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**IRON ACQUISITION BY THE DENTAL PATHOGEN *AGGREGATIBACTER
ACTINOMYCETEMCOMITANS*.**

By

**Porscha LaRai Jefferson
B.A., D.M.D, University of Louisville, 2012**

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

Master of Science

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University of Louisville, School of Dentistry
Louisville, Ky**

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A Dissertation approved on

July 25, 2012

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ABSTRACT

IRON AQUISITION IN THE DENTAL PATHOGEN *AGGERGATIBACTER ACTINOMYCETEMCOMITANS*

By

Porscha LaRai Jefferson

July 25, 2012

The dental pathogen *A. actinomycetemcomitans*, a Gram- negative organism, has been associated with aggressive forms of periodontitis. *A. actinomycetemcomitans* requires iron to grow. In the host, iron-binding proteins such as transferrin, lactoferrin, hemoglobin, and ferritin, maintain a low free-iron concentration. Microorganisms, however, have evolved complex systems to efficiently harvest iron.

Th iron acquisition systems encoded in the genome of *A. actinomycetemcomitans* are likely tailored towards the specific survival strategies needed to survive in the oral biofilm. The objectives of this project were to determine the growth of *A. actinomycetemcomitans* in different iron sources and to examine the expression of the various iron uptake systems encoded by this organism under these conditions.

A. actinomycetemcomitans was grown in chemically defined media (CDM) with or without an iron chelator (dipyridyl (DIP), and supplemented with various iron forms to determine growth.

The growth of *A. actinomycetemcomitans* was decreased in a dose-dependent manner when cultured in CDM without exogenous ferrous sulfate supplemented with DIP (CD/DIP). Growth of *A. actinomycetemcomitans* was restored most efficiently when CDM/DIP was supplemented with hemin, less so with ferric citrate, and ferric chloride, and not with ferrous sulfate. Using these growth conditions, we examined the differential regulation of the numerous iron uptake systems encoded in the *A. actinomycetemcomitans* genome.

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INTRODUCTION

Periodontal Disease

Periodontal disease is a chronic progressive disease that affects various structures of the periodontal tissues including the alveolar bone and periodontal ligament. Periodontal disease has been divided into two separate categories: Gingivitis and Periodontitis. Both diseases are inflammatory conditions that are encompassed in the term periodontal disease, however the affects of both diseases are quite different. Gingivitis, the initial stage of periodontal disease, is an inflammation of the gingival tissue characterized by swelling, redness, and bleeding upon probing of the gingiva (3). Although gingivitis is reversible following proper oral hygiene, if left untreated, it can progress into the more severe, chronic inflammatory condition, periodontitis. Periodontitis always develops from gingivitis however not all gingivitis will progress into periodontitis.

Periodontitis is defined as a condition in which there is an inflammatory reaction that occurs in the periodontium resulting in the destruction of the supporting soft tissues surrounding the teeth, loss of gingival fibers of the periodontal ligament, and resorption of the alveolar bone. Periodontitis has also been further categorized into mild, moderate, and severe periodontitis based on the patients clinical attachment loss (39,40). Mild periodontitis presents with 1-2 mm of attachment loss, moderate periodontitis

presents with 3-4 mm of attachment loss, and severe periodontitis displays attachment loss greater than 4 mm. Periodontitis is even further divided into chronic or acute forms and generalized (presenting in greater than 30% of the oral cavity) or localized (less than 30% of the oral cavity is involved).

Due to the progressive nature of this condition, if periodontitis is left untreated, loosening and/or tooth loss is usually the result. Due to the destructive nature of periodontal disease, it has been a frequent interest in many research projects. It has been concluded that periodontal disease is the clinical result of a complex interaction between the host and plaque bacteria. Destruction of periodontal supporting tissues happens as a response to very intricate host-parasite interactions. The net result of this host-parasite interaction, which in an unpredictable moment, accumulate and exceed the threshold of tissue integrity thus causing tissue destruction and ultimate bone loss.

Periodontal disease is the most prevalent of bone diseases in humans and has long been implicated as a risk factor in impairment of oral functions and tooth loss. 10-15% of adults that have periodontal disease have it severely enough that it results in tooth loss, ultimately leading to mastication malfunction and ageusia. While periodontal disease is prevalent worldwide, it has had a significant impact on the United States population (9, 18, 52). Brown *et al* estimate that about 21.8% of adults (22.6 million) exhibit signs of mild periodontitis, 9.5% (9.9 million) display signs of moderate periodontitis, and about 3.1% (about 3.2 million) exhibit signs of advanced periodontitis (9). The total amount expended for periodontal preventive procedures as well as the treatment of periodontal diseases in the U.S. was \$14.3 billion in 1999 (9). Undoubtedly, innovative methods for

the prevention and treatment of periodontal disease would have a major impact on healthcare costs.

Past research has suggested periodontal disease can be complicated by certain systemic diseases, however more recently, studies suggest that the reverse may also be true (13, 28, 29, 32, 44, 46, 50) . Current research, however controversial, proposes that periodontal disease has important systemic implications, and can influence other diseases such as cardiovascular disease (28, 29, 36, 39), pulmonary disease (44, 55) diabetes (23, 32, 50), and adverse pregnancy outcomes (11, 46, 47). Thus, a better understanding of the factors that contribute to periodontal disease can be used to develop measures to improve not only oral health of the patient, but also overall health of individuals.

