5-2017

Function of the enterobactin operon of A. actinomycetemcomitans in the presence of catecholmines and iron.

Taylor Johnson
University of Louisville

FUNCTION OF THE ENTEROBACTIN OPERON OF *A. ACTINOMYCETEMCOMITANS* IN THE PRESENCE OF CATECHOLAMINES AND IRON

By

Taylor Johnson
B.A., University of Louisville, 2014

A Thesis
Submitted to the Faculty of the University of Louisville School of Dentistry in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Oral Biology

Department of Oral Immunology and Infectious Diseases
University of Louisville School of Dentistry
Louisville, Kentucky

May 2017

FUNCTION OF THE ENTEROBACTIN OPERON OF *A. ACTINOMYCETEMCOMITANS* IN THE PRESENCE OF CATECHOLAMINES AND IRON

Taylor Johnson
B.A., University of Louisville, 2014

A Thesis Approved on

November 3, 2016

By the following Thesis Committee:

_____
Don Demuth (Thesis Mentor)

_____
Lisa Sandell (Committee Member)

_____
Rich Lamont (Committee Member)

ACKNOWLEDGEMENTS

I want to thank Dr. Demuth for introducing me to biological research as well as expanding my knowledge of oral health. I also want to thank Whitney Weigel and Blair Schneider for training and teaching me the skills I needed to conduct my research experiments. Finally I want to thank my wife, Megan, for constantly encouraging and supporting me throughout this program.

ABSTRACT

FUNCTION OF THE ENTEROBACTIN OPERON OF *A. ACTINOMYCETEMCOMITANS* IN THE PRESENCE OF CATECHOLAMINES AND IRON

Taylor Johnson

May 13, 2017

Background: *Aggregatibacter actinomycetemcomitans* (*Aa*) possesses a two-component system, QseBC, which is key in biofilm formation/virulence and is activated in the presence of iron-catecholamine complexes. *Aa* does not synthesize enterobactin, a catechol-based siderophore, yet possesses the machinery for recognition and uptake. We hypothesize that *Aa* is able to acquire iron by catecholamine mediation through the enterobactin receptor/transporter. **Methods:** By insertional mutation, we attempted to delete the enterobactin permease from the genome using suicide vector pJT1. A growth curve of truncated mutant ΔfepA was also conducted to observe growth in the presence of iron and catecholamines. Statistical significance was determined by ANOVA ($p<0.05$). **Results:** The permease deletion mutant was not achieved, but the growth curve for ΔfepA showed stunted growth in comparison to Wt by 45.4% in a chemically defined medium (CDM) supplemented with iron and a 38.4% difference in CDM supplemented with iron and norepinephrine. **Conclusions:** Given the results from the ΔfepA strain we can conclude that strains of Aa with mutated enterobactin genes have a reduction in growth phenotype due to the difficulty in iron uptake.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

A. Periodontal Disease

There are two main forms of periodontal disease, gingivitis and periodontitis. Gingivitis is a mild inflammation of the gums, characterized by swollen, bleeding and sometimes painful gingiva with subgingival pocket depths ranging from 4 to 5 mm. With proper oral hygiene, gingivitis and its symptoms can be reversed, however, if not treated properly it can progress into periodontitis [2]. Periodontitis and eventually severe periodontitis is a chronic disease, characterized by damaged soft tissue and supporting bone, tooth loss and subgingival pocket depths of 6 mm or more. The main etiological agent that causes the periodontal destruction is the buildup of microbial dental plaque.

Recent studies show that 46% of people aged 30 years or older in the United States have some degree of periodontal disease, 8.9% of the adult population suffering from severe periodontitis [3]. The prevalence correlated with age and gender and occurred more frequently in minorities in comparison to Non-Hispanic whites, displaying a variation in occurrence based on socioeconomic position.

There are several forms of adult periodontitis, from aggressive and chronic periodontitis, necrotizing periodontal diseases to periodontitis associated with genetic disorders [38]. Many factors come into consideration when classifying chronic versus aggressive periodontitis such as age of onset, the rate of disease progression, pattern of destruction and the clinical signs of the disease [39]. For example, aggressive

periodontitis is usually seen in younger patients and spreads at a much faster rate than chronic periodontitis normally present in older patients [39].

Acute and Chronic necrotizing ulcerative gingivitis is usually connected with established gingivitis and factors that may influence the host immune response such as smoking or drug use [40]. Clinical signs present necrosis of the gingival tissue as well as pain and bleeding. Other forms of periodontal disease, for example periodontitis that presents itself due to genetic disorders such as Down's syndrome, is common [41]. Morphological, anatomical and immunological aspects all play a role in the progression of the disease.

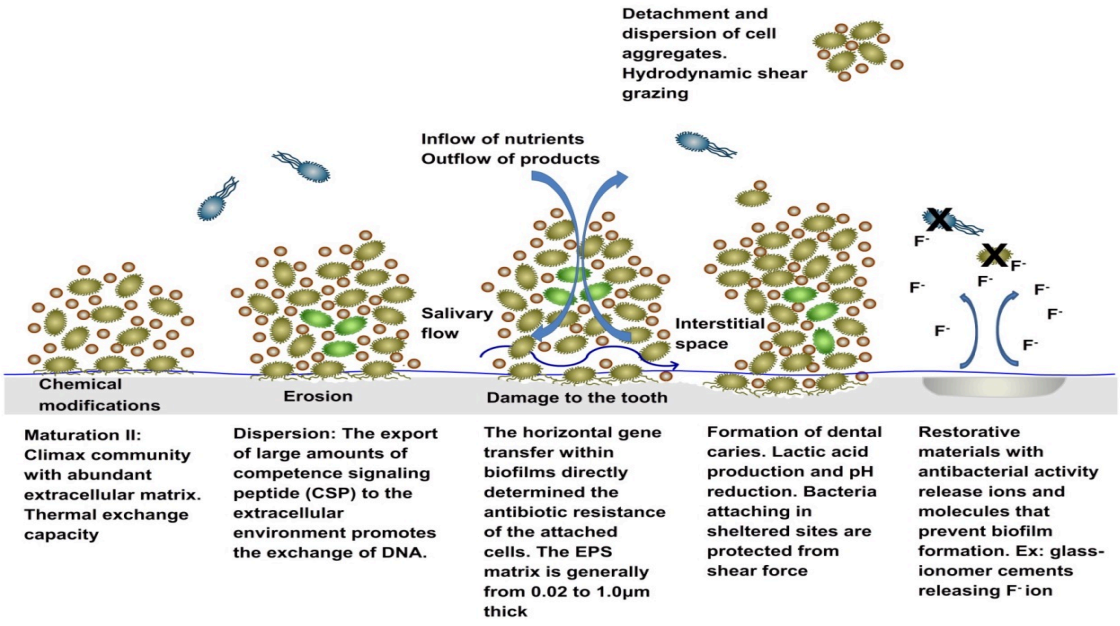
In 2014 alone, $113.5 billion dollars was spent nationally on dental care, up from $112 billion in the previous year [4]. Increasing awareness of oral health and advocating positive oral hygiene could alleviate some of the expenditure. In addition to the great expenses for dental care in the United States, there is evidence linking periodontal disease to the onset of other systemic disorders and life-threatening conditions such as atherosclerosis, osteoporosis, rheumatoid arthritis, diabetes and pregnancy complications such as preterm birth [5]. These systemic diseases will only add to the increasing cost of healthcare around the world and signifies the importance of reducing the spread of periodontal disease in the human oral cavity.

One of the main causes of the onset of periodontal diseases is the buildup of microbial plaque. If the biofilm of bacteria is not limited by mechanical means or the naturally occurring antimicrobial activity of saliva, biofilm growth can spread to the subgingival pocket. The development of a biofilm begins almost as soon as the tooth erupts during childhood. A protein film called the salivary pellicle covers the tooth to

prevent continuous deposition of calcium phosphate from the saliva and permits the attachment of early colonizing bacteria such as the oral *streptococci* [11]. Eventually, other microbes attach to the foundation of the biofilm already created by early colonizers. Coaggregation begins with successive colonization by bacteria such as *Actinomycetes*, *Prevotella, Eikenella,* and *Fusobacterium nucleatum* [12]. These communities of commensal and pathogenic bacteria reside on the teeth and are shielded and protected from antimicrobial salivary proteins and enzymes by the biofilms they create. In healthy plaque, pathogenic bacteria are usually found in relatively low numbers until some environmental stimuli triggers an expansion of their populations and expression of their virulent factors. The expression of virulence factors is hypothesized to be attributed to an imbalance of bacteria in the oral microbiome, or dysbiosis. This microbial shift is similar to an overgrowth of bacteria such as *H.* pylori in the gut leading to conditions like gastritis and peptic ulcers. Likewise, a flux of the oral bacteria residing in the oral cavity from mostly gram-positive aerobes to gram-negative anaerobes increases chances of disease [34]. Although *P. gingivalis* has been isolated from healthy oral cavities [34], its presence is usually found when the oral microbiome has shifted to an anaerobic environment where it causes the most damage [35]. Indeed, *P. gingivalis* has even earned the title of a 'Keystone' pathogen for its primary role in conditions surrounding periodontal diseases. Ultimately, expansion of the biofilm in the subgingival pocket induces an inflammatory response and immune cells such as neutrophils and macrophages migrate to the site of infection. Eventually, osteoclasts are stimulated either by the presence of the invading anaerobic bacteria or primarily by the immune response itself [6,32]. Thus, the host immune response is largely responsible for the deterioration

of the periodontal ligament and other tissues [31]. Pro-inflammatory cytokines released from responding immune cells activate osteoblasts to discharge osteoclastic factors such as IL-1, IL-6, and TNF [6]. Furthermore, components found in the outer membrane of gram-negative bacteria called lipopolysaccharide (LPS) and endotoxin proteins of bacteria are pro-inflammatory [6,7]. For example, the fimbriae of *P. gingivalis* stimulate the synthesis of chemotactic and osteolytic cytokines from monocytes and fibroblasts [8,9,10]. The functional consequences of both host and microbial factors cause resorption of the alveolar bone, leading to bone and tooth loss. There are many pathogenic bacteria associated with chronic adult periodontitis that also include *Treponema denticola, Tannerella forsythia and Aggregatibacter actinomycetemcomitans* [13,14].

Figure 1. Representation of Oral Biofilm. From J.M.F.A. Fernandes, V.A. Menezes, A.J.R. Albuquerque, M.A.C. Oliveira, K.M.S. Meira, R.A. Menezes Júnior and F.C. Sampaio (2015). Improving Antimicrobial Activity of Dental Restorative Materials, Emerging Trends in Oral Health Sciences and Dentistry, Prof. Mandeep Virdi (Ed.), InTech, DOI: 10.5772/59252.



B. *Aggregatibacter actinomycetemcomitans*

In 1996, a number of bacteria including *A. actinomycetemcomitans* were designated as etiological agents of periodontitis [15]. *Aa* is a non-motile, gram-negative facultative anaerobe of the *Pasteurellaceae* family that is commonly found in the microbial community of the human oral cavity and indeed exists as a commensal in many individuals. There are six serotypes of *A. actinomycetemcomitans* denoted from serotype 'a' to serotype 'f', with the distribution of affliction depending on the geographic location. For example, a study conducted in 1998 showed that patients in the United States displayed significantly higher levels of antibodies to the 'b' serotype of *A. actinomycetemcomitans* compared to Turkish patients, who showed significantly higher levels of anti-bodies to the 'c' serotype [16]. Recently, a study conducted in the United States in 2010 observed all serotypes in the oral cavities of the participants, in varying distributions [36]. Serotype 'c' was the dominant serotype, followed by 'a' and 'b'. Serotypes 'd' and 'e' were rare, while the prevalence of 'f' is still under investigation [37]. Additionally, patients may harbor just one serotype, or several [36].

