER STRESS

### **Introduction**

As previously shown, the two TA systems are ribosome-dependent endoribonucleases that likely cleaves mRNA to inhibit protein translation. Many examples of type II TA toxins show that recognition of mRNA is sequence specific and that these sequences are found throughout most of the genome. Since many bacteria contain a multiple type II TA systems that can differentially respond to environmental cues, it is likely that different proteins are required to adapt to the local environment. The two type II TA systems of interest in this study are from the same family of systems and they respond to some of the same environmental stressors in the similar manner, suggesting that they may affect the proteome in a similar manner under certain stress conditions.

Cultures of wild-type and both isogenic deletion mutants were grown to mid-log phase in CDM and then gently pelleted. The medium was decanted and the pellet was suspended in fresh CDM at either pH 7.0 or pH 5.0. The cultures were then allowed to grow for four hours or two doubling periods. Cultures were once again gently pelleted and suspended in a protein lysis buffer containing 8M Urea.

Samples were sent to the Proteomics core at the University of Louisville for analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an LTQ-Orbitrap. Briefly, the denatured proteins were processed within the core facility by

trypsin digestion to form smaller peptides. Peptides were first separated using liquid chromatography. After elution, the samples were then subjected to an ionization source and the particles were analyzed by mass spectroscopy. The LTQ-Orbitrap utilizes a quadrupole to process the charged particles. The first quadrupole allows certain sized particles through into the collision cell. Here, the particles are further fragmented before being sent to another quadrupole for further analysis. After the entire sample has been processed, software is able to identify the peptide fragments after comparison to the protein database and to quantitate the proteins that were present in the sample. Fold change was determined by comparing the number of times a specific protein was identified within wild-type to the number of times it was found in a mutant.

## **Results**

### **$\Delta$ 1194-1195 Mutant vs Wild-Type**

The  $\Delta$ 1194-1195 mutant had 58 proteins that were differentially represented when compared to wild-type: 43 were over-represented and 15 were under-represented (Table 11A and 11B). Of the 43 proteins that were over-represented, four were not detected within the wild-type sample. The majority of the over-represented proteins were involved in metabolism: 25% are involved in amino acid metabolism, and 25% are involved in nucleotide metabolism (Figure 21A-B). Proteins involved in virulence and transport across the membrane are the next two most common groups of proteins over-represented (19% each), followed by proteins involved in membrane formation (12%). Two of the proteins identified have no known function at this time. Since the toxins of these TA systems work by acting as endoribonucleases, it can be suggested that the activation of

the toxin results in the degradation of ribosome-associated mRNA in the wild type strain, which results in a decrease in translation of specific gene products. Our results suggest that the D11S\_1194-1195 TA system, under acidic stress, targets the mRNAs encoding the proteins identified above to enable survival.

On the other hand, there are 15 proteins that are under-represented in the mutant when compared to wild-type. Seven of the 15 proteins that were under-represented were not detected within the mutant. As with the over-represented proteins, the majority of the proteins affected are involved in metabolism; two of which are involved in the synthesis of molecules that are secreted (Figure 22A-B). Proteins involved in transcription are the second most affected by the absence of the D11S\_1194-1195 TA system. There are a variety of reasons that these proteins could be under-represented, including gene regulation of the identified proteins by the anti-toxin or the toxin/anti-toxin complex, or the activation of other TA systems to compensate for the loss in the mutant.

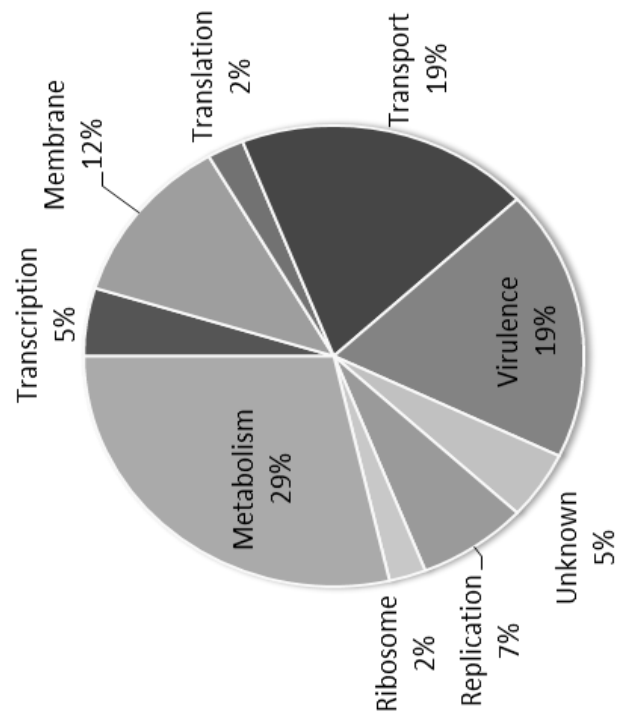
<b>Table 11A. Proteins over-represented in Δ1194-1195 vs Wild-type</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
AsnC	tr C9R706 C9R706_AGGAD	2.048150677
N-acetylmuramoyl-L-alanine amidase	tr C9R3G4 C9R3G4_AGGAD	2.04816057
tRNA-specific 2-thiouridylase MnmA	tr C9R2L0 C9R2L0_AGGAD	2.048171276
Short-chain dehydrogenase of various substrate specificities	tr C9R2U6 C9R2U6_AGGAD	2.048178364
Cytochrome D ubiquinol oxidase, subunit II	tr C9R5W5 C9R5W5_AGGAD	2.048183128
Uncharacterized protein	tr C9R2Q7 C9R2Q7_AGGAD	2.048186174
Putative GTP cyclohydrolase 1 type 2	tr C9R3S8 C9R3S8_AGGAD	2.048186591
HTH-type transcriptional regulator MalT	tr C9R480 C9R480_AGGAD	2.0481882
Conserved ABC-type transport system protein, ATPase component	tr C9R4W3 C9R4W3_AGGAD	2.048200208
MdlB protein	tr C9R1V9 C9R1V9_AGGAD	2.048200995
Molybdopterin biosynthesis MoeA protein	tr C9R3L9 C9R3L9_AGGAD	2.048203593
Tellurite resistance protein TehB	tr C9R481 C9R481_AGGAD	2.048204445
D-ribose transporter subunit RbsB	tr C9R6M8 C9R6M8_AGGAD	2.048205478
Uridine kinase	tr C9R397 C9R397_AGGAD	2.048205996
Lipoprotein, putative	tr C9R5G6 C9R5G6_AGGAD	2.048207305
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	tr C9R4D5 C9R4D5_AGGAD	2.048207976
Sodium/proline symporter	tr C9R3A6 C9R3A6_AGGAD	2.048213425
Anaerobic C4-dicarboxylate transporter	tr C9R1M8 C9R1M8_AGGAD	2.048213537
Glycerol-3-phosphate transporter (G-3-P transporter) (G-3-P permease)	tr C9R5Y5 C9R5Y5_AGGAD	2.048214244
Succinyl-diaminopimelate desuccinylase	tr C9R308 C9R308_AGGAD	2.048215854
Coproporphyrinogen-III oxidase	tr C9R6L7 C9R6L7_AGGAD	2.048220049
ABC transporter, ATP-binding protein	tr C9R4W0 C9R4W0_AGGAD	2.048223512
Integration host factor subunit alpha	tr C9R4L0 C9R4L0_AGGAD	2.048225273
AcrA protein	tr C9R4I9 C9R4I9_AGGAD	2.048228579
Alpha/beta superfamily hydrolase	tr C9R4E8 C9R4E8_AGGAD	2.048229416
Transport associated protein 4	tr D0UIW9 D0UIW9_AGGAD	2.04824494
Lipoprotein, putative	tr C9R5U7 C9R5U7_AGGAD	2.048252468

<b>Table 11A. Proteins over-represented in Δ 1194-1195 vs Wild-type, Continued</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
Opacity-associated protein OapB	tr C9R653 C9R653_AGGAD	2.048259203
Biotin synthase	tr C9R407 C9R407_AGGAD	2.048303541
50S ribosomal protein L28	tr C9R211 C9R211_AGGAD	2.048311174
Shikimate 5-dehydrogenase	tr C9R1M6 C9R1M6_AGGAD	3.072280136
Uncharacterized protein	tr C9R586 C9R586_AGGAD	3.0722832
Hemolysin	tr C9R260 C9R260_AGGAD	3.072302014
Conserved outer membrane protein	tr C9R2Q8 C9R2Q8_AGGAD	3.072350744
Inosine-5'-monophosphate dehydrogenase	tr C9R3E7 C9R3E7_AGGAD	3.072384397
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	tr C9R2Z2 C9R2Z2_AGGAD	3.072384397
Putative integrase	tr C9R439 C9R439_AGGAD	3.072408089
Adenylosuccinate lyase	tr C9R251 C9R251_AGGAD	3.584469387
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	tr C9R5N0 C9R5N0_AGGAD	4.09647951
PotD protein	tr C9R463 C9R463_AGGAD	Not Detected in WT
Fructose-1,6-bisphosphatase class 1	tr C9R630 C9R630_AGGAD	Not Detected in WT
Lipopolysaccharide assembly protein B	tr C9R5A2 C9R5A2_AGGAD	Not Detected in WT
Nodulation efficiency protein D (NfeD)	tr C9R5E8 C9R5E8_AGGAD	Not Detected in WT