The onset and progression of periodontal disease is associated with several risk factors, including but not limited to gender, age, socio-economic status, nutrition, stress, genetic makeup, and tobacco smoke. Though there are several risk factors, tobacco smoke has been considered one of the most important risk factors associated with periodontal disease (54). It is theorized that there is a positive correlation between increased tobacco usage in the population and increased numbers of periodontal infections (33, 52). A dose-dependent correlation has been demonstrated between the frequency of an individual smoker and the severity of the deteriorating periodontal health of that individual (6). Smokers are extremely more susceptible to periodontitis, with increased alveolar bone resorption (24), attachment loss, percentage of oral sites with significant attachment loss (25), tooth mobility, and tooth loss (42) than non smokers. Additionally, patients who smoke are more often refractory to treatment than non-smokers (49). Despite conflicting data that tobacco smoke may not influence the sub-

gingival microflora (22, 10), recent data strongly indicates that tobacco-induced susceptibility to periodontitis is correlated with populational shifts in the microbial composition of the oral biofilm (15, 19, 26). For example, Umeda *et al* showed that there is an increased risk of *Treponema denticola* inhabiting the oral cavity of smokers (61); while Zambon *et al* reported a higher prevalence of *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and *Porphyromonas gingivalis* in smokers (66).

While all of these factors play a role in the progression and severity of the disease, there is clear evidence that the inflammatory response in the periodontum is initiated against the resident microbial biofilm within the subgingival pocket (1). It is this inflammatory reaction caused by the host innate immune response to the bacterial pathogen that dictates the severity and progression of the periodontal infection (5).

The oral biofilm consists of several hundred bacterial species, but periodontal disease is often associated with a progressive succession of the predominant bacterial species in dental plaque from Gram-positive to Gram-negative (1, 4). In order for bacteria to colonize and persist in a host, bacterial pathogens must be able to counteract the innate and adaptive immune system of the host. Bacteria are able to evade the host immune system by maintaining a plethora of virulence factors. Many bacteria produce numerous virulence factors that have been well-characterized, including adherence proteins, cytotoxic factors, biofilm polysaccharides, chemotactic inhibitor, collagenases, LPS, and toxins to counteract the host immune system. It is these different bacterial behaviors and the resulting immune response to these pathogens that combine to promote inflammatory tissue destruction in periodontitis (5). The production by various proinflammatory

cytokines (i.e. IL-1B, IL-6, TNF α , IL-8, and RANKL) in response to specific Gram-negative bacteria within the oral cavity leads to the activation of osteoclasts (37). Activation of the bone-resorbing osteoclasts eventually results in bone resorption and alveolar bone loss—clinical symptoms of periodontitis (41, 45, 51).

Microbial Induction of Periodontal Disease

Dental plaque is the single most important risk factor associated with the onset and progression of periodontal disease (1). The formation of dental plaque begins with adsorption of early colonizing bacteria onto an acquired pellicle, a layer of salivary proteins that is mainly composed of glycoproteins that forms shortly after a dental cleaning or tooth eruption. Once an acquired pellicle is formed, early colonizers are able to manipulate the environment in order for other bacteria to be able to colonize and adhere, thus forming dental plaque. It is this dental plaque that initiates the host inflammatory reaction (5).

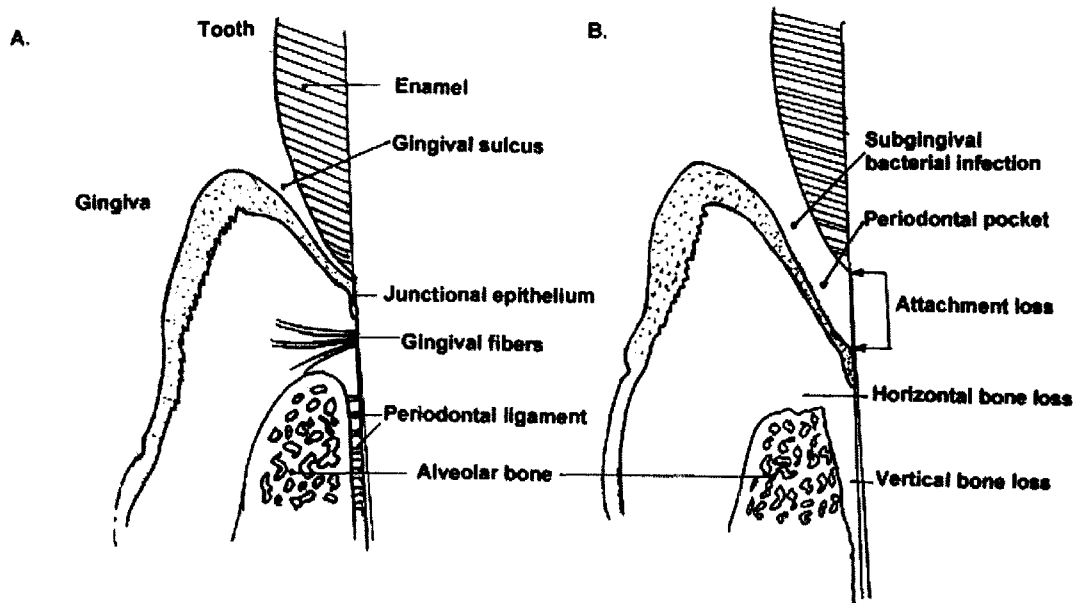


Figure 1. Anatomy of the periodontium (as reviewed by Baker, P.J. (12)).