A. actinomycetemcomitans is associated with a form of periodontal disease known as localized aggressive periodontitis (LAP), a disease that affects adolescents, more so in minority populations [16,17]. In patients of African descent LAP has been shown to be linked to a lineage of the b serotype, also known as the JP2 clone, which possesses a deletion in the leukotoxin promoter leading to increased leukotoxin production and variations in the mechanism for iron acquisition. Interestingly, this suggests that multiple forms of the disease may exists, one caused by the presence of the JP2 clone and others

by non-JP2 *A. actinomycetemcomitans* functioning as an opportunistic pathogen as seen in European Caucasian populations [16, 18]. What specifically triggers *A. actinomycetemcomitans* to exhibit pathogenic characteristics is unclear, but evidence is beginning to show that the presence of other oral bacteria enhances the virulence of *A. actinomycetemcomitans* [15]. In fact, studies have shown heightened growth and resistance to the host immune response in *A. actinomycetemcomitans* by the presence of streptococcus strains, such as *S. gordonii* [28]. Its ability to resist the host immune response can partly be attributed to the hydrogen peroxide (H_2O_2) that is produced by the streptococci. The presence of H_2O_2 that is produced as a waste product from the streptococci induces expression of genes *katA* and *apiA*. The gene *katA* aids in detoxing the H_2O_2 into oxygen and water, while *apiA* boosts auto-aggregation, host cell invasion and binds to human serum protein factor H which protects *A. actinomycetemcomitans* from the complement pathway system [28].

Additionally, *A. actinomycetemcomitans* secretes a leukotoxin (LtxA), which is a member of the repeats-in-toxin (RTX) family that specifically target the β(2)-integrin leukocyte function antigen-1 (LFA-1) that is expressed by white blood cells [30]. The targeting of this protein leads to apoptosis, or programmed cell death of the white blood cells. This is accomplished by the internalization of LtxA after binding to LFA-1, where LtxA is brought to the lysosome. The lysosomal membrane is then disrupted by the leukotoxin and the lysosomal contents are released into the cytoplasm, killing the cell [30].

Other virulence factors that allow *A. actinomycetemcomitans* to reside in the oral cavity are the fimbriae of the bacteria. Colony variants that arise within a microbial

population present different forms of fimbriae, transparent rough, transparent smooth and opaque [15]. Transparent rough and smooth fimbriae are able to bind to surfaces such as agar and glass, while the opaque cannot. Antibodies to synthetic *A. actinomycetemcomitans* fimbriae blocked binding to saliva-coated surfaces including buccal epithelial cells displaying the important role fimbriae play in colonizing a host [15]. Isolating the fimbriae exposed a protein named Flp that was part of a larger open reading frame that when analyzed revealed a protein structure similar to type IV pili [15]. Clinical isolates of *A. actinomycetemcomitans* display large fibrils branching off the organism, which are removed after passaging. These large fibrils resemble the bundle-forming fimbriae present on enteropathogenic *E. coli*, the branching fimbriae cause auto-aggregation of *A. actinomycetemcomitans* [15]. Aggregation of the bacteria allows for resistance to salivary flow and maintains the presence of the bacteria in the oral cavity. Other virulence factors responsible for host colonization include the presence of adhesin proteins on the outer-membrane that mediate cell invasion [15]. Many bacteria possess a receptor for fibronectin, a glycoprotein of the extracellular matrix of host cells that binds to components such as collagen, and are able to invade cells by receptor-mediated endocytosis [20].

There are many factors that allow *A. actinomycetemcomitans* to reside and be maintained in the oral microbiome, including the two-component system QseBC.

C. Two-Component System (QseBC)

In order for bacteria to be able to survive in the oral cavity, organisms need to quickly respond to the local environment that can be in constant flux. One factor that allows *Aa* to respond and adapt to environmental fluctuation is a signal transduction

system, comprised of a two-component system designated QseBC (Figure 2). QseBC consists of histidine kinase QseC, which is comprised of a periplasmic sensor domain, two trans-membrane domains and a kinase domain located in the cytoplasm. Binding of the signal by QseC activates the kinase domain and initiates a cascade that results in the phosphorylation of QseB. QseB responds to the activation of QseC via a receiver domain and a helix-turn-helix DNA binding motif, which in turn influences gene expression (Figure 2) [21].

Different bacteria possess QseBC and rely on its role in virulence [35]. Studies have shown that QseBC in *E. coli* is activated by the presence of catecholamines [22]. This activation leads to increased expression of genes responsible for biofilm growth, the expression of flagella and motility as well as other virulence genes [21]. Furthermore, QseBC expressed by *H. influenzae* responds to ferrous iron, but not catecholamines [23].

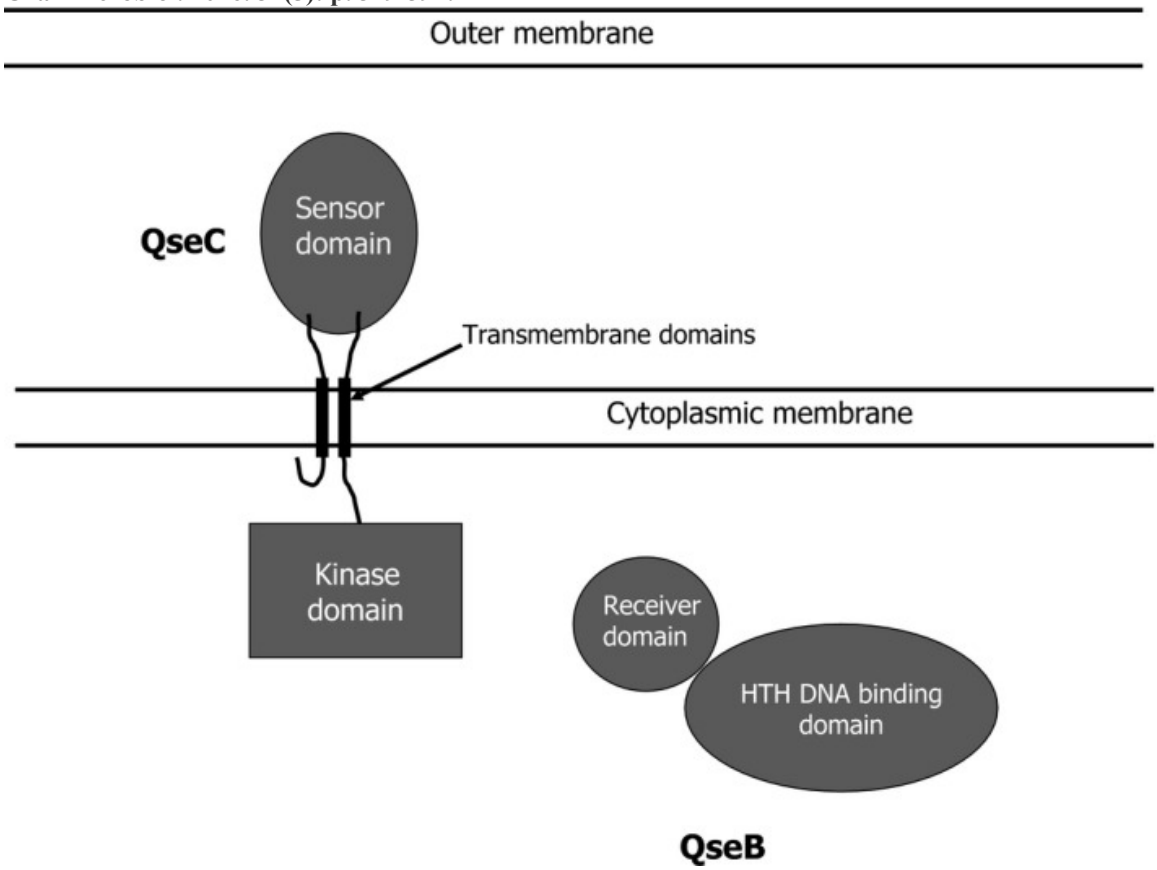
A. actinomycetemcomitans is unique in that QseBC may be activated by the presence of iron-catecholamine complexes. Strains of *A. actinomycetemcomitans* lacking QseC showed altered phenotypes compared to the wild-type strain, such as significantly lowered levels of biofilm mass [25] and a reduced capacity of inducing bone loss in a mouse model of periodontitis [35].

Catecholamines such as norepinephrine or epinephrine act as pseudosiderophores and are able to strip iron away from host iron-containing proteins such as lactoferrin and transferrin [24]. Indeed, the role of transferrin is to deliver iron to iron-dependent cells throughout the body via the circulatory system [19]. Transferrin and lactoferrin are also associated with the innate immune system. As a result of the high affinity for iron, exhibited by transferrin and lactoferrin, there are relatively low levels of free iron in the

body, making it inaccessible to bacteria. However, the presence of stress hormones in response to inflammation such as gingivitis may result in an environment that is rich in catecholamines. Since lactoferrin is also released by neutrophils, bacteria may then be able to utilize these stress hormones to acquire an essential nutrient via by catecholamine-mediated iron acquisition.

In order to acquire iron, bacteria secrete siderophores into the extracellular space of their environment. Siderophores have a high affinity towards iron and bacteria are able to transport the iron into the cell once the siderophore receptors on the outer membrane recognize the complex. *E. coli* and other enteric bacteria utilize the siderophore enterobactin. Studies have shown that enterobactin is vital as the final acceptor of iron from iron-catecholamines complexes [26]. Mutant strains of *E. coli* defective in either enterobactin synthesis or siderophore uptake were placed in the presence of transferrin and norepinephrine. Results showed significant decreases in iron uptake, indicating that enterobactin is required for obtaining iron from catecholamines [24,26]. When the mutations were complemented back into the bacteria, the ability to accept iron stolen from transferrin in the presence of norepinephrine was restored. Additionally, research showed the shuffle of iron from loaded transferrin to unloaded transferrin in the presence of norepinephrine, supporting that the catecholamines are able to stimulate shuffling of iron bound to molecules such as transferrin [26].

Figure 2. TCS QseBC. From Weigel, W.A. and Demuth, D.R. *QseBC, a two-component bacterial adrenergic receptor and global regular of virulence in Enterobacteriaceae and Pasteurellaceae.* Mol Oral Microbiol. 2016. 31(5): p. 379-397.