<b>Table 11B. Proteins underrepresented in <math>\Delta</math>1194-1195 vs Wild-type</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
Cell division protein ZapA	tr C9R1Y4 C9R1Y4_AGGAD	0.256027213
RNA polymerase sigma factor	tr C9R2V1 C9R2V1_AGGAD	0.341358857
ATP-dependent RNA helicase SrmB	tr C9R7E4 C9R7E4_AGGAD	0.341363892
Methionine import ATP-binding protein MetN	tr C9R779 C9R779_AGGAD	0.341368982
30S ribosomal protein S15	tr C9R6D9 C9R6D9_AGGAD	0.341384509
Outer membrane protein assembly factor BamD	tr C9R1Y6 C9R1Y6_AGGAD	0.409630534
Macrolide export ATP-binding/permease protein MacB	tr C9R4F0 C9R4F0_AGGAD	0.409646276
Ferric uptake regulation protein (Ferric uptake regulator)	tr C9R597 C9R597_AGGAD	0.438899439
Amylovoran biosynthesis glycosyltransferase AmsE	tr C9R5I9 C9R5I9_AGGAD	Not Detected
PEBP family protein	tr C9R782 C9R782_AGGAD	Not Detected
Shikimate 5-dehydrogenase	tr C9R3U7 C9R3U7_AGGAD	Not Detected
DNA-binding protein	tr C9R4U2 C9R4U2_AGGAD	Not Detected
Sugar efflux transporter	tr C9R399 C9R399_AGGAD	Not Detected
UDP-3-O-acylglucosamine N-acyltransferase	tr C9R1I0 C9R1I0_AGGAD	Not Detected
Phosphohistidine phosphatase SixA	tr C9R3E0 C9R3E0_AGGAD	Not Detected

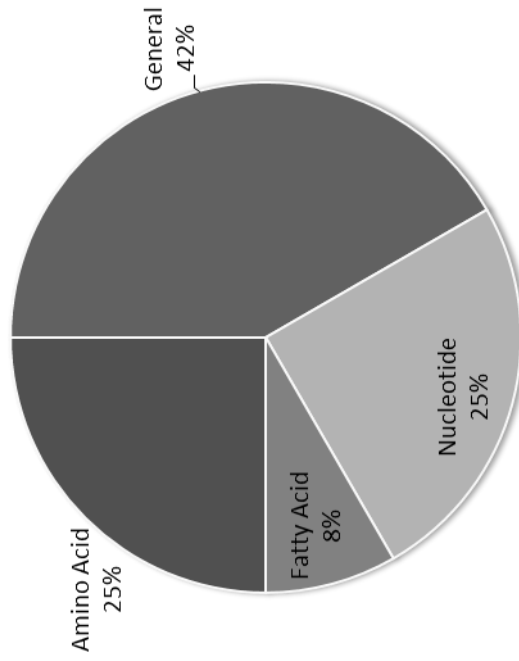


## Proteins Over-Represented In $\Delta$ 1194-1195 vs Wild-type



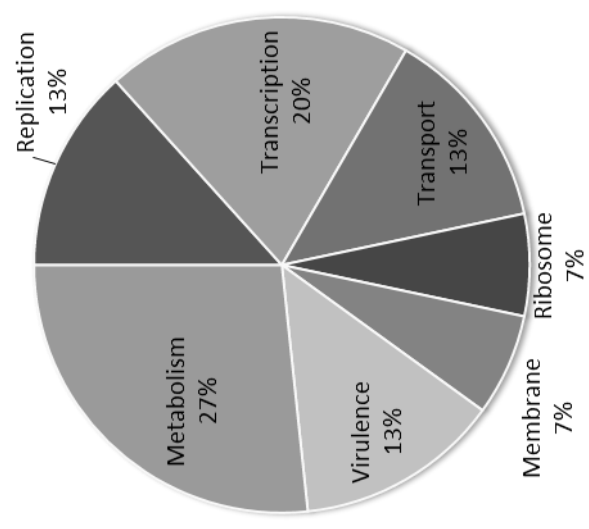
**Figure 21A. Proteins Over-Represented in  $\Delta$ 1194-1195.** The proteins that were identified as being over-represented when compared to wild-type are grouped based on the function of the protein.

## Metabolism Proteins Over-Represented in $\Delta 1194-1195$



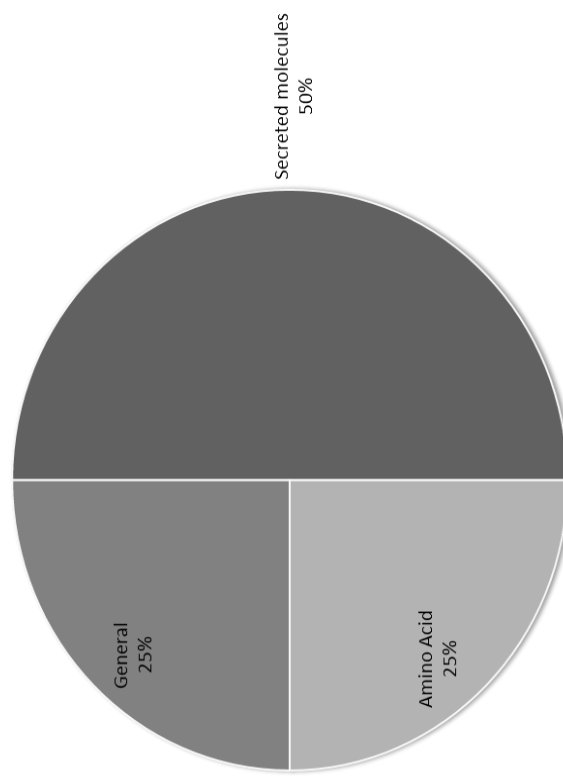
**Figure 21B. Breakdown of the over-represented metabolism proteins in  $\Delta 1194-1195$ .** The proteins identified as being involved in metabolism are further characterized based on the metabolic process involved.

**Proteins Underrepresented In  $\Delta$ 1194-1195 vs  
Wild-type**



**Figure 22A. Proteins underrepresented in  $\Delta$ 1194-1195 vs Wild-Type.** Proteins identified as underrepresented when compared to the wild-type sample grouped by general protein function.

### Metabolism Proteins Underrepresented In $\Delta 1194-1195$



**Figure 22B. Breakdown of the underrepresented metabolism proteins in  $\Delta 1194-1195$ .** The proteins identified as being involved in metabolism are further characterized based on the metabolic process involved.

### **$\Delta$ 1718-1719 vs Mutant**

The  $\Delta$ 1718-1719 isogenic mutant had 92 identified proteins as being differentially represented when compared to the wild-type. Unlike the  $\Delta$ 1194-1195, many of the proteins identified were underrepresented rather than over-represented. Of the 92 proteins, 38 are over-represented and 54 were underrepresented (Table 12A-B). Of the 38 proteins that are over-represented, six were not detected in the wild-type sample. As seen in the  $\Delta$ 1194-1195 mutant, the majority of the over-represented proteins identified are involved in metabolism: 22% involved in nucleotide metabolism and 21% involved in amino acid metabolism (Figure 23A-B). Unique to the  $\Delta$ 1718-1719 mutant, is the involvement of nitrogen metabolism. The protein that was identified is related to the cytochromes c-type protein, NrfB, in *E. coli*. NrfB is a protein that is involved in nitrite reduction and is involved in the electron transport chain under anaerobic conditions. This is followed by proteins involved in virulence (18%), transport across the membrane (11%), membrane formation (8%), and translation (8%). There are also two proteins identified that have no known function at this time. It is highly probable that the activation of the D11S\_1718-1719 TA system results in the degradation of the ribosome-associated mRNA for these proteins to enhance survival. This is also consistent with the real-time PCR data which suggests that the two TA systems respond to some of the same environmental conditions. Importantly, the D11S\_1718-1719 system was responsive to anaerobic stress whereas the D11S\_1194-1195 system was not, which is consistent with the inclusion of nitrogen metabolism proteins seen in the  $\Delta$ 1718-1719 mutant.