A) In health, gingival fibers connect the gingival soft tissue to the root of the tooth, and the alveolar bone and cementum are connected by periodontal ligament fibers. **B)** In periodontal disease, subgingival infection by Gram-negative bacteria results in soft tissue damage, producing attachment loss and deepening the sulcus into the periodontal pocket. Alveolar bone resorption moves the bone surface away from the tooth root (vertical bone loss) and reduces the height of the alveolar bone crest (horizontal bone loss). Copyright permission to reproduce figure granted by Elsevier and (5).

As mentioned previously, bacteria are able to adhere to the acquired pellicle on hard tissue to form a biofilm. The ability of bacteria to adhere tightly to underlying substrata impedes the efficient removal of biofilms by physical or chemical means. In the oral cavity, biofilms form on the surfaces of both hard and soft tissues, and the development of the biofilm depends on interactions between both bacterial cell-surface adhesions and host receptors and bacteria-bacteria interactions (12). Without the ability to adhere to surfaces in the oral cavity, bacteria are swallowed with the saliva and enter the digestive tract, where they may not survive.

The last stage of bacterial colonization in the oral cavity involves late colonizers, whose presence is often associated with periodontal disease progression. A hallmark of inflammatory periodontal disease is the increased subgingival colonization by several Gram-negative species, including *A. actinomycetemcomitans*, *Prevotella intermedia*, *Campylobacter rectus*, and a group of three other bacteria designated 'the red complex' bacteria (*T. denticola*, *T. forsythia*, and *P. gingivalis*) (40). It is the bacteria previously mentioned that are considered the late colonizers or the anaerobic bacteria that cause this inflammatory reaction in the gingival tissues. The red complex is a group of microorganisms that are most intimately associated with adult periodontal disease. *A. actinomycetemcomitans*, another late colonizer, is associated with an aggressive form of periodontitis primarily found in juveniles, however this bacterial species can also be found in chronic periodontitis as well (58).

Aggregatibacter actinomycetemcomitans

A. actinomycetemcomitans (formerly *Actinobacillus actinomycetemcomitans*) is a small, fastidious, CO₂-requiring coccobacillus (34). It is a capnophilic, facultative anaerobic Gram-negative bacterium that belongs to the family Pasteurellaceae (34). *A. actinomycetemcomitans* was initially isolated from actinomycotic oral lesions along with *Actinomyces israelii*, leading to its species name (30). *A. actinomycetemcomitans* was first isolated in 1912 (38), however it was never recognized as a member of the oral microbial environment until 1975 (35). *A. actinomycetemcomitans* has been associated with several systemic infections including chronic periodontitis, but the pathogen is most known for its strong association with localized aggressive periodontitis, an extremely destructive form of periodontal disease that is most common in adolescents and young adults (58). The fact that *A. actinomycetemcomitans* has also been recovered from subgingival microflora of periodontally healthy patients suggest that *A. actinomycetemcomitans* may also represent an opportunistic pathogen. Due to *A. actinomycetemcomitans* strong association with localized aggressive periodontitis, most studies of *A. actinomycetemcomitans* have focused on its role in this disease. If the disease is left untreated, localized aggressive periodontitis results in rapid destruction of the alveolar bone and all supporting structures ultimately causing tooth loss. The disease specifically targets the mandibular incisors and first molars (65).

A. actinomycetemcomitans, like other bacterial pathogens, utilizes a plethora of virulence factors to cause disease and evade the host immune response. *A. actinomycetemcomitans* produces numerous virulence factors that have been well-characterized, including adherence proteins, cytotoxic factors, biofilm polysaccharides,

chemotactic inhibitor, collagenases, LPS, and toxins. There is also strong evidence that leukotoxin (LtxA), a 116-kDa protein that belongs to the repeats-in-toxin (RTX) family of pore forming toxins, expression affects disease progression (8, 31). Studies have shown that the target of LtxA is quite specific, which is unusual among RTX toxins, killing only polymorphonuclear leukocytes, monocytes and T-cells, all cells responsible for the innate immune response, in humans, apes, and Old World monkeys (60). Previous studies have also demonstrated the ability of *A. actinomycetemcomitans* to bind and invade epithelial (43) and endothelial cells (56), which may be an important mechanism for evading the host immune system and disseminating beyond the initial site of infection (17). Furthermore, fresh clinical isolates of *A. actinomycetemcomitans* express fimbriae (Figure 2), which allow them to form tenacious biofilms on a variety of solid surfaces, such as hydroxyapatite, glass, and plastic (53). Fimbriae expression may be important in biofilm pathogenesis *in vivo*, however it is not the sole determinant in biofilm formation, as isogenic smooth strains are capable of forming biofilms (57). However fimbriae are thought to enable *A. actinomycetemcomitans* to colonize the tooth surface, persist in the oral cavity, and initiate infection in the presence of salivary flow, thus classifying them as a virulence factor.