D. Enterobactin Operon

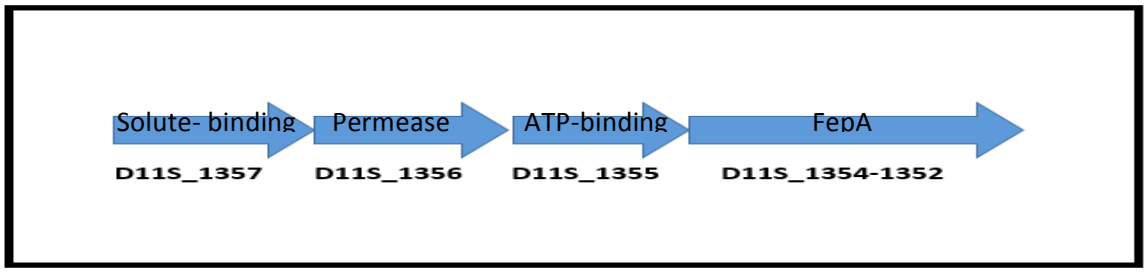
Interestingly, *A. actinomycetemcomitans* does not synthesize enterobactin, a catechol-based siderophore, yet possesses an enterobactin operon that codes for the recognition and uptake of it. This may suggest that *A. actinomycetemcomitans* has evolved to be an opportunistic pathogen, taking advantage of the host environment. With the evidence that QseBC is activated in the presence of iron-catecholamine complexes and the possession of enterobactin machinery for the uptake of enterobactin, this raises the question of the relationship between the enterobactin operon and stress hormone mediated iron acquisition.

The enterobactin operon consists of six genes, encoding proteins that make up the machinery for recognition and uptake of enterobactin (figure 3). The first set of genes

encoded on the operon is an iron ABC-family transporter. Specifically, the first operon gene, annotated as 1357 of serotype c D11S-1 codes for a solute-binding protein of the ABC transporter, D11S_1356 codes for the permease protein and D11S_1355 codes for an ATP-binding protein. The enterobactin ABC transporter for bacteria that synthesize the siderophore is important for transport of iron into the cell. ABC transportation is a form of active transport, which requires adenosine triphosphate (ATP) to translocate solutes across the cell membrane. The solute is then imported through a transmembrane protein permease from the periplasm to the cytoplasm.

Genes D11S_1354 codes for a TonB-dependent outer membrane siderophore receptor protein designated as *fepA*. This receptor is present on the outer membrane of gram-negative bacteria and is a member of the beta-barrel protein family. FepA is an essential outer membrane porin protein that is involved in the import of iron from the extracellular space to the periplasm of gram-negative bacteria, mediated by siderophores. Thus the flow of iron acquisition is the recognition of ironbound enterobactin by outer membrane receptor FepA, which brings the molecule into the periplasm where it is recognized by the ABC transporter and transported into the cytoplasm.

Figure 3 [42]. Enterobactin Operon. From Weigel, Whitney, "Functional characterization of Aggregatibacter actinomycetemcomitans QSEBC : a bacterial adrengeric receptor and global regulator of virulence." (2015). *Electronic Theses and Dissertations.* Paper 2296.



E. Significance

Microbial endocrinology is an emerging field in which the relationship between bacterial organisms and host hormones present during infection are observed [27]. When the body is under some kind of stress, like the inflammation that occurs during periodontal disease, stress hormones are released. Stress stimulates the release of neurotransmitter catecholamines like epinephrine or norepinephrine by the sympathetic nervous system, putting the body in the "flight or fight" state.

The combination of the release of stress hormones and the immune response puts bacteria in a perfect position to take advantage of the environment. As cells associated with the immune system, e.g., neutrophils, respond to the area of infection, additional stress hormone and iron-chelating molecules are released. Thus the presence of both stress hormone and iron-complexes allow for catecholamine mediated iron acquisition for any inhabiting bacteria, benefiting from both the stress and immune response.

As previously stated, although *A. actinomycetemcomitans* does not synthesize the siderophore enterobactin, it does encode the transportation system for recognition and uptake of the molecule. There is also evidence that the two-component system QseBC is activated in the presence of iron-catecholamine complexes, leading to an induction of genes responsible for anaerobic metabolism and respiration and a down regulation in genes encoding iron acquisition [21]. This is possibly a result of the bacteria sensing an iron rich environment and spends less energy in trying to bring in large amounts of iron.

The importance of QseBC in virulence and the ability of pseudosiderophores may suggest that *A. actinomycetemcomitans* has evolved to utilize stress hormone mediated iron acquisition without having to spend the energy in producing enterobactin. Therefore,

the hypothesis of this thesis is that *A. actinomycetemcomitans* acquires iron by stress hormone mediation through the enterobactin receptor/transporter.

CHAPTER II

METHODS

PART I: INSERTIONAL MUTAGENESIS

To study the role between the enterobactin operon, the acquisition of iron and its possible influence on the expression of QseBC, mutant strains of *Aggregatibacter actinomycetemcomitans (Aa)* lacking genes responsible for iron acquisition were analyzed. Specifically, studies will observe strains of *Aa* with inhibited outer membrane receptor FepA or deletion of the intermembrane permease.

A. Preparation

Stocks of *Aggregatibacter actinomycetemcomitans* wild-type strain 652 serotype 'c' were grown overnight in 50 mL of Brain Heart Infusion medium (BHI: 37g BHI powder per liter, pH 7.4 ± 0.2), pelleted, resuspended in 1 mL BHI and 1 mL glycerol and stored in a -80°C freezer. Wild-type strain 652 was grown in 10 mL brain heart infusion overnight and genomic DNA was obtained from the culture via Promega 'DNA Purification System Miniprep' following the manufacturer's protocol. The insertional mutation was carried out using the suicide vector pJT1 (Figure 4). Strains of XL1-Blue *Escherichia coli,* electrocompetent cells containing the plasmid pJT1, were grown overnight in 50 mL Luria Bertani medium (25g LB powder per liter, pH 7.0 ±0.2) and stored in a -80°C freezer. To acquire the plasmid, the pJT1 containing *E. coli* were inoculated into 10 mL LB and placed in an incubated shaker overnight at 37°C. The plasmid was then isolated via plasmid prep kit 'QIAprep Spin

Miniprep Kit' by Qiagen following the provided protocol. Both *A. actinomycetemcomitans* genomic DNA and pJT1 are rehydrated with nuclease free water and stored in a 4°C refrigerator.

B. Polymerase Chain Reaction (PCR)

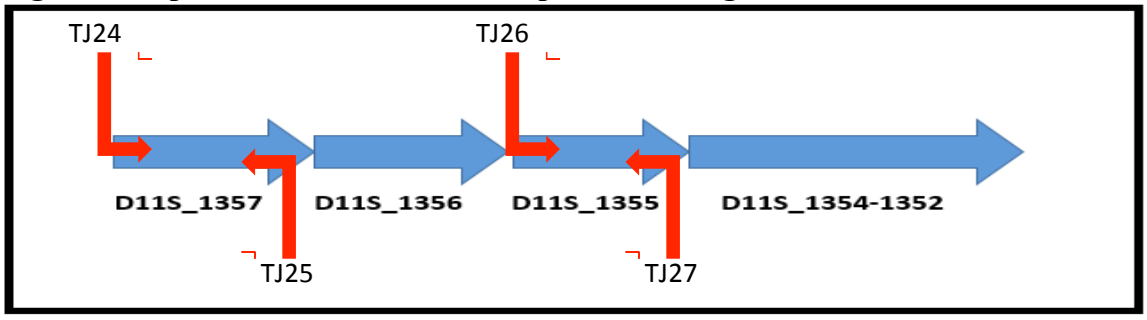
Standard polymerase chain reactions were carried out via the use of Radiant 'HiFi ultra DNA Polymerase' following the protocol provided using the BioRad Mycycler Thermal Cycler. This was used to amplify the flanking genes (D11S_1357 and 1355) of the enterobactin permease (D11S_1356) of the Genomic DNA of *A. actinomycetemcomitans* D11S strain. The primers used in the reaction are listed in the table below.

Table 1. PCR Primer Sets

Primer Name	Sequence 5'- 3'
TJ24 (F*)	GGAAATTCCCATGGCGGCCGCAAAAAATGG
TJ25 (R*)	GCCGGCAAACTCGAGATCCCCCG
TJ26 (F*)	GGGGCAGACGATGCTCGAGAATTTAGTGG
TJ27 (R*)	GTACGTCCTTGGGCTGCAGGTTGAGTAAAAAC

*F denotes Forward Primer; R denotes Reverse Primer; underlines portions represent restriction enzyme sequences

Figure 4. Representation of Primer Amplification Regions.



The PCR reaction mix was as follows:

- 10µL Radiant 5X HiFI Ultra Reaction Buffer
- 2µL Forward Primer (10µM)
- 2µL Reverse Primer (10µM)
- 1µL gDNA
- 0.5uL Radiant HiFi Ultra Polymerase (2u/µL)
- 34.5µL PCR-grade water

The following table displays the PCR program settings:

Table 2. PCR Settings.

Cycles	Temperature	Time	Notes
1	95°C	1 minute	Initial Denaturation
25-35	95°C 55°C to 65°C 72°C	15 seconds 15 seconds 30 seconds per Kb	Denaturation Annealing Extension

The PCR product was observed by gel electrophoresis, purified using a PCR clean up kit and stored in a 4°C refrigerator.

Gel Electrophoresis Preparation:

1% agarose gel was prepared by dissolving 1 gram of molecular biology grade agarose powder in 100 mL of 1X Tris-acetate-EDTA (TAE) buffer. The mixture was heated in a conventional microwave until all agarose powder has melted into solution. It was allowed to cool before adding 5 µL of Midori Green Advance DNA stain. Once the gel had set, the gel was carefully transferred to an electrophoresis chamber (1X TAE buffer). 10 µL of 1KB ladder (Figure 4**)** was then added to the first well to help quantify the size of the PCR product. Samples (10 µL) were mixed with some dye (5 µL) and

subsequently added to the neighboring wells. A current was applied to the chamber (≅100V) for 50 minutes and visualized under UV light.

C. Digestion

Amounts of up to 1 μg of PCR product were used for the digestion process. Digestions occur to make specific cuts along the genomic DNA and plasmid pJT1 DNA for ligation later in the experiment using specific restriction enzymes. The upstream and downstream PCR product portions and pJT1 are digested with the appropriate restriction enzymes based upon the flanking genes (D11S_1357 and D11S_1355) and the MCS portion of pJT1 (Figure 14) and incubated at 37°C overnight. To prevent self-ligation of the plasmid during the ligation phase, terminal 5'-phosphate groups are removed by a phosphatase and incubated at the same temperature for an additional hour.

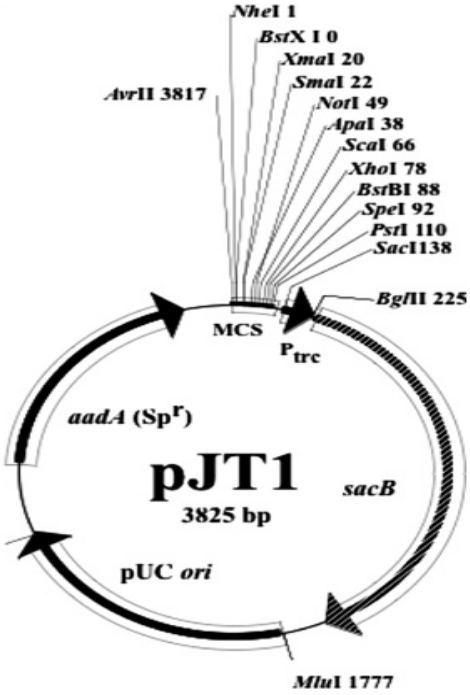
Table 3. Digestion Set-up.