The  $\Delta$ 1718-1719 deletion mutant exhibited many more proteins that are underrepresented compared to the over-represented. Of the 54 proteins identified, 20

were present in wild type but were not detected in the mutant sample. These proteins are involved with many different pathways including iron transport, LPS synthesis, cell formation, stationary phase survival, and type IV secretion. The majority of the identified proteins were involved in metabolism: 16% involved in amino acid metabolism and 11% each for nucleotide and carbohydrate metabolism (Figure 24A-B). As seen in the over-represented proteins, we also see the involvement of nitrogen metabolism. This protein is identified as another cytochrome c-type protein, but instead of being involved in the electron transport chain, this protein is involved in the denitrification pathway, which is the reduction of nitrate to dinitrogen, which can in turn act as a terminal acceptor for electron transport in place of oxygen.

The lack of so many metabolism genes, compared to the  $\Delta 1194-1195$ , strain is consistent with the loss of metabolic activity seen over time in stationary phase. Following metabolism, proteins involved in transport across the membrane (13%), virulence (11%), and replication (9%) are the next most identified proteins. Also of interest, 9% of the underrepresented proteins in this strain have no known function. Taken together, these results appear to be consistent with the phenotype seen in mutant's ability to survive stationary phase and resist acidic stress.

<b>Table 12A. Proteins over-represented in Δ1718-1719 vs Wild-Type</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
HTH-type transcriptional regulator MalT	tr C9R480 C9R480_AGGAD	2.33323847
tRNA-specific 2-thiouridylase MnmA	tr C9R2L0 C9R2L0_AGGAD	2.33326575
Cytochrome c nitrite reductase, pentaheme subunit	tr C9R6A6 C9R6A6_AGGAD	2.333270941
Molybdopterin biosynthesis MoeA protein	tr C9R3L9 C9R3L9_AGGAD	2.3332763
Tellurite resistance protein TehB	tr C9R481 C9R481_AGGAD	2.33328287
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	tr C9R4D5 C9R4D5_AGGAD	2.33329127
Mannonate dehydratase	tr C9R506 C9R506_AGGAD	2.33329162
Putative GTP cyclohydrolase 1 type 2	tr C9R3S8 C9R3S8_AGGAD	2.3332931
Anaerobic C4-dicarboxylate transporter	tr C9R1M8 C9R1M8_AGGAD	2.33330228
Conserved ABC-type transport system protein, ATPase component	tr C9R4W3 C9R4W3_AGGAD	2.33330528
Alanine racemase	tr C9R3J0 C9R3J0_AGGAD	2.33330792
7-cyano-7-deazaguanine synthase	tr C9R316 C9R316_AGGAD	2.333308559
Chaperone protein HscA homolog	tr C9R384 C9R384_AGGAD	2.333311488
Methyltransferase domain family	tr C9R693 C9R693_AGGAD	2.333315617
Putative N-acetylmannosamine-6-phosphate 2-epimerase	tr C9R562 C9R562_AGGAD	2.333316888
Trk system potassium uptake protein TrkA (K(+)-uptake protein trkA	tr C9R719 C9R719_AGGAD	2.33331717
Succinyl-diaminopimelate desuccinylase	tr C9R308 C9R308_AGGAD	2.33332003
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	tr C9R5N0 C9R5N0_AGGAD	2.3333477
Guanylate kinase	tr C9R4V0 C9R4V0_AGGAD	2.333348296
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	tr C9R2Z2 C9R2Z2_AGGAD	2.33335056
Phosphohistidine phosphatase SixA	tr C9R3E0 C9R3E0_AGGAD	2.333355213
50S ribosomal protein L31	tr C9R205 C9R205_AGGAD	2.333358038
50S ribosomal protein L28	tr C9R211 C9R211_AGGAD	2.33338839
HemY protein	tr C9R2R4 C9R2R4_AGGAD	2.333406739
ABC transporter, ATP-binding protein	tr C9R4W0 C9R4W0_AGGAD	2.91662946
MdlB protein	tr C9R1V9 C9R1V9_AGGAD	3.49985374

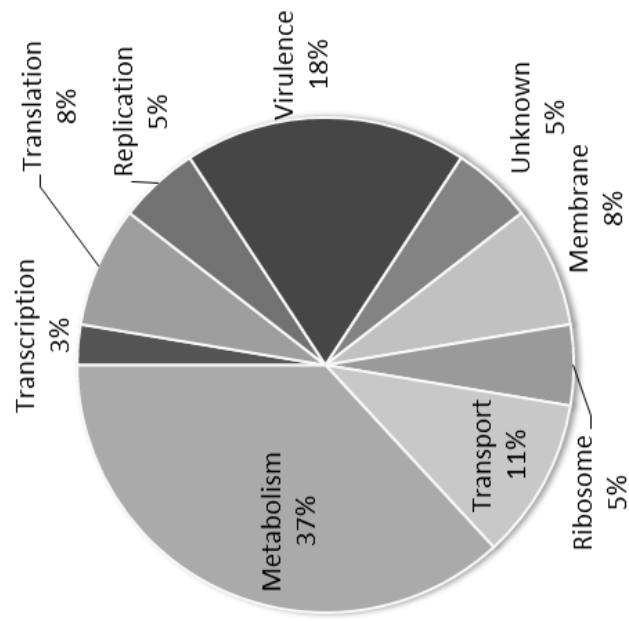
<b>Table 12A. Proteins over-represented in <math>\Delta</math>1718-1719 vs Wild-Type (continued)</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
Uncharacterized protein	tr C9R586 C9R586_AGGAD	3.49990112
Conserved outer membrane protein	tr C9R2Q8 C9R2Q8_AGGAD	3.49996723
Hemolysin	tr C9R260 C9R260_AGGAD	3.49997777
Inosine-5'-monophosphate dehydrogenase	tr C9R3E7 C9R3E7_AGGAD	3.50002583
Heavy metal-binding protein, putative	tr C9R6G6 C9R6G6_AGGAD	3.50003706
Phenylalanine--tRNA ligase alpha subunit	tr C9R4L2 C9R4L2_AGGAD	4.666829217
Capsular polysaccharide synthesis	tr C9R202 C9R202_AGGAD	Not Detected in WT
Excinuclease ABC subunit B	tr C9R1Y9 C9R1Y9_AGGAD	Not Detected in WT
UvrABC system protein B	tr C9R568 C9R568_AGGAD	Not Detected in WT
Fructose-1,6-bisphosphatase class 1	tr C9R630 C9R630_AGGAD	Not Detected in WT
Lipopolysaccharide assembly protein B	tr C9R5A2 C9R5A2_AGGAD	Not Detected in WT
Nodulation efficiency protein D (NfeD)	tr C9R5E8 C9R5E8_AGGAD	Not Detected in WT



<b>Table 12B. Proteins under-represented in Δ1718-1719 vs Wild-Type</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
Macrolide export ATP-binding/permease protein MacB	tr C9R4F0 C9R4F0_AGGAD	0.2333297
UPF0319 protein D11S_1646	tr C9R5C4 C9R5C4_AGGAD	0.233334914
Ribulose-phosphate 3-epimerase	tr C9R1U2 C9R1U2_AGGAD	0.291658268
Cytochrome c-type protein	tr C9R7G9 C9R7G9_AGGAD	0.291662013
ATP-dependent Clp protease ATP-binding subunit ClpX	tr C9R6G1 C9R6G1_AGGAD	0.291667122
UPF0250 protein D11S_2139	tr C9R6Q4 C9R6Q4_AGGAD	0.291669942
Putrescine-binding periplasmic protein	tr C9R1Q1 C9R1Q1_AGGAD	0.3181806
30S ribosomal protein S15	tr C9R6D9 C9R6D9_AGGAD	0.38887493
Glycerol-3-phosphate transporter	tr C9R1W9 C9R1W9_AGGAD	0.388877596
RNA polymerase sigma factor	tr C9R2V1 C9R2V1_AGGAD	0.3888784
Uncharacterized protein	tr C9R1K0 C9R1K0_AGGAD	0.388879949
Opacity-associated protein OapA	tr C9R654 C9R654_AGGAD	0.388880892
Regulator of ribonuclease activity B	tr C9R717 C9R717_AGGAD	0.388880984
Membrane-bound lytic murein transglycosylase A (Murein hydrolase A) (Mlt38)	tr C9R632 C9R632_AGGAD	0.388883054
Putative aldolase YneB	tr C9R4N8 C9R4N8_AGGAD	0.388883164
Aldo/keto reductase	tr C9R2N9 C9R2N9_AGGAD	0.38888338
Uridine phosphorylase	tr C9R707 C9R707_AGGAD	0.388883454
Uncharacterized protein	tr C9R3M9 C9R3M9_AGGAD	0.388883823
Possible AAA+ superfamily ATPase	tr C9R317 C9R317_AGGAD	0.388884928
TadZ	tr C9R2X0 C9R2X0_AGGAD	0.388886689
Chromosomal replication initiation protein	tr C9R6K4 C9R6K4_AGGAD	0.388887077
Phosphatidylserine decarboxylase proenzyme	tr C9R6S9 C9R6S9_AGGAD	0.388887189
Uncharacterized protein	tr D0UIV3 D0UIV3_AGGAD	0.38888734
GTP cyclohydrolase-2	tr C9R5H0 C9R5H0_AGGAD	0.388888038
Cell division protein ZipA homolog	tr C9R520 C9R520_AGGAD	0.388888889
Queuine tRNA-ribosyltransferase	tr C9R5T0 C9R5T0_AGGAD	0.437498099
Outer membrane protein assembly factor BamD	tr C9R1Y6 C9R1Y6_AGGAD	0.46664817
Ribonucleoside-diphosphate reductase	tr C9R3K2 C9R3K2_AGGAD	0.466655995
Phosphate transport regulator	tr C9R5Q5 C9R5Q5_AGGAD	0.466660286