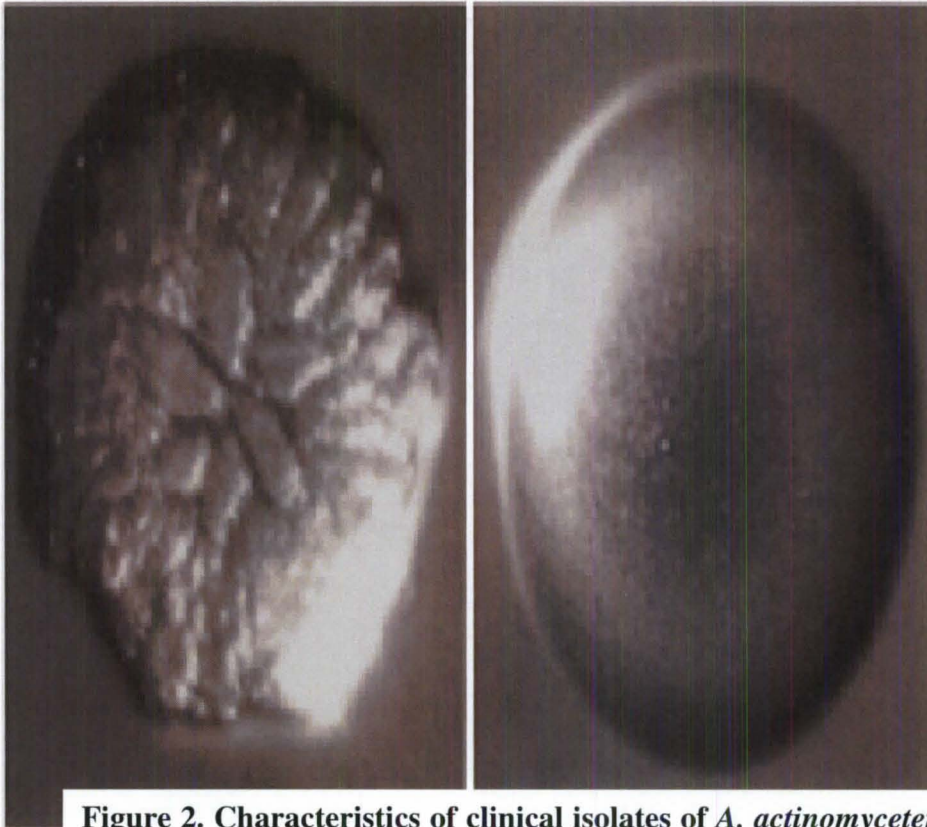


Figure 2. Characteristics of clinical isolates of *A. actinomycetemcomitans*

(16). A) 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 in the center. **B)** As the bacterium is passaged in liquid broth, it loses its ability to express fimbriae due to a spontaneous mutation in the *flp* promoter region, and the broth becomes turbid with the growth of isogenic non-adherent variants. When plated on solid medium, these variants form large, smooth colonies (16). Copyright permission to reproduce figure granted by Society for General Microbiology and (16).

Necessity of Iron

Iron is the most abundant metal in nature, and as such, plays a major role in the life processes of all organisms (62). Iron is the central atom of the heme group in hemoglobin, which is necessary for oxygen transport in blood. Iron is also present in myoglobin which allows oxygen to diffuse through muscle cell), cytochromes (proteins involved in the electron transport chain), proteins involved with DNA synthesis and cell division. Furthermore, iron is used to help produce the connective tissues in the human body, it is incorporated in the production of neurotransmitters, as well as playing an integral role in the maintenance of human immune system. Due to the fact that iron plays such a integral role in life processes it is tightly regulated. Too little iron can lead to iron deficiency anemia and present with symptoms of constant fatigue, palor, and severe weakness. Too much iron is known as hemochromatosis and can present as abdominal pain, fatigue, and generalized darkening of the skin.

The paucity of free iron in mucous membranes, tissues, or body fluids acts as a host defense mechanism against bacterial infections by creating a bacteriostatic environment, because iron is an essential nutrient for all life (22). Host iron binding proteins such as transferrin, lactoferrin, hemoglobin, ferritin all act collectively to maintain a low free iron concentration, thus inhibiting bacterial growth (63). As mentioned before all organisms require iron to survive. A critical component of bacterial virulence is the ability to obtain iron from their host. Several bacteria have evolved iron-sequestering mechanisms such as the secretion of siderophores, which compete with host

iron binding molecules for iron. Previous research has shown that *A. actinomycetemcomitans* is not capable of binding transferrin (22, 64). In addition, this dental pathogen is not known to excrete siderophores (64). However, it has been shown that *A. actinomycetemcomitans* is capable of binding both lactoferrin and hemoglobin (2, 21), two iron-containing compounds that serve as iron sources for other bacteria (48). The exact sources that *A. actinomycetemcomitans* utilizes in the oral cavity are not known.

Research to date has shown that *A. actinomycetemcomitans* has at least 15 iron acquisition genes encoded in its genome (54). The amount of *A. actinomycetemcomitans*' genome involved in iron acquisition suggests the importance of iron availability to the survival of this organism. With all of this capability that *A. actinomycetemcomitans* has to obtain iron, there must be some discussion or priority in the specific type of iron that *A. actinomycetemcomitans* prefers to utilize. The iron acquisition systems encoded in the genome of *A. actinomycetemcomitans* are tailored towards the specific survival strategies needed to survive in the oral biofilm. Due to *A. actinomycetemcomitans* genome and the the fact that *A. actinomycetemcomitans* is a facultative anaerobe, *A. actinomycetemcomitans* is expected to be able to utilize inorganic and organic as well as ferric and ferrous iron sources both in planktonic culture and biofilms. The most common iron transport systems utilized by bacteria seem to be the ferric iron transport complexes such as ABC transporters. However other bacteria such as *H. pylori*, *E. coli*, *P. gingivalis* and *V. cholera* also employ ferrous iron transport through *Feo* (Ferrous Iron Transport) systems. Due to relatively soluble nature of ferrous iron (0.1 M for Fe²⁺ cf 10⁻¹⁸ M for Fe³⁺ at pH 7), it would seem that ferrous iron would be the preferred iron source for