Digestion 1	Digestion 2	Digestion 3
X μL TJ24+25	X μL TJ26+27	X μL pJT1
1 μL *Not*I	1 μL *Xho*I	1 μL *Not*I
1 μL *Xho*I	1 μL *Pst*I	1 μL *Pst*I
10% total vol. Buffer 3.1	10% total vol. Buffer 3.1	10% total vol. Buffer 3.1
Remaining μL H_2O	Remaining μL H_2O	Remaining μL H_2O
= Total μL	= Total μL	= Total μL

X is determined by ng/μL

Nucleic acid quantitation was determined by a nanodrop spectrophotometer machine and used to calculate the amount of the digestion product used for the ligation portion of the experiment. Following the clean up of the digestion product, the three samples were combined for ligation.

Figure 5. Plasmid pJT1. From Juárez-Rodríguez MD, Torres-Escobar A, Demuth DR. *Construction of new cloning, lacZ reporter and scarless-markerless suicide vectors for genetic studies in Aggregatibacter actinomycetemcomitans*. Plasmid, 2013. 69(3): p. 211–22.



D. Ligation

The equation below was used to calculate the amount from each of the digestion products needed for the ligation. It is used to determine the amount of each digestion product needed (X ng) for successful ligation:

X = (10ng vector)(bp insert)/(3.8bp pJT1)

Once the amount needed was calculated, the three samples were then added together in a microcentrifuge tube, incubated at 55°C for 5 minutes and T4 ligase, T4 ligase buffer and water were then added to the mixture. The ligation was placed in a 4°C refrigerator for at least 48 hours.

E. Electroporation

The ligation mix was then electroporated into 50 µL electrocompetent *E. coli* using the BioRad 'Micropulser' on the setting 'EC1' in a 0.1 cm cuvette (1.8kV). The cells were quickly nourished with 500 µL of Super Optimal broth with added Catabolite repression (SOC, see Table 4 below) and placed in a shaking incubator at 37°C for one hour. After incubation, the culture was then distributed to LB/spectinomycin agar plates in 3 µL droplets and incubated at 37°C for 24 hours.

Table 4. Stock Solution of 1X SOC Media.

Amount	Compound	Concentration
100 µL	NaCl	5M
125 µL	KCl	1M
500 µL	$MgCl_2$	1M
500 µL	$MgSO_4$	1M
1 mL	Glucose	1M

F. Plasmid Extraction

Spectinomycin resistant *E. coli* colonies were collected individually and grown over night in 10 mL of LB supplemented with 10 µL of spectinomycin. The complete plasmid construct was isolated by using the Qiagen 'Spin Miniprep Kit', nucleic acid quantitation was determined by nanodrop and stored in a 4°C refrigerator. To verify that the correct cuts were made during the digestion phase of the experiment, an additional digestion was carried out to identify both the plasmid pJT1 and the two flanking gene fragments of genes D11S_1357 and D11S_1355. To do so, 1 µg of each plasmid was digested with the appropriate restriction enzymes (Table 5).

Table 5. Construct Digestion

Digestion 1	Digestion 2	Digestion 3	Digestion 4	Digestion 5	Digestion 6	Digestion 7
1 µg Sample 1	1 µg Sample 2	1 µg Sample 3	1 µg Sample 4	1 µg Sample 5	1 µg Sample 6	1 µg Sample 7
1 µL *Not*I	1 µL *Not*I	1 µL *Not*I	1 µL *Not*I	1 µL *Not*I	1 µL *Not*I	1 µL *Not*I
1 µL *Pst*I	1 µL *Pst*I	1 µL *Pst*I	1 µL *Pst*I	1 µL *Pst*I	1 µL *Pst*I	1 µL *Pst*I
1/10 total µL Buffer 3.1	1/10 total µL Buffer 3.1	1/10 total µL Buffer 3.1	1/10 total µL Buffer 3.1	1/10 total µL Buffer 3.1	1/10 total µL Buffer 3.1	1/10 total µL Buffer 3.1
Remaining µL Water	Remaining µL Water	Remaining µL Water	Remaining µL Water	Remaining µL Water	Remaining µL Water	Remaining µL Water

G. Sequencing

Using the same conditions referenced in part B; gel electrophoresis is conducted with the samples and observed under UV light. Samples showing DNA bands at both 3.8 KB (indicating pJT1) and 1400 base pairs (indicating fragments TJ24/25 and TJ26/27) were selected and prepared for sequencing. For sequencing, 400 ng of the plasmid were prepared as well as primer for the DNA sequencing process, MDJR 263.

Table 6. Set-up for Plasmid Sequencing

Sample
400 ng Construct
1 µL Primer MDJR 263
Remaining µL Water
= 12 µL Total

Data from The Center for Genetics and Molecular Medicine Sequencing Service of the University of Louisville will suggest whether or not there was successful ligation of plasmid pJT1 and genomic DNA of *A. actinomycetemcomitans* by identifying specific genomic sequences unique to the flanking genes D11S_1357 and D11S_1355 and the plasmid pJT1. Stocks of *E. coli* containing the full construct were grown overnight,

prepared for storage and stocked in an -80°C freezer.

H. Electroporation of Plasmid Construct into *A. actinomycetemcomitans*

Plasmid pJT1 was isolated from the strains of *E. coli* containing the complete construct using the Qiagen 'Spin Miniprep Kit' following the protocol provided by the manufacturer. Amounts of up to 20 μg of the plasmid were electroporated into competent *A. actinomycetemcomitans* using the BioRad 'Micropulser' on the setting 'EC1' in a 0.1 cm cuvette (1.8kV). The cells were then quickly nourished with 500 μL of SOC media (Table 4), incubated at 37°C for one hour under anaerobic conditions and distributed onto a BHI/Spectinomycin agar plate in 3 μL droplets. 48 hours later, spectinomycin resistant colonies were collected, grown overnight in 50 mL of BHI supplemented with spectinomycin, prepared for storage and stocked in a -80°C freezer.

I. Passaging

Successfully electroporated *A. actinomycetemcomitans* will present spectinomycin resistant growth. To verify successful integration, the genomic DNA of a colony was analyzed with diagnostic primers (Table 7) via PCR (Figure 8). Spectinomycin resistant *Aa* was inoculated into 1 mL of BHI supplemented with 1 μL of spectinomycin and incubated overnight at 37°C. The following day, 5 μL was taken from the overnight culture, placed into a fresh microcentrifuge tube containing the BHI/Spectinomycin and incubated overnight. The next 3 days, 5 μL was taken from each previous passage and passaged into just 1 mL of BHI and incubated overnight. After the third passage in BHI, 5 μL was passaged into 1 mL of Tryptone Yeast Extract (TYE) broth and the process was repeated for the next 3 days. On the ninth day, 5 μL was taken from the previous passage and inoculated into 1 mL TYE containing 1 μL, or 1%,

Isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG induces the transcription of the *sacB* gene of pJT1, which encodes levanesucrase. Levanesucrase is lethal to gram-negative bacteria. Any growth after the addition of IPTG provides a strong selection for *A. actinomycetemcomitans* cells that have undergone double recombination to eliminate *sacB*. To select the bacteria that have undergone this double recombination, cultures were diluted to $1x10^{-5}$ the original amount and streaked onto TYE/1% IPTG/40% sucrose agar plates and incubated at 37°C under anaerobic conditions for 48 hours.

J. Double Recombinant Selection

Sucrose resistant colonies were replica plated onto to TYE/1% IPTG/40% Sucrose agar plates and BHI/Spectinomycin agar plates. The purpose of doing so was to confirm and select the desired phenotype observed on the primary plate, in this case sucrose resistant/spectinomycin sensitive *Aa* colonies. The plates were incubated again for 48 hours at 37°C under anaerobic conditions. Sucrose-resistant/spectinomycin sensitive colonies were collected, grown overnight in 10 mL BHI, prepared for storage and placed in a -80°C freezer.

Colonies were grown overnight in 1 mL of BHI, centrifuged and resuspended in 1 mL of purified water three times to obtain usable genomic DNA of any potential mutant. The PCR conditions were similar to the protocol described in part B, however the initial denaturation phase is increased to two minutes to verify cell lysis of the colony samples obtained.

Colony PCR:

- 2 μL Colony gDNA
- 2 μL M7 (F)

- 2 µL M6 (R)
- 10 µL Radiant 5X HiFI Ultra Reaction Buffer
- 0.5 µL Radiant HiFi Ultra Polymerase (2u/µL)
- 33.5 µL Water

The PCR products were observed under UV light after gel electrophoresis (same conditions as part B). Diagnostic primers were created to observe the possibility of the deletion of gene D11S_1356. Forward primer M7 began shortly upstream of the original upstream fragment of D11S_1357 and reverse primer M6 began shortly downstream of the original downstream fragment of gene D11S_1355. The wild type strain should show a size of around 2800 base pairs, any colony PCR that resulted in a reduced size indicates a mutation and will be collected, grown up overnight in BHI and stocked in a -80° freezer.

Table 7. Diagnostic Primers

Primer Name	Sequence 5'-3'
M7 (F)	GACGATTTTGCACCCGAAGTGCAA
M6 (R)	GGTGCAAATAAATCTTCTAAAAACACAAATGCC

PART II

WT/ΔFEPA GROWTH STUDIES

Outer membrane receptor, FepA, recognizes iron in the extracellular spaces and imports the resource into the periplasmic space of *Aggregatibacter actinomycetemcomitans*. Observing the effects on the growth of ΔfepA strains of *Aa* in an environment supplemented with iron and stress hormone may support the hypothesis of the role of the enterobactin operon in iron acquisition.

A. Preparation

Stocks of ΔfepA strains were inoculated into 50 mL of BHI and incubated

overnight at 37°C under anaerobic conditions. They were then centrifuged, resuspended in 1 mL BHI/1 mL glycerol and stored in a -80°C freezer.

B. Growth Study

Twelve 50 mL conical tubes will be prepared for each strain with triplicates of each of the four conditions. The four conditions are: CDM, CDM supplemented with 100 μM ferrous chloride, CDM supplemented with 50 μM norepinephrine, and CDM supplemented with both 100 μM ferrous chloride and 50 μM norepinephrine. Each conical tube will receive 200 μL of the appropriate strain of *A. actinomycetemcomitans*, 20 μL of 100 μM ferrous chloride into each tube that requires iron and 20 μL of 50 μM norepinephrine into each tube that calls for catecholamine.

The growth of each strain of *A. actinomycetemcomitans* in each of its conditions was checked at 4, 12, 24, 36, and 48 hours by a spectrophotometer at wavelength of 600 nm. To do so, 4 mLs from each conical tube was taken and placed into a 10 mL conical tube, centrifuged and resuspended in 1 mL of BHI. The bacterial concentrations were then measured at a setting of OD600. Each of the triplicates from the two strains in their four conditions were compared to a blank of 1 mL BHI. The data for each triplicate was averaged and compared to the other strain.

C. ΔFepA Growth With Transferrin

Wild type and *ΔfepA* strains of *A. actinomycetemcomitans* were grown over night in 10 mL of BHI, centrifuged and resuspended in 10 mL of CDM. Both strains of *A. actinomycetemcomitans* were inoculated into a solution of CDM and 100 μM/mL holo-Transferrin (1,200 – 1,700 ppm iron content) and CDM supplemented with both 100 μM holo-Transferrin and 50 μM norepinephrine. A 48-well plate was used for this study.

Each well will receive 1 mL of each environment for both wild type and ΔfepA strains of *A. actinomycetemcomitans,* in triplicate. Readings were taken by a plate reader and incubated at 37°C under anaerobic conditions and observed at 4, 12, 24, 36, and 48 hours.