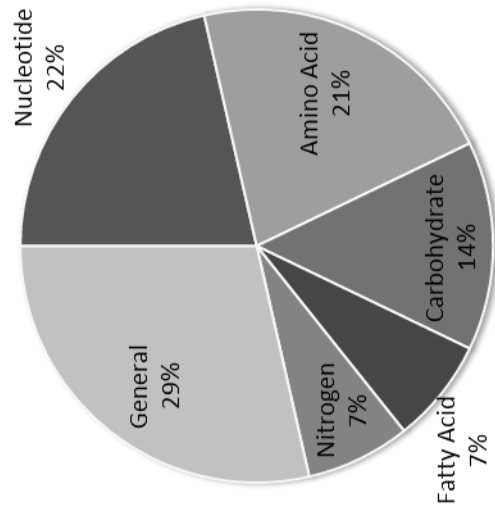
<b>Table 12B. Proteins under-represented in Δ1718-1719 vs Wild-Type (continued)</b>		
<b><u>Gene Name</u></b>	<b><u>Accession Number</u></b>	<b><u>Fold Change</u></b>
tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	tr C9R1N8 C9R1N8_AGGAD	0.466663115
Aspartate--ammonia ligase	tr C9R705 C9R705_AGGAD	0.466668996
Lipoprotein, putative	tr C9R2X8 C9R2X8_AGGAD	0.466673217
30S ribosomal protein S6	tr C9R1W3 C9R1W3_AGGAD	0.466684313
Mannose-specific PTS system protein IID	tr C9R1Q9 C9R1Q9_AGGAD	0.5
Aminotransferase, class-V	tr C9R5E2 C9R5E2_AGGAD	Not Detected
Apolipoprotein N-acyltransferase	tr C9R373 C9R373_AGGAD	Not Detected
Cluster of Binding-protein-dependent transport systems inner membrane component	tr C9R394 C9R394_AGGAD	Not Detected
Cystathionine beta-lyase	tr C9R3C3 C9R3C3_AGGAD	Not Detected
D-3-phosphoglycerate dehydrogenase	tr C9R2I2 C9R2I2_AGGAD	Not Detected
Enoyl-[acyl-carrier-protein] reductase [NADH]	tr C9R3S7 C9R3S7_AGGAD	Not Detected
Iron-sulfur cluster insertion protein ErpA	tr C9R533 C9R533_AGGAD	Not Detected
L-asparaginase, putative	tr C9R6E4 C9R6E4_AGGAD	Not Detected
Long-chain-fatty-acid--CoA ligase	tr C9R5F5 C9R5F5_AGGAD	Not Detected
Mce related protein	tr C9R5N9 C9R5N9_AGGAD	Not Detected
N utilization substance protein B homolog	tr C9R3J3 C9R3J3_AGGAD	Not Detected
Nicotinamide-nucleotide adenylyltransferase	tr C9R2F0 C9R2F0_AGGAD	Not Detected
Possible heptosyltransferase II (Inner core)	tr C9R207 C9R207_AGGAD	Not Detected
Putative transport protein D11S_0239	tr C9R7K3 C9R7K3_AGGAD	Not Detected
DNA-binding protein	tr C9R4U2 C9R4U2_AGGAD	Not Detected
Sugar efflux transporter	tr C9R399 C9R399_AGGAD	Not Detected
UDP-3-O-acylglucosamine N-acyltransferase	tr C9R1I0 C9R1I0_AGGAD	Not Detected
Glycerol-3-phosphate transporter (G-3-P transporter) (G-3-P permease)	tr C9R5Y5 C9R5Y5_AGGAD	Not Detected
Alpha/beta superfamily hydrolase	tr C9R4E8 C9R4E8_AGGAD	Not Detected
Transport associated protein 4	tr D0UIW9 D0UIW9_AGGAD	Not Detected

## Proteins Over-Represented In $\Delta 1718$ -1719 vs Wild-Type



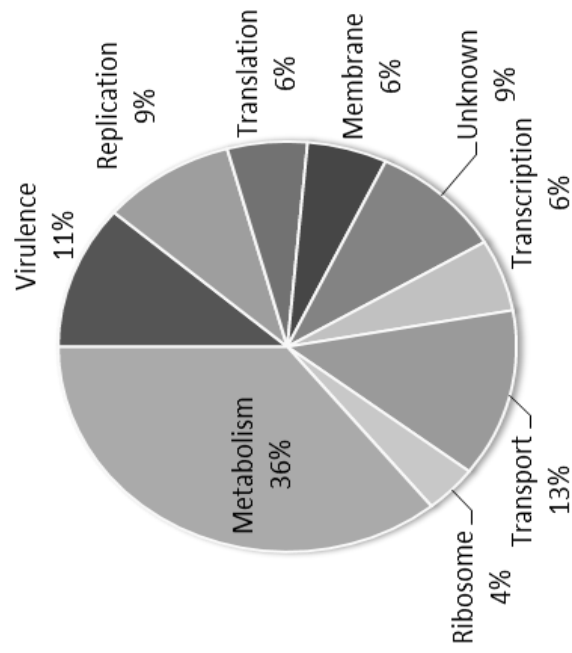
**Figure 23A. Proteins Over-Represented in  $\Delta 1718$ -1719.** The proteins that were identified as being over-represented when compared to wild-type are grouped based on the function of the protein.

## Metabolism Proteins Over-Represented In $\Delta 1718-1719$



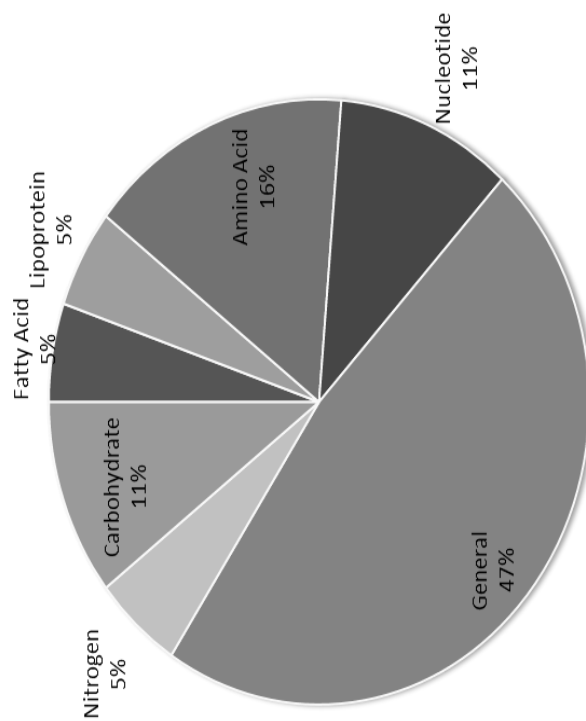
**Figure 23B. Breakdown of the over-represented metabolism proteins in  $\Delta 1718-1719$ .** The proteins identified as being involved in metabolism are further characterized based on the metabolic process involved.

## Proteins Underrepresented In $\Delta 1718-1719$ vs Wild-Type



**Figure 24A. Proteins underrepresented in  $\Delta 1718-1719$ .** The proteins that were identified as being over-represented when compared to wild-type are grouped based on the function of the protein.

## Metabolism Proteins Underrepresented In Δ1718-1719



**Figure 24B. Breakdown of the underrepresented metabolism proteins in Δ1718-1719.** The proteins identified as being involved in metabolism are further characterized based on the metabolic process involved.