most bacteria (68). However only under anaerobic conditions, or low pH environments does ferrous iron transport seem predominant. This fact lead us to believe that *A. actinomycetemcomitans* is able utilize ferrous iron as well as ferric iron transport in the oral cavity (68, 69, 70). Our hypothesis is that *A. actinomycetemcomitans* primarily utilizes ferric iron and hemin as iron sources in the oral biofilm due to the nature of *A. actinomycetemcomitans* genome. In this study, we investigate the planktonic growth of *A. actinomycetemcomitans* in an iron-limited and iron-chelated environment as well as analyze the ability of *A. actinomycetemcomitans* to grow in an iron-chelated environment supplemented with different iron sources and investigate what particular iron acquisition genes are being upregulated in each particular iron environment.

| Gene Name | Gene ID | Definition |
|----------------------------|-------------------|---|
| hmsHF | AA00490,0 0491 | hemin transport and storage |
| afuABC | AA00696-0 0700 | iron(III) ABC transporter |
| hgpA | AA00762 | hemoglobin binding protein A |
| fecBCDE | AA00795-0 0799 | iron(III) dicitrate transporter |
| hitABC (afuABC) | AA01048-0 1051 | iron (III) ABC transporter |
| afuABC | AA01642-0 1645 | iron(III) ABC transporter |
| fatBDCE | AA01824-0 1827 | enterochelin transporter |
| ftnAB | AA02120,0 2121 | nonheme ferritin |
| hemU, fecD | AA02144,0 2145 | iron(III) ABC transporter |
| sidABCD | AA02151-0 2154 | ferric enterobactin transporter |
| tbpA | AA02201 | transferrin-binding protein |
| fecB | AA02380 | ferric hydroxamate (chelatin) periplasmic-binding protein |
| fur | AA02516 | Fur, ferric uptake regulator |
| AfeABCD | AA02549-0 2552 | iron (chelated) ABC transporter |
| hasR | AA02782 | Outer membrane hemophore receptor |

Table 1. Iron Uptake Operons in *A. actinomycetemcomitans*. Iron acquisition operons encoded in *A. actinomycetemcomitans* genome (54). There is a redundancy of ferric iron transporters (ABC transporters), hemin and hemoglobin transporters. *A. actinomycetemcomitans* even seems to be able to utilize siderophore like molecules secreted from other bacteria through utilization of its enterochelin transport system.

CHAPTER TWO: MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. actinomycetemcomitans* bacterial strain employed in this study was *A. actinomycetemcomitans* 652. *A. actinomycetemcomitans* 652 is an afimbriated, smooth colony morphotype strain and was grown at 37°C under microaerophilic conditions in Chemically Defined Media (CDM; Sigma-Aldrich, St. Louis, MO) and CDM supplemented with 150 uM of dipyriddy (DIP; Sigma-Aldrich, St. Louis, MO) per liter. Ferric Chloride, Ferric Citrate, Ferrous Sulfate, Hemin (Sigma-Aldrich, St. Louis, MO) were added to CDM with DIP at a 250 uM concentration per liter.

Table 2. Primers* used in this study.

| Primers | Primer Sequence (5' — 3') | Target Gene | Size (kbp) |
|------------|--------------------------------|-----------------|------------|
| 5' 00696 | CGGATTACTCGCCTCACAAGCG | afuA 00696 | 0.318 |
| 3' 0696 | CCTTTGGTTTTTGCCGGATCGC | afuA 00696 | 0.318 |
| 5' 00795 | GCGCAGAAAATCGGTGATGTCGTGG | fecB 00795 | 0.317 |
| 3' 00795 | GCGATACTTTCCTGACGATAGTGTGC | fecB 00795 | 0.317 |
| 5' 5S rRNA | GCGGGGATCCTGGCGGTGACCTACT | 5S-2-rRNA | 0.089 |
| 3' 5S rRNA | GCGATCTAGACCACCTGAAACCATAACC | 5S-2-rRNA | 0.089 |
| 5' 01642 | CGCAGGCGGATTTTTGGTATGGCG | afuA 01642 | 0.191 |
| 3' 01642 | CCGGCAGTTGAACCTCGCCTTTGTA | afuA 01642 | 0.191 |
| 5' AA02120 | GCAAGGTTATGAAGGTGCTGCGGC | ftnA/rsgA 02120 | 0.351 |
| 3' AA02120 | GCAGTGCCGCTTTTGCCAAGAAGTTTA | ftnA/rsgA 02120 | 0.351 |
| 5' AA00762 | CCATGGAATTGAGACAACAAGCAACGTTAG | hgpA AA00762 | 0.263 |
| 3' AA00762 | TCTAGATAGTCCAATTCCTGCGGTA | hgpA AA00762 | 0.263 |

Achievement of an Iron Limited Environment. *A. actinomycetemcomitans* was grown in a chemically defined media (CDM) in order to regulate the amount and type of iron that the bacteria could use for growth. *A. actinomycetemcomitans* was grown in CDM that contained FeSO₄ (CDM regular), and was then passaged into CDM that lacked an iron source (FeSO₄), creating an iron limited environment.