E. Apo-Transferrin Control

Wild type and *ΔfepA* strains of *A. actinomycetemcomitans* were grown over night in 10 mL of BHI, centrifuged and resuspended in 10 mL of CDM. Both strains of *A. actinomycetemcomitans* were inoculated into a solution of CDM and 100 μM apo-Transferrin (<0.005% iron content) and CDM supplemented with both 100 μM apo-transferrin and 50 μM norepinephrine. A 48-well plate was used to harbor the bacteria, each well receiving 1 mL of each environment for both wild type and ΔfepA strains, in triplicate. The 48-well plate was incubated at 37°C under anaerobic conditions and measurements of growth were obtained by plate reader at 4, 12, 24 and 36 hours.

CHAPTER III

RESULTS

PART I: INSERTIONAL MUTATION

The first step in constructing the pJT1 construct was amplifying the flanking genes of the gene of interest, D11S_1356, by PCR. Following the PCR protocol using *Aa* gDNA template and designed primers, the concentration of the PCR product after purification was: TJ24 + TJ25 = 207.3 ng/µL (Figure 5); TJ26 + TJ27 = 106.3 ng/µL (Figure 6).

Figure 6. Upstream PCR Product (≈550 bp)

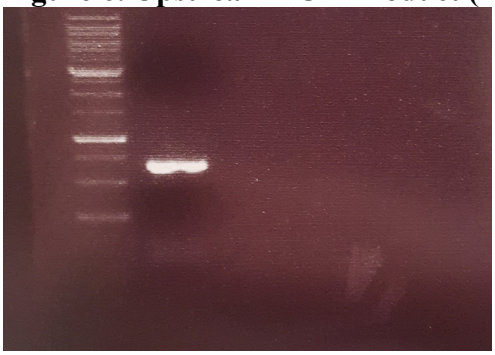


Figure 7. Representation of Upstream PCR Amplification.

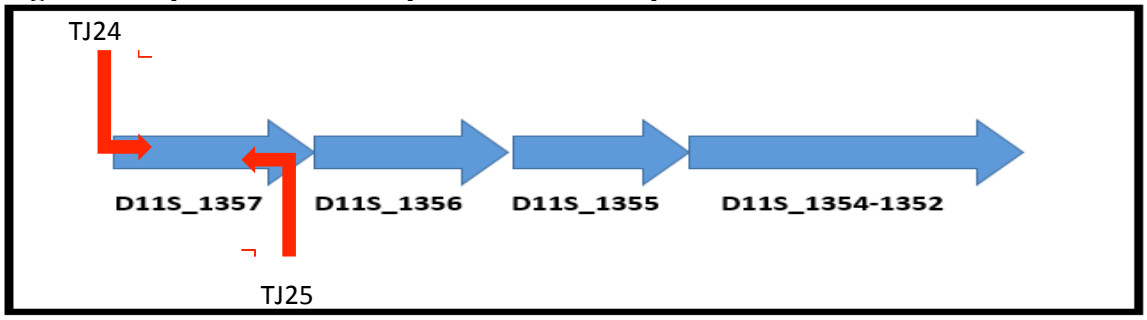


Figure 8. Downstream PCR Product (≈850 bp)

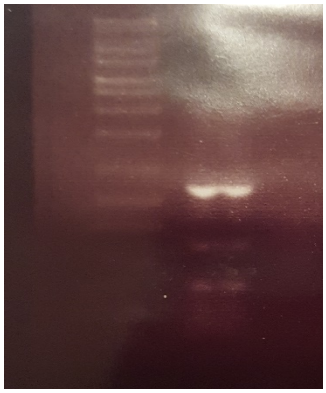
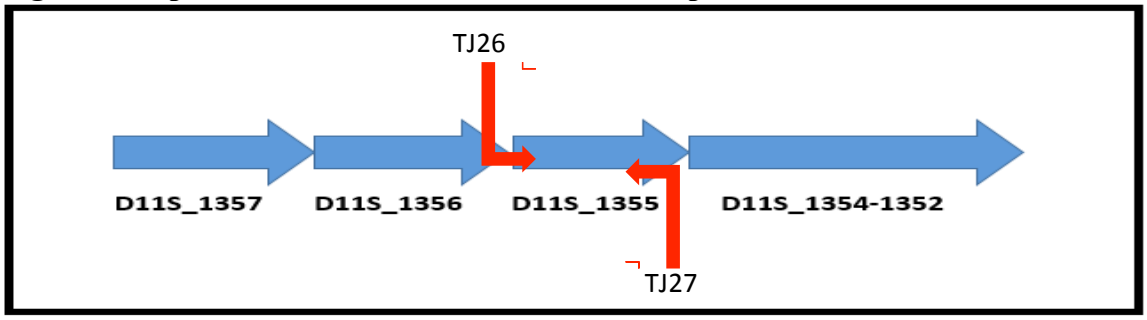


Figure 9. Representation of Downstream PCR Amplification



Following the PCR process, pJT1 and the PCR products were digested with specific restriction enzymes, which make cuts in targeted sequences of the DNA. Amounts of up to 1 µg of PCR product were digested with the restriction enzymes *Not*I and *Xho*I for the upstream region amplified by primers TJ24,25 and *Xho*I and *Pst*I for the downstream region amplified by primers TJ26,27. A 1µg concentration of pJT1 was also used and digested with restriction enzymes *Not*I and *Pst*I. This proposes two possible insertion points of plasmid pJT1 once the products are ligated together, pJT1 inserted into either upstream gene D11S_1357 or downstream gene D11S_1355.

Results from the digestion process were computed into the equation used for setting up the ligation process: X = (10ng vector)(bp insert)/(3.8bp pJT1). The

concentration of the digestion product of pJT1 was 37.4 ng/μL. Taking 1μL of the digestion into 3.74 μL of water diluted it to 10ng/μL. The fragment of TJ24,25 was a size 547 base pairs, which computed a 1.44 ng requirement. To increase the chances of a successful ligation, a high flanking region to plasmid ratio was used. Any concentration up to a 100:1 ratio that did not result in successful ligation would require repeating the PCR and digestion processes, even redesigning PCR primers. The concentrations obtained allowed for up to an 80:1 ratio and thus, 4.88μL (115 ng) of Digestion 1 was taken for the ligation. Likewise, the fragment of TJ26,27 was 856 base pairs, computing a 2.25 ng requirement. Again, an 80:1 concentration was used, calculating to 6.29 μL (179.89 ng) of Digestion 2 for ligation.

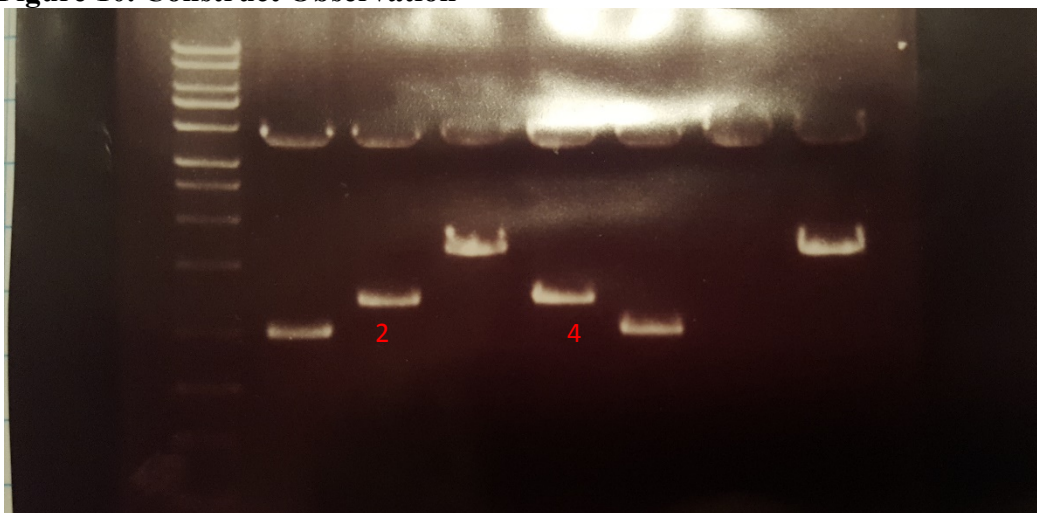
The components were combined to give a total volume of 15 μL as follows:

- 80:1 TJ24+25 4.88 μL
- 80:1 TJ26+27 6.29 μL
- 1:3 pJT1 1 μL
- T4 DNA Ligase 0.8 μL
- T4 DNA Ligase Buffer 1.5 μL
- Water 0.53 μL

The ligation was electroporated into competent *E. coli* plated onto LB/spec agar plates where seven colonies grew, indicating successful integration. Plasmid pJT1 is not suicidal in *E.coli* and allowed for quantities of the construct to be accumulated and stored. The plasmids that were isolated from the spectinomycin resistant colonies were re-digested with the upper restriction enzyme used, *Not*I, and the lowermost restriction enzyme that was used, *Pst*I. This would identify the genomic quantity between the cuts

that were made in the primary digestion. The product of this digestion was then observed under UV light.

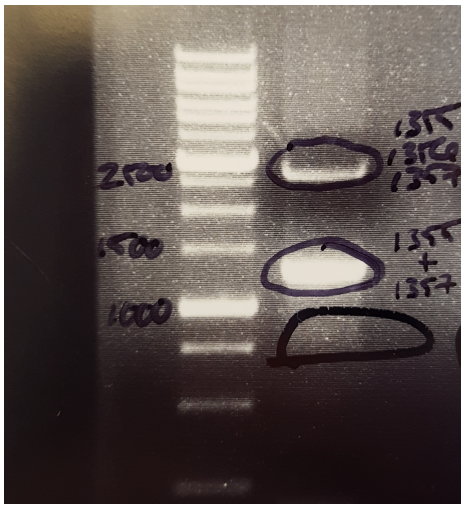
Figure 10. Construct Observation



The upper DNA marker displays the appropriate genomic quantity for plasmid pJT1 at 3,800 nucleotide base pairs. Of the seven samples, samples numbers 2 and 4 (table 10) were identified displaying insert fragments consistent with the anticipated DNA insert of around 1,400 base pairs. These constructs were prepared and sent for sequencing. The data received from the sequencing department identified the upstream and downstream fragments D11S_1357 and D11S_1355, suggesting a successful ligation with plasmid pJT1.

This construct was then electroporated into electrocompetent *A. actinomycetemcomitans* resulting in five spectinomycin resistant colonies, indicating a single recombination. Genomic DNA of the spectinomycin resistant colonies was obtained using Promega 'DNA Purification System Miniprep' and diagnostic primers were used to sequence the DNA within the parameters (Table 7). The PCR identified both the D11S_1357/1355 fragment at approximately 1,400 base pairs, and the homologous chromosome of the host, D11S_1357/1356/1355 (Figure 8).