### **$\Delta 1194-1195$ vs $\Delta 1718-1719$**

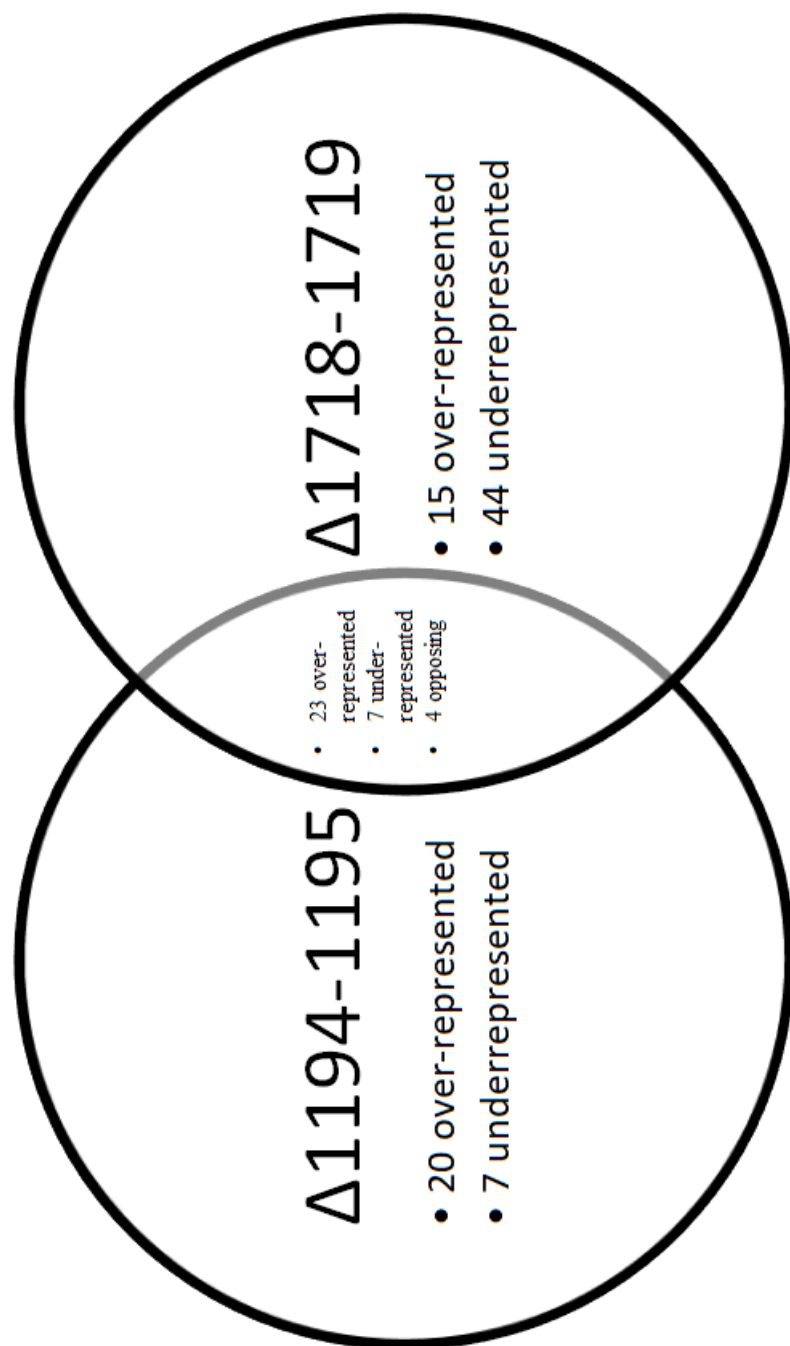
Since the real-time PCR data suggests that some functional redundancy between the two putative TA systems, the proteomics data was also analyzed to compare the two mutant strains to each other (Figure 25). In total, 120 proteins were identified as being differentially represented when compared to wild-type. Of these, 34 were common to both mutant strains: 23 over-represented, 7 underrepresented, and four differentially represented. This data further supports the hypothesis that TA systems in the same family may exhibit some level of functional redundancy. It is also interesting that 59 differentially represented proteins are unique to the  $\Delta 1718-1719$  mutant strain, of which 15 are over-represented and 44 are under-represented relative to the wild type strain. Twenty-seven differentially represented proteins are unique to the  $\Delta 1194-1195$  mutant strain, of which 20 are over-represented and 7 are under-represented relative to the wild type. These results confirm that functional redundancy exists for these TA systems but also clearly suggests that each TA system may also have specific functions, which is consistent with the phenotypic differences seen in these strains.

Looking at just the proteins that are represented in both isogenic deletion strains, proteins involved in metabolism and virulence are the most prevalent, and represent more than 50% of the differentially represented proteins when compared to wild-type. Of the metabolism proteins identified, amino acid metabolism was the most common pathway affected. It is also interesting to point out that all of the proteins that are found in both mutant strains have known functions.

Examining the unique proteins to each strain, the majority (42%) of the proteins identified in the  $\Delta 1718-1719$  strain are involved in metabolism (Figure 27A-B). The

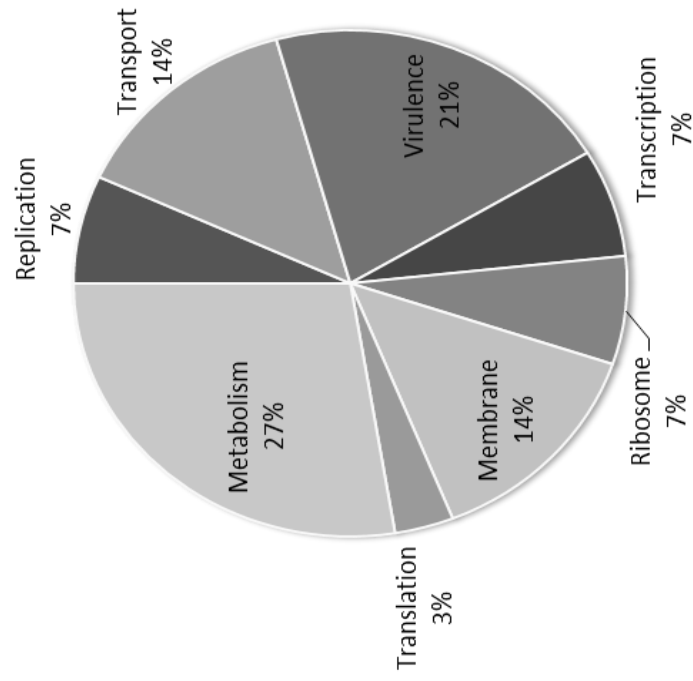
majority of these metabolism proteins are involved in various general pathways, but nucleotide metabolism is the most common affected. The next most prevalent group is proteins with no known function (12%), followed by transport across the membrane and virulence (9% each). While metabolism is still the majority of identified proteins in the  $\Delta 1194-1195$  strain, transport across the membrane is the next largest group, followed by proteins involved in transcription and membrane formation (Figure 28A-B). The majority of the metabolism proteins in the  $\Delta 1194-1195$  strain are general enzymes, there's only two other groups identified: amino acid and nucleotide metabolism. Both of which are equally represented.





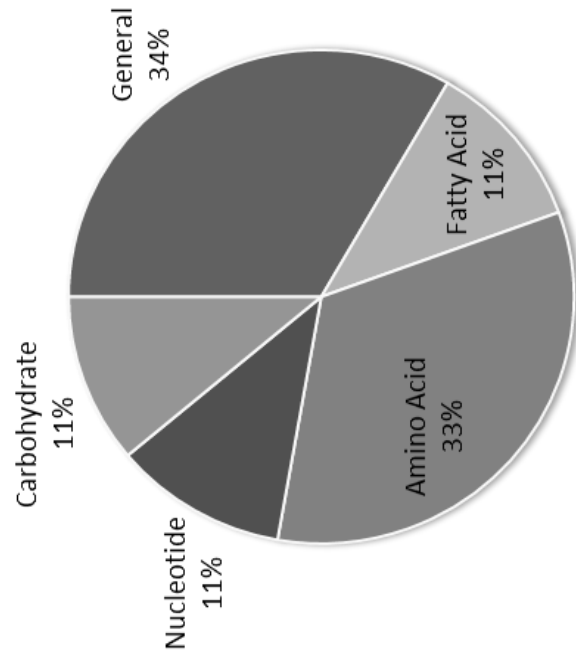
**Figure 25. Comparison of proteomics data between two isogenic mutants.** Total proteins identified as being differentially expressed were compared to determine similarities between the two isogenic mutants.

## Identified Proteins In Common Between Two Isogenic Mutants



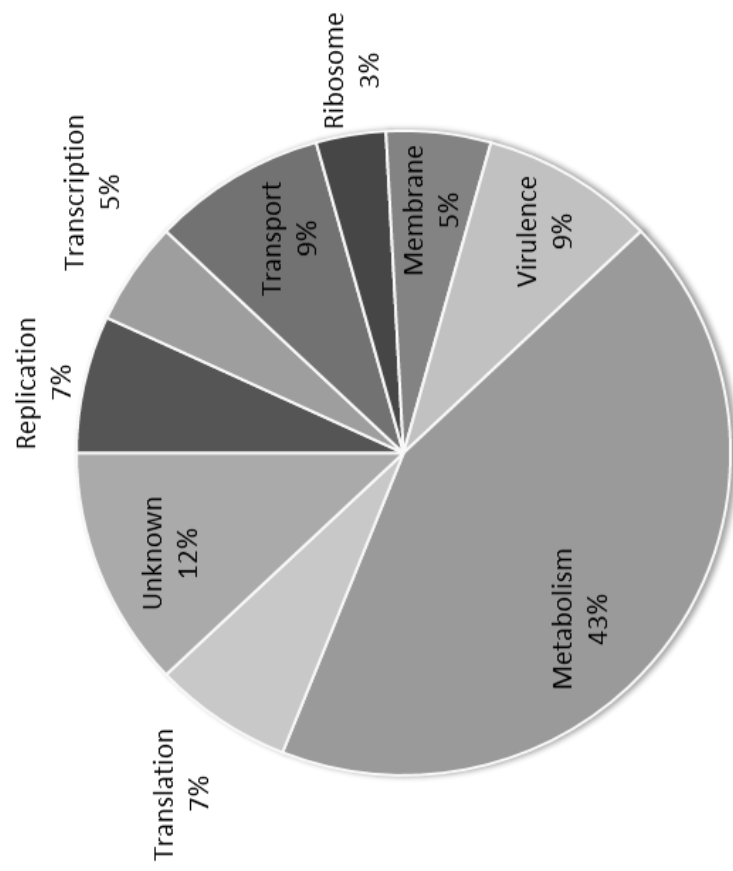
**Figure 26A. Total proteins identified.** The total number of unique proteins identified through the proteomics analysis broken down by protein function.