Chemically Defined Media (CDM)

| Amino Acids | 1L of 20x (g) |
|----------------------------------|---------------|
| L-Glutamic Acid HCl | 27.2 mM |
| Glycine | 26.6 mM |
| L-Threonine | 16.79 mM |
| L-Serine | 19.03 mM |
| L-Lysine HCl | 10.95 mM |
| L-Arginine HCl | 11.49 mM |
| L-Histidine HCl H ₂ O | 14.09 mM |
| L-Glutamine | 8.38 mM |
| L-Asparagine H ₂ O | 15.12 mM |
| L-Proline | 13.23 mM |
| L-Asparatic Acid | 15.03 mM |
| L-Ornithine HCl | 2.37 mM |
| L-Hydroxyproline | 3.05 mM |

| Amino acids (Hydrophobic) | 1L of 20x (g) |
|---------------------------|---------------|
| L-Alanine | 22.45 mM |
| D-Alanine | 22.45 mM |
| L-Leucine | 15.25 mM |
| L-Valine | 17.07 mM |
| L-Tryptophan | 9.79 mM |
| L-Methionine | 13.40 mM |
| L-Isoleucine | 15.25 mM |
| L-Phenylalanine | 12.11 mM |
| L-Tyrosine | 2.21 mM |
| L- Cystine | 416.2 μ M |

| Purines/Pyrimidines | 1L of 20x (g) |
|---------------------|---------------|
| Adenine | 1.85 mM |
| Guanine | 1.32 mM |
| Cytosine HCl | 2.43 mM |
| Thymine | 1.59 mM |
| Xanthine | 1.31 mM |
| Hypoxanthine | 1.47 mM |
| Uracil | 1.78 mM |

| Inorganic Salts | 1L of 20x (g) |
|---------------------------------|---------------|
| MnSO ₄ | 830.8 μM |
| NaCl | 34.2 mM |
| K ₂ HPO ₄ | 22.9 mM |
| KH ₂ PO ₄ | 146.9 mM |
| KNO ₃ | 19.78 mM |

| Inorganic Salts | 1L of 20X (g) |
|-------------------------------------|---------------|
| KI | 12.05 μM |
| CuSO ₄ 5H ₂ O | 5.206 μM |
| Boric Acid | 161.7 μM |
| ZnSO ₄ 7H ₂ O | 56.8 μM |
| Sodium molybdate | 48.6 μM |

| Vitamins/Factors | 1L of 1000x (g) |
|--------------------|-----------------|
| Choline chloride | 358.1 mM |
| Beta-alanine | 112.2 mM |
| Pyridoxal | 4.04 mM |
| Pyridoxine HCl | 5.91 mM |
| Pyridoxamine diHCl | 4.15 mM |

| | |
|--------------------------------------|---------------|
| Spermidine HCl | 6.88 mM |
| Nicotinic acid | 8.12 mM |
| Nicotinamide | 8.19 mM |
| Calcium pantothenate | 4.56 mM |
| Spermine tetraHCl | 2.87 mM |
| Thiamine HCl | 3.32 mM |
| Myo-Inositol | 55.5 mM |
| Nicotinamide adenine dinucleotide | 1.51 mM |
| p- Aminobenzoic acid | 729.2 μ M |
| Vitamin B12 | 7.38 μ M |

| | |
|--------------------|-----------|
| | 1L of 20x |
| NaHCO ₃ | 23.8 mM |

| | |
|-----------------|-----------|
| | 1L of 20x |
| L- Cysteine HCl | 10.7 mM |

| | |
|-------------------------------------|--------------|
| | 1L of 1x (g) |
| MgSO ₄ 7H ₂ O | 28.4 μ M |

| | |
|-------------------------------------|--------------|
| | 1L of 1X (g) |
| FeSO ₄ 7H ₂ O | 17.9 mM |

| | |
|-------------------------------------|---------------|
| | 1L of 1x (mg) |
| CaCl ₂ 2H ₂ O | 680.2 μM |

| | |
|-----------------|----------|
| | 1L of 1x |
| Pimelic acid | 624.2 nM |
| D- Biotin | 409.3 nM |
| DL-6,8-thioctic | 484.6 nM |
| Folic acid | 2.265 μM |

| | |
|------------|---------------|
| | 1L of 1X (mg) |
| Riboflavin | 2.66 μM |

Table 6. *A. actinomycetemcomitans* Chemically Defined Media. All chemicals used were from Sigma-Aldrich, St. Louis, MO.

Achievement of an Iron Chelated Environment. A.

Actinomycescomitans growth was achieved by passaging the bacteria in an iron limited environment (a media in which no exogenous iron source was added) and an iron chelated environment (a media in which a chelator was added). On Day 1, *A. actinomycescomitans* strain 652 was inoculated into regular CDM from a frozen stock. This culture was incubated at 37°C overnight under microaerophilic conditions. On Day 2, the bacteria from the overnight culture of regular CDM was inoculated into CDM with no iron plus 100 uM, 150 uM or 200 uM of dipyriddy (DIP) to achieve a 1st passage in an iron chelated environment. This 1st passage was incubated at 37°C overnight under microaerophilic conditions. On Day 3, the bacteria from the overnight culture of CDM no iron 1st passage plus 100 uM, 150 uM, or 200 uM of DIP was inoculated into fresh media of CDM no iron plus 100 uM, 150 uM, or 200 uM of DIP to achieve a 2nd passage in an iron chelated environment. CDM no iron is an iron-limited environment. CDM no iron supplemented with DIP (iron chelator) is an iron-chelated environment. Growth of *A. actinomycescomitans* was accessed by measuring optical densities of the bacteria at 600 nM utilizing spectrophotometer. Figure 3 shows how this was accomplished.