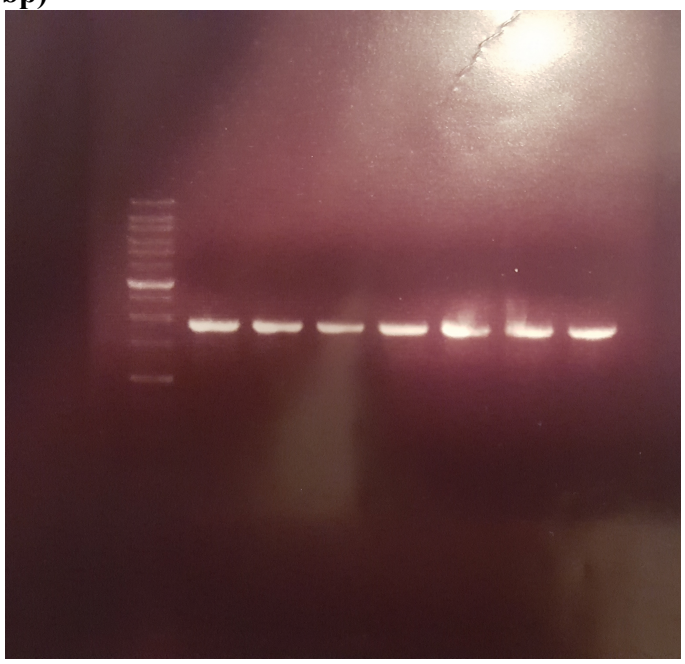
Figure 11. Colony PCR After Integration Displaying Host Chromosome and Construct.



.

These colonies then began the passaging phase to select for the colonies that had undergone a double recombination by presence of growth in sucrose. The colonies that grew in the presence of sucrose were diluted and plated on a TYE/IPTG/40% sucrose plate. After incubation, the colonies that grew were replicate plated onto another TYE/IPTG/40% sucrose plate and a BHI/spectinomycin plate. Again, after incubation, 48 colonies that expressed spectinomycin sensitivity and sucrose resistance were collected [insert picture]. The diagnostic primers M6 and M7 were used (Table 7), with the forward primer M7 shortly upstream of the original upstream fragment and the reverse primer M6 shortly after the original downstream fragment to verify that gene D11S_1356 had been deleted. However, colony PCR had revealed the same DNA amount as the wild type, signifying that the colonies had reverted to wild type (Figure 9).

Figure 12. Colony PCR Post Passaging Process, Displaying Wt DNA Size (≈2800 bp)



To determine whether the plasmid had been maintained in these clones, sucrose resistant colonies were grown in BHI supplemented with spectinomycin. Interestingly, growth did occur, meaning that spectinomycin resistance was being retained in these strains of *A. actinomycetemcomitans*. A plasmid miniprep was conducted, resulting in a low concentration of 10 ng/µL of plasmid. To verify that the plasmid pJT1 had not mutated, the same stock used in the ligation process above and a new stock of pJT1 was electroporated into competent *A. actinomycetemcomitans*. Indeed, no growth occurred, which verifies that the plasmid pJT1 had not been altered before its electroporation into *A. actinomycetemcomitans*.

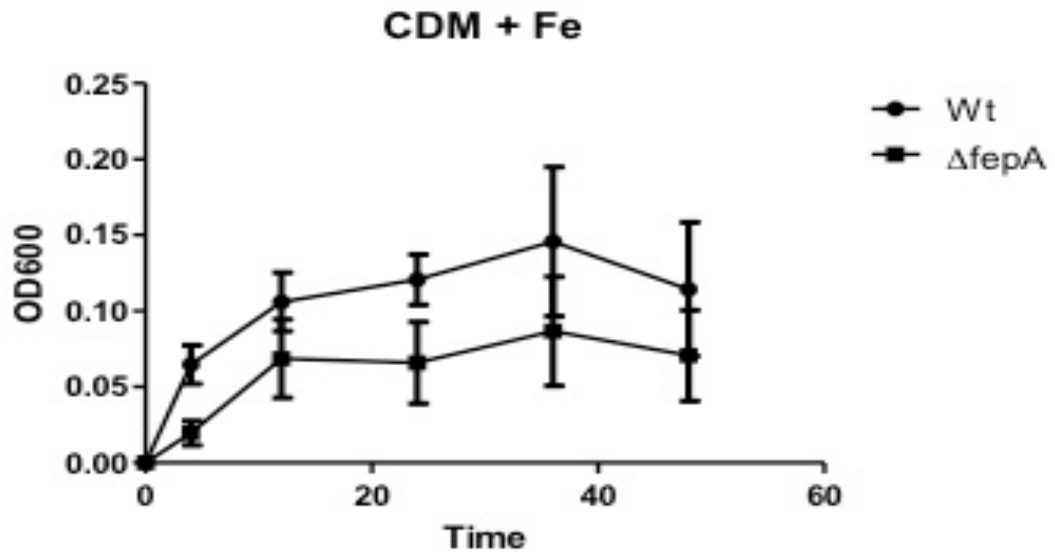
PART II: Wt/ΔfepA Growth Study

Although Δpermease strains of *A. actinomycetemcomitans* were not obtained, this study was intended to help answer the question of the relationship between the enterobactin operon and the presence of iron-catecholamine complexes. With a mutation in the FepA outer membrane receptor, the results are anticipated be similar to Δpermease strains due to the inability to import and utilize the complex. Comparing the growth of the wild type strain of *A. actinomycetemcomitans* and the ΔfepA strain, results indicate that the ΔfepA strain is effected by the mutation.

A. *Aa* Strains Supplemented with Iron Source

Over the course of the 48 hours that the growth curves were observed, the wild type strain grew on average 45.4% more than the ΔfepA strain. A repeated measure ANOVA (RM-ANOVA) was conducted to observe the means between the two strains. Overall, there was significance ($p < 0.05$) between the two strains with a p-value of 0.0247. To analyze each time point, a two-way ANOVA test was also conducted, which confirmed significance to take place at the 4-hour (p-value = 0.007) and 24-hour (p-value = 0.039) mark. These results suggest that the ΔfepA strain is not as effective in acquiring free iron compared to the wild type strain and indicates the role FepA serves in the cell's vitality.

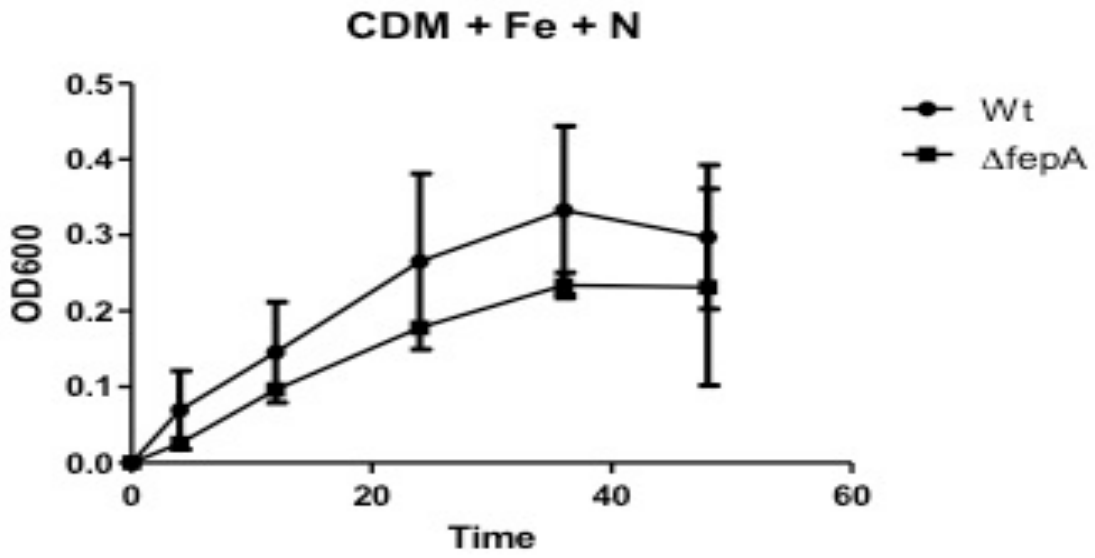
Figure 13. Growth Curve: CDM + 100μM Fe



B. *Aa* Strains Supplemented with Iron Source and Stress Hormone

A RM-ANOVA was also conducted on the growth curves that were both supplemented with iron and norepinephrine. Over the course of 48 hours, there was no significance in the means (p-value = 0.22). To further analyze, a two-way ANOVA test was conducted on each of the five time points and confirmed that there was no significance in the variances of the samples. Although there was no significance between the two strains over the 48-hour period, the wild type strain grew 38.4% better than the ΔfepA strain, further demonstrating the important role FepA may play in iron acquisition [Figure 11].

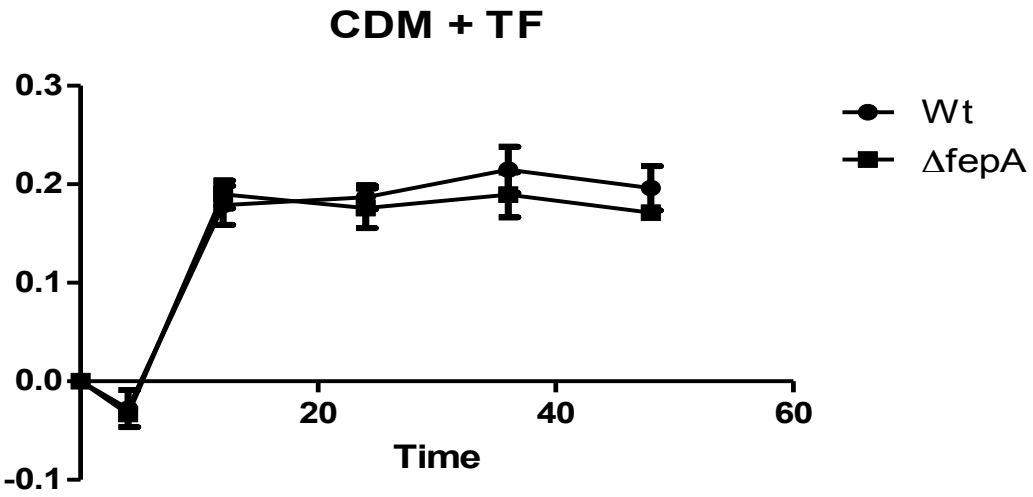
Figure 11. CDM + 100 µM Fe + 50 µM Norepinephrine



C. *Aa* Strains Supplemented with Loaded Iron Source, holo-Transferrin

In vivo, bacteria rarely come across free iron ions. Instead, ironbound molecules such as lactoferrin and transferrin circulate through the blood stream. To create an environment similar to this, the iron source from the previous experiment was replaced with iron-loaded holo-Transferrin. A RM-ANOVA was conducted on the growth curve of wild-type strain *A. actinomycetemcomitans* versus the ΔfepA strain, resulting in no significance difference in the means (p-value = 0.28) between the two strains. To further analyze the data at each time point, a two-way ANOVA was also conducted to confirm no significance in the variation between the two strains over the course of the 48-hour period. This was expected, as seen in other studies [26], with no stress hormone to encourage the shuffling of iron bound to transferrin, neither strain of *A. actinomycetemcomitans* was able to acquire the ion.