## Identified Metabolism Proteins In Common Between Two Isogenic Mutants



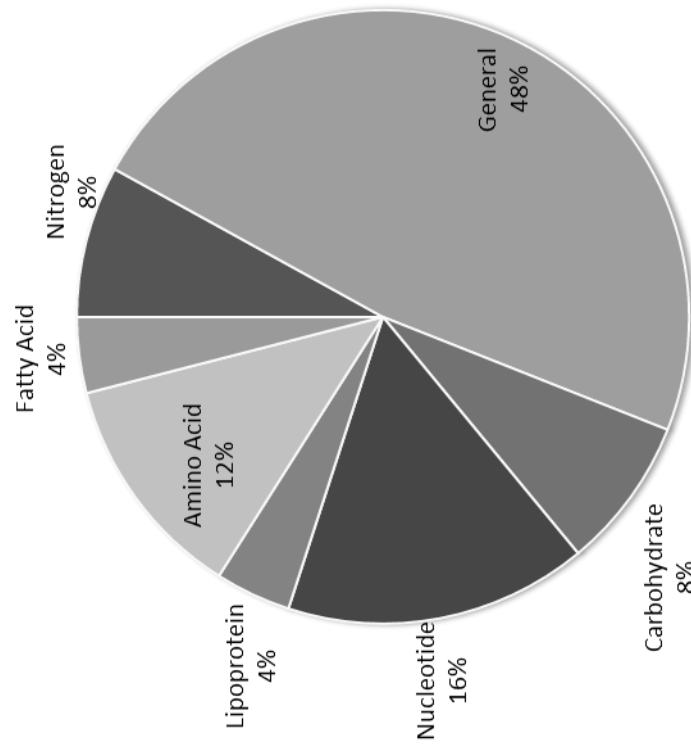
**Figure 26B. Breakdown of metabolism proteins in common between two isogenic mutants.** Among the identified proteins that were identified as being shared between the two mutants, the metabolism proteins were further grouped based on pathways involved.

## Identified Proteins Unique To $\Delta 1718-1719$



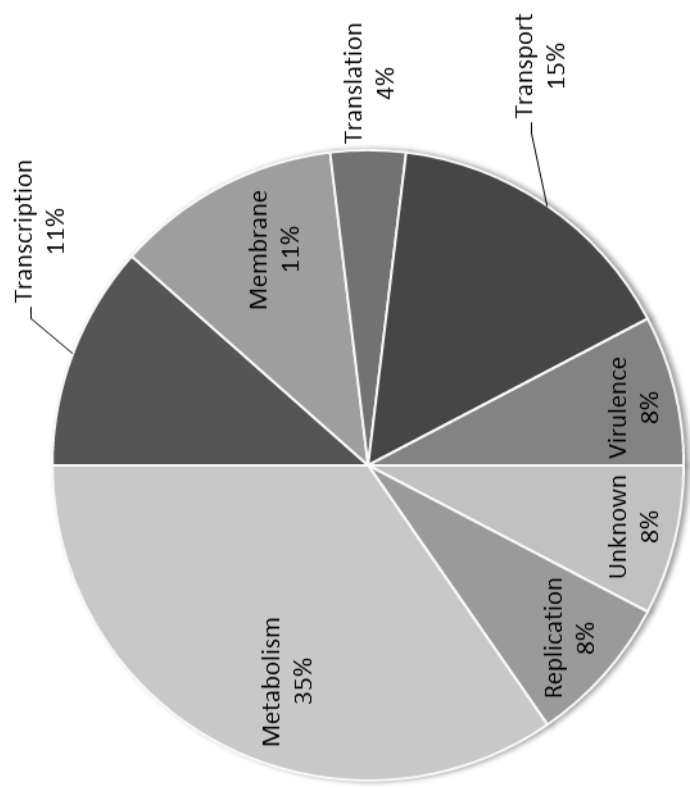
**Figure 27A. Identified proteins unique to  $\Delta 1718-1719$ .** Proteins identified as being unique to  $\Delta 1718-1719$  are grouped based on function of the protein.

## Identified Metabolism Proteins Unique To $\Delta 1718-1719$



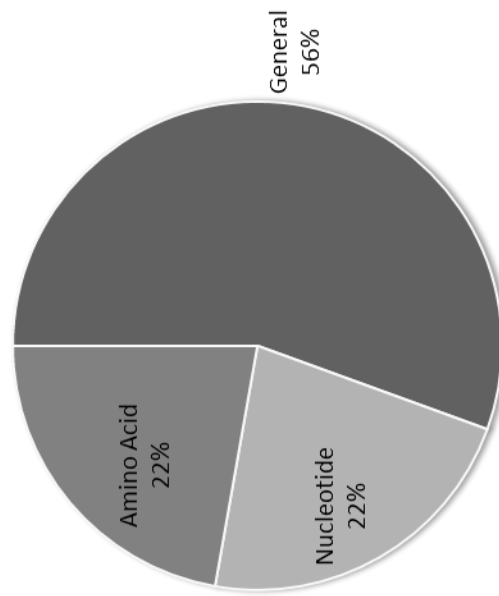
**Figure 27B. Breakdown of metabolism proteins unique to  $\Delta 1718-1719$ .** Identified metabolism proteins that are unique to the  $\Delta 1718-1719$  isogenic mutant were further grouped based on pathways involved.

**Identified Proteins Unique to  $\Delta$ 1194-1195**



**Figure 28A. Identified proteins unique to  $\Delta$ 1194-1195.** Proteins identified as being unique to  $\Delta$ 1194-1195 are grouped based on protein function.

## Identified Metabolism Proteins Unique to $\Delta 1194-1195$



**Figure 28B. Breakdown of identified metabolism proteins unique to  $\Delta 1194-1195$ .** Identified proteins that are unique to the  $\Delta 1194-1195$  isogenic deletion mutant are further grouped based on pathway involved.

## CHAPTER SEVEN: DISCUSSION

Type II TA systems are one way that bacteria and archaea have evolved to respond rapidly to changing environments. The oral cavity is a constantly changing environment that is affected by everything that we eat and drink, saliva, and oral hygiene products. In order to sustain life in this environment, bacteria will grow in biofilms attached to the tooth surface. Biofilms are able to limit the effects of some of these stress conditions by creating gradients that make the bacteria resistant to changes. There is also substantial evidence that bacteria that are susceptible to a particular antibiotic in planktonic culture may exhibit resistance in a biofilm, yet the role that type II TA systems play in the response of oral organisms to stress is largely unknown.

Here, we showed that an oral commensal with potential to be an opportunistic pathogen, *A. actinomycetemcomitans*, contains 14 putative TA loci that represent at least three of the six known families of type II TA systems. Three of these loci possess pseudogenes that have experienced partial gene deletion or rearrangement and these three systems are likely non-functional. Two of the systems identified in strain D11S-1 were not widely conserved across all seven serotypes. Of the 33 genomes examined, all three contained an intact HipAB-like system and 22 of the remaining genomes contained only the anti-toxin. Interestingly, most the 33 genomes examined were derived from human isolates, but many of the identified putative TA systems are conserved in the RhAA1



strain, which was isolated from the oral cavity of a rhesus monkey. The RhAA1 strain also contains more copies of the type II TA systems than any of the other strains, which may reflect the ability of this strain to adapt to environmental stress conditions that exist within the rhesus monkey oral cavity.