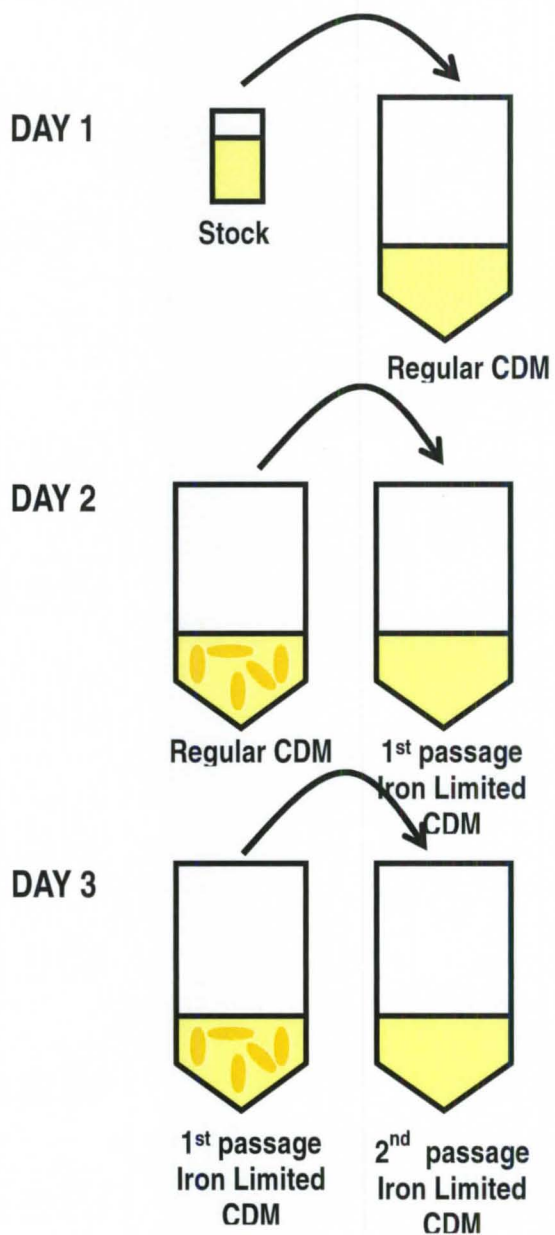


Figure 3: Achievement of an iron limited and iron chelated environment.

Diagram shows how *A. actinomycetemcomitans* was passaged in order to achieve an iron limited environment. The same mechanism was utilized in order to obtain an iron chelated environment except in an iron chelated environment an iron chelator, dypridyl (DIP), was added to the CDM No Iron at 100 uM, 150 uM, and 200 uM concentrations respectively.

RNA Isolation. Overnight cultures of the appropriate *A. actinomycetemcomitans* incubated at 37°C until the mid-exponential growth phase (O.D. of .08 for 1st passage CDM No Iron with 150 uM DIP and .028 for 2nd passage CDM No Iron with 150 uM DIP) for RNA isolation. Total RNA was isolated from *A. actinomycetemcomitans* cells using the 5 Prime PerfectPure RNA Cell & Tissue kit (5 Prime Inc., Gaithersburg, MD) according to the manufacturer's instructions. To ensure that the samples were free of contaminating genomic DNA, the RNA preparation was digested with RQ RNase-free DNase I (Promega Corporation, Madison, WI). The concentration and purity of each RNA sample were measured via spectrophotometry (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc, Wilmington, DE) and was also assessed by gel electrophoresis. Samples were checked for contamination of genomic DNA by real-time PCR using *A. actinomycetemcomitans* 5s rRNA primers (Table 2) RNA samples were considered free of significant genomic DNA if no amplification product was detected by real-time PCR after at least 30 cycles of amplification. RNA that was not immediately utilized for a reverse-transcription reaction was aliquoted into 1.5 ml Eppendorf tubes and stored at -80°C until future use.

cDNA synthesis and qPCR for iron acquisition gene expression. First-strand cDNA was prepared by using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with the Smart Cycler system (Cepheid, Sunnyvale, CA) in a final reaction volume of 25 μ l that contained 100 ng of cDNA, appropriate iron acquisition primers (5' Induction and 3' Induction; \sim 71 μ M final concentration), and 1x FastStart SYBER Green Master (Roche, Indianapolis, IN). The amplification conditions for qPCRs were as follows: denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds for 35 cycles. The threshold cycle for each qPCR was determined from a second derivative plot of total fluorescence as a function of cycle number by using the software package supplied with the Smart Cycler system. All gene-specific threshold values were normalized against threshold values from primers specific for the *A. actinomycetemcomitans* 5S rRNA gene (\sim 60 μ M final concentration). qPCR reactions were carried out in triplicate with consistent results. Each qPCR end-point amplification product was visualized by electrophoresis on 2% agarose gels. Primers utilized in this study included *afuA 1642* (iron III ABC transporter), *afuA 0696* (periplasmic iron-compound binding protein), *hgpA 0696* (hemoglobin binding protein A), *fecB 0795* (iron III dicitrate-binding protein), and *ftnA 2120* (nonheme ferritin) (54).