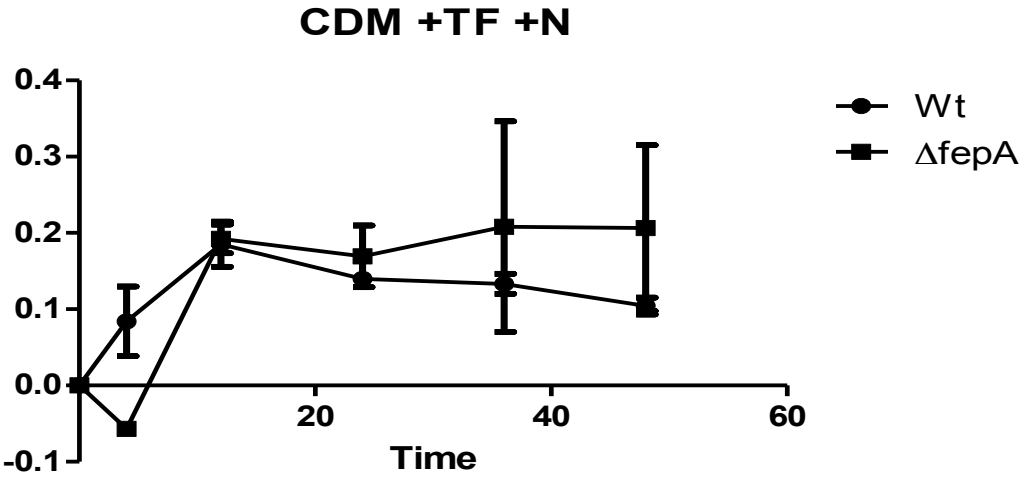
Figure 12. CDM + 100 µM holo-Transferrin



D. *Aa* Strains Supplemented with Loaded Iron Source, holo-Transferrin and Stress Hormone

The stress hormone norepinephrine was then added to the environment. A RM-ANOVA was conducted on the two strains both supplemented with holo-transferrin and the catecholamine norepinephrine resulting in no significance (p-value = 0.63) between the two strains. To further analyze the strains at each time point, a two-way ANOVA was conducted. It identified significance in variance at the 4-hour mark with a p-value of 0.02, however, all other time points were not significant. Further experimentation with ΔfepA strains of *A. actinomycetemcomitans* is encouraged, but the results show that for at least the first twelve hours, the wild type strain was more able to thrive in that environment then the ΔfepA strain.

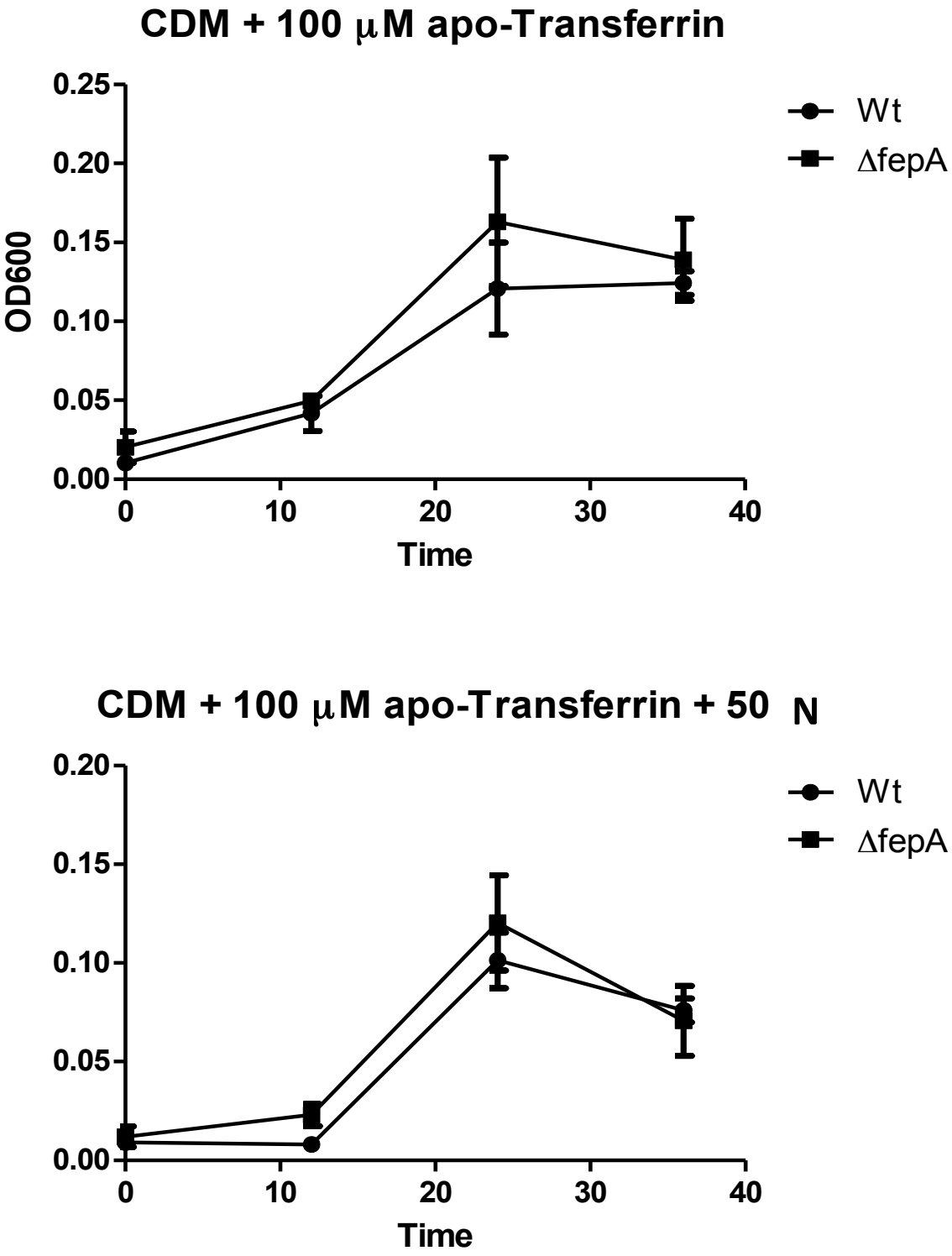
Figure 13. CDM + 100 µM holo-Transferrin + 50 µM Norepinephrine



E. Unloaded Iron Source Control

A RM-ANOVA was conducted on the two environments to observe the controlled experiments. The two environments of CDM supplemented with 100 µM apo-Transferrin and CDM supplemented with both 100 µM apo- transferrin and 50 µM norepinephrine displayed p-values of 0.7326 and 0.7613, respectively. There was also no significance in the means, which was expected with a control group. For the 36 hours that were recorded, both strains of *A. actinomycetemcomitans* grew similarly in both environments (Figure 15).

Figure 15. Apo- Transferrin Control Graphs



CHAPTER IV

DISCUSSION

The two-component system called QseBC in *A. actinomycetemcomitans* is stimulated in the presence of iron-catecholamine complexes. Without QseBC, biofilm growth is significantly decreased, displaying the importance the two-component system plays in the pathogenicity of the bacteria.

Bacteria are able to acquire iron through the synthesis and secretion of siderophores, molecules that bind to iron and bring it into the cell to be utilized. One of the common siderophores synthesized is enterobactin, a catechol based siderophore.

Studies have shown that catecholamines, such as norepinephrine, may act as pseudosiderophores and are able to strip iron from ironbound molecules like transferrin or lactoferrin. This iron-catecholamine complex can serve as a mediator for iron acquisition for bacteria with enterobactin acting as the final iron acceptor. However, those studies show that enterobactin must be present for the bacteria to be able to acquire the iron [26].

Interestingly, *A. actinomycetemcomitans* does not synthesize enterobactin, but possesses and encodes for the machinery that recognizes enterobactin and transports it into the cell. This lead to the hypothesis that *A. actinomycetemcomitans* is able to acquire iron through the enterobactin receptor and transporter it possesses without having to synthesize enterobactin itself, by stress hormone mediation. To study this, attempts were made to create mutant strains of *A. actinomycetemcomitans*, in the hopes of creating a

deletion mutation in the gene that encoded the permease of the enterobactin ABC family transporter. However, we encountered complications in constructing the Δpermease strain. After the electroporation of plasmid pJT1 into competent *A. actinomycetemcomitans,* spectinomycin resistance was observed. This suggested that successful insertion of the plasmid occurred into the *A. actinomycetemcomitans* genome because the plasmid codes for the resistance to spectinomycin, while wild type *A. actinomycetemcomitans* does not and pJT2 should not be maintained in the recombinant clones.

Double recombination was selected for by the observation of growth in the presence of sucrose. Sucrose activates the *SacB* gene in plasmid pJT1, creating polymers of carbohydrates that are lethal to gram-negative bacteria. Growth did occur, as well as on the agar plates containing sucrose and IPTG. This phenotype suggests that a recombination event occurred that deleted plasmid sequences including *sacB*. However, after analyzing the sucrose resistant colonies, colony PCR indicated they had reverted back to wild type, but also displayed a resistance to spectinomycin they did not display before. The reasoning behind this phenomenon and acquiring true Δpermease strains of *A. actinomycetemcomitans* will require additional research.

Strains of a truncated mutant strain of *A. actinomycetemcomitans* in the FepA outer membrane receptor were acquired by another student in the lab. This mutation should lead to an alteration in the recognition of iron-catecholamine complexes, thereby displaying a phenotype similar to a deletion of the permease. To study this, wild-type and ΔfepA strains of *A. actinomycetemcomitans* were inoculated into media containing free iron as well as iron and norepinephrine. The results showed that the ΔfepAstrains had a

more difficult time growing in both environments. At some time during the study of the growth curve, the wild type grew significantly better than the ΔfepA strain. Although at other times there was no significance between the two strains, overall the wild type strain grew 45.4% more in CDM supplemented with iron and 38.4% more in CDM supplemented with both iron and norepinephrine over the 48-hour period. There are other systems for *A. actinomycetemcomitans* to acquire iron [33], however the enterobactin operon is the most peculiar, because of its relationship with the siderophore it doesn't synthesize as mentioned above. The growth reduction that occurred in the ΔfepA strain supports the hypothesis of the efficient role of the enterobactin operon in *A. actinomycetemcomitans* iron acquisition and the presence of stress hormone.

However, free iron is not usually present in vivo. To create an environment similar to the oral cavity, the iron source was replaced with transferrin. Comparing the growth of wild type and mutant strains in the presence of just holo-Transferrin, there was little difference between them. This is expected, as there is no stress hormone present to encourage the transferrin to unload its iron content. When the stress hormone norepinephrine was present, there was a significant difference in growth observed in the first twelve hours, specifically at the 4-hour mark. This suggests that the wild-type strain is also able to better utilize the iron-catecholamine complexes than the ΔfepA strain.

These results can lead to other experiments concerning the disruption of the enterobactin operon. *A. actinomycetemcomitans* is in a great position when the host responds to its presence with the immune response. Inflammation causes stress hormones to be present and cells related to the immune response such as neutrophils release additional stress hormones and ironbound molecules. With *A. actinomycetemcomitan's*

ability to evade the immune response, the presence of both stress hormone and ironbound molecules puts the bacteria in a perfect position for catecholamine-mediated iron acquisition.

Furthermore, the relationship between the enterobactin operon, iron acquisition and the two-component system QseBC can be further studied once a successful deletion of the permease gene is achieved. With the evidence that QseBC is activated in the presence of iron-catecholamine complexes and is needed for virulent properties of *Aggregatibacter actinomycetemcomitans,* studying the expression of *qseBC* may be the link between the enterobactin operon and iron acquisition. Certain genes are affected when QseBC is activated, with iron uptake genes down regulated. This may be because the bacteria has sensed an iron rich environment and diverts energy to expressing other useful genes, such as metabolism and respiration [21]. A rt-PCR comparing the expression of *qseBC* in wild type and Δpermease strains of *A. actinomycetemcomitans* in the presence of iron and stress hormone may reveal one of the roles of the enterobactin operon.