*A. actinomycetemcomitans*, is able to respond to environmental stress. Some environmental conditions effect the growth of the bacteria. As with many human pathogens, *A. actinomycetemcomitans* has an iron requirement and iron starvation slows growth. Since *A. actinomycetemcomitans* prefers to utilize lactate as a carbon source (Brown *et al* 2007) and that lactic acid is produced by many of the oral streptococci in the oral cavity, it is interesting that when an iron chelator was added to the medium, it appeared to have no effect on growth when lactate was the carbon source. When the iron chelator bipyridyl, which is used in many commercially available colorimetric assays to determine concentration of iron in samples, is added to a medium that contains iron, the medium changes color as the iron is bound. The bipyridyl interacts with iron (II) to form a complex containing one iron ion connected covalently to 3 bipyridyl molecules that results in a red color being observed: the more intense the color, the more iron that is present in the solution (Kaes *et al* 2000). When bipyridyl was added to CDM containing lactate and iron, no color change was observed, but when it is added to CDM containing glucose and iron, the medium turns bright pink. One possible explanation for this is that lactate can complex with iron (Ali *et al* 2000) and thus lowers the concentration of iron that is available to the chelator. Also of interest is the effect that carbon dioxide has on growth since *A. actinomycetemcomitans* needs carbon dioxide to grow on plates.

The presence of type II TA systems in the genome of *A. actinomycetemcomitans* represents one mechanism for bacteria to respond and adapt quickly to the environmental changes. The presence of multiple systems in the genome is not unusual, but many species do not contain any of these systems and some species have dozens of copies. Previous studies have shown that TA systems can cross-talk by a variety of mechanisms including activating the transcription of other systems, anti-toxin inhibition of non-cognate toxins, or enhancing the activity of other systems (Yang *et al* 2010, Kasari *et al* 2013). When considering the real-time PCR data to determine transcription levels of the putative TA systems, there were several TA systems that appeared to be largely unresponsive to the conditions tested. Two of the TA systems that were unique to *A. actinomycetemcomitans* exhibited little to no activation of transcription when exposed to the environmental conditions tested. One explanation for this result is that these systems respond to an environmental stimulus that was not tested in our approach. A more common pattern observed across all families of the type II TA systems identified was that each system responded highly to only one or two stress conditions, but was largely unresponsive to others. This suggests that specificity exists in the response of various members of the TA system repertoire of *A. actinomycetemcomitans* in that specific TA systems respond to the greatest extent to certain environmental conditions. However, our data also shows that many of the putative type II TA systems respond at least partially to many of the stress conditions tested. Interestingly, putative TA systems within a family appeared to exhibit functional redundancy. For example, the two systems in the MazEF family exhibited similar levels of activation across all conditions. This functional redundancy would protect against the loss of one system to ensure survival, which also

suggests these systems are important for the survival and adaption to the environment within the oral cavity.

It is not currently clear how this specificity to environmental stress exists. Briefly, the inhibition of the type II TA system is due to the presence of the anti-toxin, which can inhibit the toxin's activity and represses the systems transcription by binding to the promoter region (Muthuramalingam *et al* 2016, Hayes *et al* 2014). To activate the system, general ATP-dependent bacterial proteases, such as Lon and Clp, will readily degrade the anti-toxin, thus depleting the cellular concentration of it and causing the activation of the toxin (Muthuramalingam *et al* 2016, Hayes *et al* 2014, Gerdes 2000). However, these are general proteases and they are responsible for a large percentage of the protein degradation within the cell (Muthuramalingam *et al* 2016, Van Melderren *et al* 2009). Interestingly, anti-toxins have been experimentally been shown to be substrates for Lon, however, it is has not been demonstrated *in vivo*. These same studies also indicate the activation of type II TA systems is more complex than a single protease degrading the anti-toxin, and more importantly, the reason for specificity of a response to a stressor has not been elucidated at present (Sat *et al* 2001, Christensen *et al* 2001, Muthuramalingam *et al* 2016). It is possible that the anti-toxin only interacts with the protease under certain circumstances, such as stress, and cell density, or that the stressor causes the anti-toxin to become unfolded, thus activating the proteases (Micevski *et al* 2013, Muthuramalingam *et al* 2016, Yang *et al* 2016, Gur 2013).

Wild-type bacteria exhibit reduced metabolic activity over the course of a week at stationary phase; the loss in activity was fairly steady from day to day. The  $\Delta 1194$ -1195 mutant was able to survive at stationary phase, but experienced a significant drop in

metabolic activity after 96 hours. This suggests that the loss of this TA system may affect viability at stationary phase. Similar results were obtained with the  $\Delta 1718-1719$  mutant but to a greater extent. This isogenic deletion mutant was much more susceptible to acidic pH in stationary phase when compared to wild-type; loss of metabolic activity was observed as early as 24 hours and was very evident at 72 hours. One possible explanation for this observation is that the activity of the D11S\_1718-1719 TA system may effectively compensate for the loss of the D11S\_1194-1194 system, however, the activity the D11S\_1194-1194 system may not fully compensate for deletion of D11S\_1718-1719. This is consistent with functional redundancy between the two systems and the loss of the D11S\_1194-1195 system is not sufficient to greatly affect the overall metabolic activity of the bacteria. Similar functional redundancy has been observed in TA systems of other organisms. It has been shown in a strain of *E. coli* that possesses five TA systems that the loss of a single TA system had no impact on the fitness of the bacteria (Tsilibaris *et al* 2007). However, another study went a step further and deleted all five TA systems and observed a phenotype when the bacteria were grown in a biofilm (Wang *et al* 2011). In *A. actinomycetemcomitans*, the generation of a double mutant was attempted multiple times, but was unsuccessful, which suggests that these systems are important for survival and that deletion of both may be lethal. It is also interesting to note that the D11S\_1718-1719 TA system was present in all 33 genome sequences of *A. actinomycetemcomitans* that were analyzed for presence of TA systems, but the D11S\_1194-1195 was not present in all genomes. This could also indicate the importance of the D11S\_1718-1719 in the overall fitness of the bacteria since its deletion was difficult to achieve and the deletion showed the greatest phenotype.

Both mutant strains also exhibited reduced biofilm biomass relative to the wild type when mature biofilms were exposed to acidic conditions and this phenotype was complemented with functional copies of the TA systems. Importantly, initial biofilm formation of the mutant strains was similar to wild-type, indicating that the mutants are not deficient in the ability to form a biofilm. This is consistent with our earlier results showing that both systems may function to preserve metabolic activity during stationary phase.

To further examine the function of the acid responsive TA systems, the activity of the individual toxin and anti-toxin proteins was determined. Since the TA systems chosen for further study are both RelBE-like homologs, we surmised that these putative systems may represent endoribonucleases that are dependent on the ribosome for activity. *In vitro* translation experiments using a control plasmid with a known protein product showed that both purified toxins inhibited translation and that the anti-toxins had no effect on translation. In addition, when toxin and anti-toxin were mixed together in equimolar amounts, the activity of the toxin was inhibited and no effect on translation was observed. In many cases, the anti-toxin is encoded by the first gene in the operon and the toxin by the second. However, Figure 20 clearly shows that 1718 behaves as a toxin and 1719 behaves as an anti-toxin. This is further supported by the mRNA degradation assay using MS2 bacteriophage genomic RNA (Figure 21). There are a few examples of this in the literature. It does not appear that function is affected by this reversal and phenotypes that have been seen are very similar to the phenotypes seen in the “normal” TA systems. (Budde *et al* 2007, Christensen-Dalsgaard *et al* 2009, Jorgensen *et al* 2009, Jurenas *et al* 2017) The most noted example of this rearrangement is seen in *higBA*, where *higB* is the

toxin. HigBA belongs to a family of TA systems that resemble RelBE because it is an endoribonuclease that is dependent on the ribosome to function. Budde *et al* showed that deletion of *higB* only was possible, but deletion of *higA* was not possible. They also showed that activation of the HigB toxin resulted in slowed growth, but was not associated with cell death. Christensen-Dalsgaard *et al* also suggests that *higBA* is *relBE*, but in reverse. Taken together, our results suggest that the putative toxins degrade mRNA in the presence of ribosomes and inhibit translation. This activity is prevented by the cognate anti-toxin proteins and thus, both systems appear to function as RelBE-like TA systems, regardless of the gene order.

Proteomic analyses of the mutant and wild type strains identified differentially represented proteins that were common to the two mutants, but also a significant number of polypeptides that were unique to each mutant strain. This further supports the idea that multiple TA systems within a family may have functional redundancy, but are also able to differentially respond to different environmental cues. This is highlighted, in part, by the presence of the nitrogen metabolism proteins being identified in the  $\Delta 1718-1719$  mutant and not the  $\Delta 1194-1195$  mutant. Furthermore, the real-time PCR data indicated that the D11S\_1718-1719 TA system was responsive to anaerobic stress while the D11S\_1194-1195 TA system was not.

There are a wide variety of pathways that are affected by the presence of these systems, including virulence factors such as leukotoxin, which was over-represented in the mutants. This suggests that the expression of the leukotoxin may be regulated in part by the endoribonuclease activity of the toxins in the wild-type strain. Since the leukotoxin is an important virulence factor associated with aggressive periodontitis, it is possible that

exploitation of the TA system could be used to potentially target leukotoxin expression and reduce the potential for the bacteria to cause disease. It is also interesting that autoinducer-2 aldolase produced by *A. actinomycetemcomitans* is only affected by the  $\Delta 1718$ -1718 isogenic mutant and was less abundant in the mutant. Since quorum sensing is important in the formation of mature biofilms, this may explain in part why the  $\Delta 1718$ -1719 mutant exhibited reduced biofilm biomass after exposure to acidic conditions than the  $\Delta 1194$ -1195 mutant. Another interesting finding was the presence of a type IV secretion system component that was differentially expressed in the two mutants. BLAST results suggest that this particular component has homology to the VirB5 protein family, and that this particular protein is important for protein-protein interactions for pilus assembly (Yeo *et al* 2003). This protein was not detected in the  $\Delta 1194$ -1195 mutant, but was over-represented in the  $\Delta 1718$ -1719 mutant. Type IV secretion has not been well studied in *A. actinomycetemcomitans*, but of the available literature, we know that *A. actinomycetemcomitans* has a CagE-homolog, which is a protein that is commonly associated with type IV secretion in other organisms and that this CagE protein may be necessary to cause inflammation and tissue destruction in the host (Teng *et al* 2005). A more recent study looked at the prevalence of type IV secretion systems in periodontitis patients in Taiwan, and found that 90% of the samples with a complete type IV secretion system module were from patients with localized aggressive periodontitis (Liu *et al* 2017). This suggests that the wild-type strain may also utilize the D11S\_1718-1719 TA system to aid in the invasion of surrounding host tissues and enable disease progression.

It is also important to note the proteomic analyses presented here were done on planktonic cells, not biofilms. It is possible that different proteins may be affected during

biofilm growth and thus it may be necessary to conduct the proteomic approach using biofilm cells to fully understand how these TA systems alter the proteome of *A.*

*actinomycetemcomitans* exposed to acidic conditions. This is especially important since type II TA systems have been directly implicated in the formation and stability of biofilms in other bacterial species.

Targeting type II TA systems as a way to control bacterial growth has been widely considered to result in the killing of the bacteria, without the need for antibiotics. This would prevent the evolution of antibiotic resistance strains of bacteria, and hosts may experience fewer side effects since humans appear to have no homologous proteins (Park *et al* 2013, Unterholzner *et al* 2013, Williams *et al* 2012). It is possible that by understanding how these systems respond to environmental stress and how they respond within the cell, novel therapies that exploit these systems could be developed. Some proposed methods of doing this involve chronic activation of the toxin, chronic activation of the proteases, or complete inhibition of the toxin to prevent survival. These therapies have the potential to be cost-effective to the consumer, in addition to being less painful than current methods. These novel therapies may also be able to target multiple species at once without difficulty.



## CHAPTER EIGHT: FUTURE DIRECTIONS

### **Persister Cell Formation and Anti-toxin Characterization:**

One of the biggest questions in the literature of TA systems is: why do some bacteria have multiple copies? Fasani *et al* suggest that one reason pathogenic bacteria have so many copies is to increase the likelihood that persister cells will develop. Persister cells cannot be identified through normal genomic methods since there is not a gene associated with the ability to form this type of cell. Persister cells can be assayed for by monitoring cell cultures for viability rather than metabolic activity. Many commercially available kits exist that allow the identification of viable cells that may otherwise be viewed as dead or dying. Preliminary tests within the laboratory suggest that viability between wild-type strain and the two mutants may not be greatly affected over time, but the assays still need to be optimized for use with *A. actinomycetemcomitans*.

Fasani *et al* also suggest that bacteria increase the likelihood for developing persister cells by utilizing TA systems at different times and that they may use growth rate as the signal for the activation. It is possible that this is why some of the putative TA systems identified did not appear to be responsive to any of the environmental stressors tested. It may be necessary to stress the bacteria for longer (or shorter) times or stress the bacteria at different stages of growth. Initial tests in the laboratory attempted to collect total RNA from early-log phase, mid-log phase, and late log phase for analysis. Unfortunately, early and late log phase were difficult to isolate enough usable mRNA for

analysis using the cesium chloride method. Since these initial tests, a commercially available kit for the isolation of mRNA from high lipid tissue culture has been successfully used with better yields from less starting sample than the cesium chloride method. It may be useful to repeat the real-time PCR assay using more time points, and more primer sets for the untested putative TA systems.

The anti-toxin of the TA pair and the TA complex has been shown in many cases to autoregulate the expression of the TA system, and in some cases to regulate the expression of other genes within the genome. Examining the promoter region of the TA operon and identifying the binding site for regulation will facilitate determining the regulon for each of the TA systems and highlight how TA complexes affect gene expression even when the toxin itself is not activated. TA-mediated regulation at the transcriptional level may in part explain why the levels of some proteins were reduced in the mutant strains.

### **Novel Therapies:**

To exploit TA systems to develop potential novel therapies, we first need to understand how the anti-toxin interacts with the toxin. Some 3D crystal structures have already been determined, which can provide a starting point to determine what is actually necessary for the toxin to be inhibited (Brown *et al* 2009, Francuski *et al* 2009, Shinohara *et al* 2010, Boggild *et al* 2012, Heaton *et al* 2012, Schureck *et al* 2014). After the interaction of the toxin and anti-toxin has been elucidated more thoroughly, it may be possible to develop small molecules or peptides that could potentially mimic this binding. Since the anti-toxin is labile and is degraded easily, a presence of the mimic could

prevent the activation of the toxin even in the absence of anti-toxin, thus making the bacteria more susceptible to killing through biofilm disruption, antibiotics, or other environmental stressors. We anticipate that this approach would inhibit adaptation of *A. actinomycetemcomitans* to acidic conditions and may prevent the formation of persister cells. By inhibiting toxin activation under acidic stress it is possible that infection could be contained or eliminated without the need for an antibiotic or painful debridement.

It may also be possible to develop small molecules that inhibit the Lon and Clp proteases, which initiate the activation of TA systems. This could prove to be a more universal treatment pathway since more than one family of TA systems are activated by these proteases and may be more conserved across species than the individual TA systems. On the other hand, it may be possible to cause chronic activation of the toxin, which would cause the bacteria to stop growing, and since it would not be turned off under favorable conditions, it would eventually die. Small molecules may be developed that can inhibit the activation of the TA operon, thus preventing more anti-toxin from being transcribed and translated. Small molecules may also be able to developed that cause the activation of the general proteases, which would keep the supply of the anti-toxin low.

#### **Other TA Systems and Stress:**

Previous work in *E. coli* showed that a strain deficient in five TA systems was deficient in antibiotic resistance, but only in the biofilm. It may be necessary to repeat the proteomic assay done in this study with bacteria stressed in a biofilm. Optimization would need to be done to determine how long to stress the biofilm for optimal results.

There are multiple other TA systems that were not characterized during the initial real-time PCR experiments. More insight into how these systems work and when they work could be determined by repeating the assay here with more primer sets. Of the systems that were characterized, there are many others that can be examined as well. Of notable interest:

1. Iron Starvation
  - a. D11S\_2133-2134
2. Anaerobic
  - a. D11S\_2133-2134
  - b. D11S\_1068-1069
  - c. D11S\_0905-0906
  - d. D11S\_0919-0920
  - e. D11S\_1798-1799
3. Lactate as a carbon source
  - a. D11S\_0905-0906
  - b. D11S\_0919-0920
4. Microaerophilic Conditions
  - a. D11S\_0905-0906
  - b. D11S\_0919-0920

These conditions are important for survival in the oral cavity and should be examined as well. Since only a handful of stress conditions were tested here, it is highly probable that some of the unresponsive systems, such as D11S\_0499-0500, and D11S\_1023-1024 have very specific stress conditions to which they respond. It is

possible that one or more of these TA systems are utilized for the invasion of epithelial cells and/or specifically when in a biofilm.

This will enhance our understanding of why some bacteria have multiple copies of TA systems and will enable us to see how these systems cooperate with each other.

**Other strains of *A. actinomycetemcomitans*:**

Since a BLAST search showed that the type II TA systems found in strain D11S were conserved across various serotypes, it may be of interest to examine the effects of environmental stress in other serotypes, such as serotype A and B, which are also associated with disease in humans. It would be important for the development of novel, non-invasive therapies to determine if the type II TA systems being targeted are responsive in all serotypes and how they are responsive.

Of interest as it relates to this study, serotypes D, E, F, and G all contain a HipAB homolog when compared to the sequence in *E. coli*, but they do not contain the HipAB homolog found in D11S. Interestingly, these serotypes, while they can be found in human oral cavities, are not typically associated with disease. The sequence in D11S appears to have experienced a deletion event that broke the protein into two different genes. It would be interesting to see what would happen with a full HipAB being introduced into the strain used here. It would also be interesting to see how the D11S HipAB system affects other strains with a full length HipAB system.

This will prove to be important when we look at the studies showing prevalence of serotypes across a population around the world. Different serotypes are prevalent in certain populations whereas others may not be found in certain areas.

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