Strains of *A. actinomycetemcomitans* with a disruption in the enterobactin operon such as ΔfepA or Δpermease strains could also be studied in a mouse model to determine its effect on periodontal disease. If it could be shown that the onset or degree of localized aggressive periodontitis is decreased, the outer membrane receptor FepA or the intermembrane permease could be the target of therapeutic agents.

REFERENCES

1. Juárez-Rodríguez MD, Torres-Escobar A, Demuth DR. *Construction of new cloning, lacZ reporter and scarless-markerless suicide vectors for genetic studies in Aggregatibacter actinomycetemcomitans*. Plasmid, 2013. 69(3): p. 211–22.
2. Armitage, G.C., *Clinical evaluation of periodontal diseases.* Periodontol 2000, 1995. 7: p. 39-53.
3. Eke, Paul I. et al. *Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 – 2012*. J Periodontol, 2015. 86(5): p. 611–622.
4. Wall T, Vujicic M. *U.S. dental spending continues to be flat. Health Policy Institute Research Brief.* American Dental Association. 2015.
5. Kinane, D. and Marshall, G. *Periodontal manifestations of systemic disease.* Australian Dental Journal, 2001. 46: p. 2–12.
6. Nair, S.P., et al., *Bacterially induced bone destruction: mechanisms and misconceptions.* Infect Immun, 1996. 64(7): p. 2371-80.
7. Wilson, M. *Biological activities of lipopolysaccharides from oral bacteria and their relevance to the pathogenesis of periodontitis*. Sci. Prog, 1995. 78:19–34.
8. Hanazawa, S., K. Hirose, Y. Ohmori, S. Amano, and S. Kitano. *Bacteroides gingivalis fimbriae stimulate production of thymocyte-activating factor by human gingival fibroblasts*. Infect. Immun, 1988. 56: p. 272–274.
9. Hanazawa, S., Y. Murakami, K. Hirose, S. Amano, Y. Ohmori, H. Higuchi, and S. Kitano. *Bacteroides (Porphyromonas) gingivalis fimbriae activate mouse peritoneal macrophages and induce gene expression and production of interleukin-1*. Infect. Immun, 1991. 59: p. 1972–1977.
10. Hanazawa, S., Y. Murakami, A. Takeshita, H. Kitami, K. Ohta, S. Amano, and S. Kitano. *Porphyromonas gingivalis fimbriae induce expression of the neutrophil chemotactic factor KC gene of mouse peritoneal macrophages: role of protein kinase C*. Infect. Immun, 1992. 60: p. 1544–1549.
11. Kolenbrander PE, London J. *Adhere today, here tomorrow: oral bacterial adherence.* Journal of Bacteriology, 1993. 175(11): p. 3247-3252.
12. Li, J., Helmerhorst, E.J., Leone, C.W., Troxler, R.F., Yaskell, T., Haffajee, A.D., Socransky, S.S. and Oppenheim, F.G. *Identification of early microbial colonizers in human dental biofilm*. Journal of Applied Microbiology, 2004. 97: p. 1311–1318.
13. Holt, S. C. and Ebersole, J. L. *Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis*. Periodontology 2000, 2005. 38: p. 72–122.
14. Stillman, N., Tanner, A. *Oral and dental infections with anaerobic bacteria: clinical features, predominant pathogens, and treatment*. Clin Infect Dis, 1993. 16 (Suppl 4): S304-S309.
15. Henderson, B., Ward, J., Ready, D. *Aggregatibacter (Actinobacillus) actinomycetemcomitans: a triple A* periodontopathogen?* Periodontol 2000, 2010. 54: p. 78-105.

16. Celenligil H. and Ebersole J.L. *Analysis of serum antibody responses to periodontopathogens in early-onset periodontitis patients from different geographical locations*. J Clin Periodontol, 1998. 25: p. 994-1002.
17. Fine, Daniel H., Markowitz, Kenneth., Furgan, David., Fairlie, Karen., Ferrandiz, Javier., Nasri, Cebile., McKiernan, Marie., Gunsolley, John. *Aggregatibacter actinomycetemcomitans and its Relationship to Initiation of Localized Aggressive Periodontitis: Longitudinal Cohort Study of Initially Healthy Adolescents*. J. Clin. Microbiol. 2007. Vol. 45. 12: p. 3859-3869.
18. Haubek D., Dirienzo, JM., Tinoco EM., Westergaard J., Lopez, NJ., Chung, CP., Poulsen, K., Kilian, M. *Racial Tropism of a Highly Toxic Clone of Actinobacillus actinomycetemcomitans Associated with Juvenile Periodontitis*. J Clin Microbiol, 1997. 35: p. 3037-3042.
19. Sandrini, Sara M., Shergill, Raminder, Woodward, Jonathan, Muralikuttan, Remya, Haigh, Richard D., Lyte, Mark, Freestone, Primrose P. *Elucidation of the Mechanism by Which Catecholamine Stress Hormones Liberate Iron from the Innate Immune Defense Proteins Transferrin and Lactoferrin*. J Bacteriol. 2010, p. 587-594.
20. Rajvir, Malik, Radha, Changela, Prema, Krishan, Shalini, Gugnami, Deepika, Bali. *Virulence factors of Aggregatibacter actinomycetemcomitans – A status update.* J ICDRO, 2015. 7(2): p.137-145.
21. Weigel, W.A. and Demuth, D.R. *QseBC, a two-component bacterial adrenergic receptor and global regular of virulence in Enterobacteriaceae and Pasteurellaceae.* Mol Oral Microbiol. 2016. 31(5): p. 379-397.
22. Clarke, M., Hughes, D., Zhu, C., Boedeker, E., Sperandio, V. *The QseC sensor kinase: A bacterial adrenergic receptor*. PNAS, 2006. 103(27): p. 10420-10425.
23. Steele, K., O'Connor, L., Burpo, N., Kohler, K., Johnston, J. *Characterization of a Ferrous Iron-Responsive Two-Component System in Nontypeable Haemophilus influenzae*. J Bacteriol, 2012. 194(22): p. 6162-6173.
24. Sandrini, S., Shergill, R., Woodward, J., Muralikuttan, R., Haigh, R., Lyte, M., Freestone, P. "*Elucidation of the Mechanism by Which Catecholamine Stress Hormones Liberate Iron from the Innate Immune Defense Proteins Transferrin and Lactoferrin*." J Bacteriol, 2010. 192(2): p. 587-594.
25. Juárez-Rodríguez, M., Torres-Escobar, A., Demuth, D. *ygiW and qseBC are co-expressed in Aggregatibacter actinomycetemcomitans and regulate biofilm growth*. Microbiology, 2013. 159: p. 989-1001.
26. Freestone, Primrose P.E., Haigh, Richard D., Williams, Peter H,, Lyte, Mark. *Involvement of enterobactin in norepinephrine–mediated iron supply from transferrin to enterohaemorrhagic Escherichia coli.* FEMS Microbiology Letters, 2003. 222(1): p. 39-43.
27. Lyte, M., Freestone, P. *Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health*. New York: Springer, 2010. p. 1-14.
28. Ramsey, M.M., Whiteley, M. *Polymicrobial interactions stimulate resistance to hose innate immunity through metabolite perception*. Proc Natl Acad Sci USA, 2009. 106: p. 1578-1583.
29. Schmid-Hempel P. *Immune defense, parasite evasion strategies and their relevance for "macroscopic phenomena" such as virulence*. Philosophical

Transactions of the Royal Society B: Biological Sciences, 2009. 364(1513): p. 85-98.
30. DiFranco, K.M., Gupta A., Galusha, L.E., Perez, J., Nguye, T.V., Fineza, C.D., Kachlany S.C. *Leukotoxin (Leukothera®) targets active leukocyte function antigen-1 (LFA-1) protein and triggers a lysosomal mediated cell death pathway*. *J Biol Chem*, 2012. 287(21): p. 17618-27.
31. Hienz, Stefan A., Paliwal, Sweta, Ivanoovski, Saso. *Mechanisms of Bone Resorption in Periodontitis*. J Immunol Res, 2015. 2015:615486.
32. Jiang Y, Mehta CK, Hsu T-Y, Alsulaimani FFH. *Bacteria Induce Osteoclastogenesis via an Osteoblast-Independent Pathway*. Infection and Immunity, 2002. 70(6): p. 3143-3148.
33. Rhodes, E. R., Tomarus, A. P., McGiIlivary, G., Connerly, P. L., Actis, L,A. *Genetic and Functional Analyses of Actinobacillus actinomycetemcomitans AfeABCD Siderophore-Independent Iron Acquisition System*. Infection and Immunity, 2005. 73: p. 3758-3763.
34. Nath SG, Raveendran R. Microbial dysbiosis in periodontitis. *Journal of Indian Society of Periodontology*. 2013;17(4):543-545.
35. Novak, Elizabeth A., Shao, HanJuan, Daep, Carlo Amorin, Demuth, Donald R. *Autoinducer-2 and QseC Control Biofilm Formation and In Vivo Virulence of Aggregatibacter actinomycetemcomitans*. Infection and Immunity. 2010, p. 2919-2926.
36. Chen, C., Wang, T., Chen, W. *Occurrence of Aggregatibacter actinomycetemcomitans serotypes in subgingival plaque from United States subjects.* Mol Oral Microbiol. 2010. 25(3): 207- 214.
37. Brigido, Jandenilson A., Silveria, Virginia R.S. da., Rego, Rodrigo, O., Nogueira, Nadia A. P. *Serotypes of Aggregatibacter actinomycetemcomitans in relation to periodontal status and geographic origin of individuals – a review of the literature.* Med Oral Pathol Oral Cir Bucal. 2014. 19(2): e184-e191.
38. Armitage, Gary C. *Development of a Classification System for Periodontal Disease and Conditions.* Ann Periodontol. 1999. Vol. 4: p. 1-6.
39. Nath SG, Raveendran R. *"What is there in a name?": A literature review on chronic and aggressive periodontitis*. Journal of Indian Society of Periodontology. 2011. 15(4): p.318-322.
40. Weigt, D., Kleber, B.M. *Acute and Chronic Necrotizing Ulcerative Gingivitis.* Stomatol DDR. 1990. 40(3): p. 117-9.
41. Reuland-Bosma, W., van Dijk, J. *Periodontal disease in Down's syndrome: a review.* J Clin Periodontol. 1986. 13(1): p. 64-73.
42. Weigel, Whitney. *Functional characterization of Aggregatibacter actinomycetemcomitans QSEBC : a bacterial adrengeric receptor and global regulator of virulence.* (2015). Electronic Theses and Dissertations. Paper 2296.
43. J.M.F.A. Fernandes, V.A. Menezes, A.J.R. Albuquerque, M.A.C. Oliveira, K.M.S. Meira, R.A. Menezes Júnior and F.C. Sampaio. *Improving Antimicrobial Activity of Dental Restorative Materials, Emerging Trends in Oral Health Sciences and Dentistry*, Prof. Mandeep Virdi (Ed.), InTech, 2015. DOI: 10.5772/59252.

CURRICULUM VITAE

NAME:	Taylor Johnson
ADDRESS:	Dept. of Oral Immunology and Infectious Disease University of Louisville School of Dentistry 501 S Preston Louisville, KY 40202
DOB:	Elizabethtown, KY – August 22, 1991
EDUCATION: & TRAINING	B.A., Biology/Psychology (Minor) University of Louisville Louisville, KY 2010 -2014
AWARDS:	National Scholar Award – University of Louisville