CHAPTER THREE: EXPRESSION OF IRON ACQUISITION GENES

Introduction:

Dental plaque forms on surfaces of teeth and is composed of a dynamic microbial community. As this microbial community matures, there is an increase in gram negative bacteria and decrease in gram positive bacteria. As gram negative bacteria proliferate in the oral biofilm, the host immune system reacts to the microbes, and eventually leads to periodontal disease. *A. actinomycetemcomitans*, is a gram negative coccibacillus, that is associated with the aggressive forms of periodontal disease. However in order for bacteria such as *A. actinomycetemcomitans*, to proliferate inside a human a host, iron is needed. Iron is an essential element needed for growth and normal metabolic functions in all organisms. Because the human host regulates iron, thus keeping a bacteriostatic environment, bacteria must overcome this iron sequestration by encoding in its genome iron acquisition systems, in order to obtain iron from the host environment so that the bacteria may thrive and proliferate. Research to date has shown that *A. actinomycetemcomitans* has at least 15 iron acquisition genes encoded in its genome. The iron acquisition systems encoded in the genome of *A. actinomycetemcomitans* are tailored towards the specific survival strategies needed to survive in the oral biofilm. Due to all of the capability that *A. actinomycetemcomitans* has to obtain iron, there must be some discussion or priority in what type of iron *A. actinomycetemcomitans* prefers to utilize for

growth. Due to the redundancy of ferric iron transporters in *A. actinomycetemcomitans* genome and the major iron sources that *A. actinomycetemcomitans* will encounter to in the oral cavity lead us to our hypothesis that *A. actinomycetemcomitans* primarily utilizes ferric iron, obtained from iron chelators or host proteins, and hemic as iron sources in the oral cavity.

Results:

***A. actinomycetemcomitans* is capable of growing in an iron-limited environment.** In order for us to test *A. actinomycetemcomitans*' ability to grow in an environment that lacked an iron source, *A. actinomycetemcomitans* was first grown in an iron rich media (CDM regular) overnight in an incubator at 37C. One milliliter of overnight planktonic cultures of *A. actinomycetemcomitans* strain 652 grown in CDM regular was inoculated into fresh CDM 19 mL. Growth was analyzed for 12 hours by measuring the Optical Density at 600 nm every hour. The first passage with no iron culture was achieved by inoculating from an overnight culture of *A. actinomycetemcomitans* grown in CDM regular into fresh CDM with no added iron source (CDM No Iron). The second passage CDM No Iron culture was achieved by inoculating from an overnight culture of *A. actinomycetemcomitans* grown in the first passage of CDM No Iron. The third passage CDM No Iron culture was achieved by inoculating from an overnight culture of *A. actinomycetemcomitans* grown in the second passage of CDM No Iron. Results shown in Figure 4 are averages of O.D. \pm standard deviations.

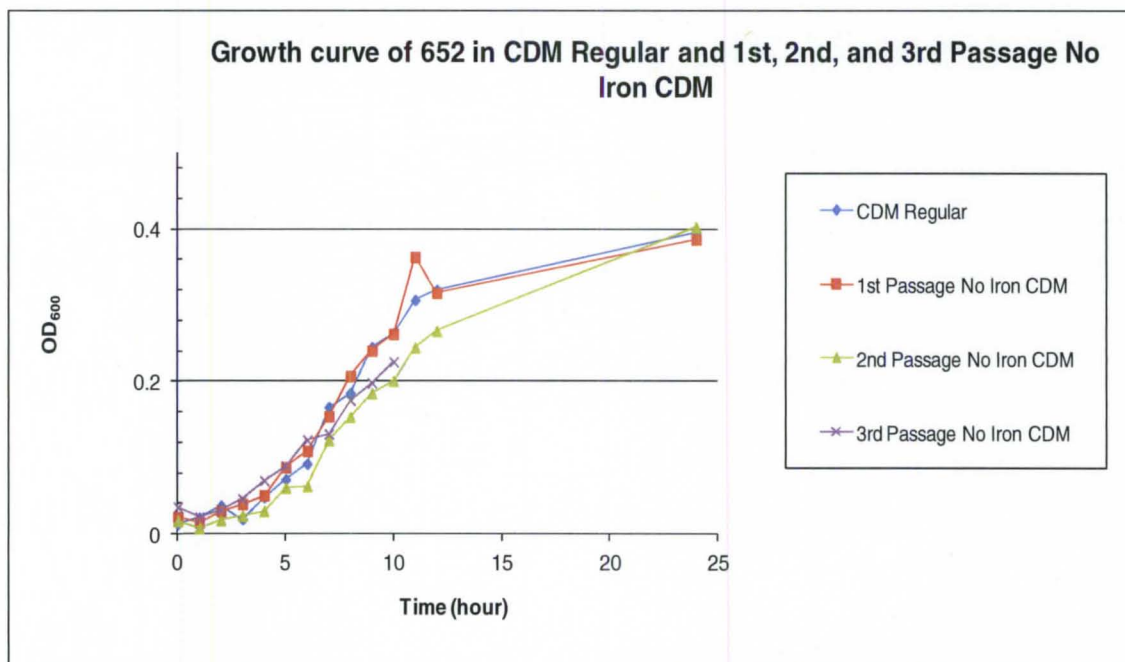


Figure 4. Growth of *A. actinomycetemcomitans* in an iron-limited environment. Growth of *A. actinomycetemcomitans* measured by spectrometry O.D. 600 nm. Growth was measured in triplicates, results of growth were averages \pm standard deviations.

Results showed that *A. actinomycetemcomitans* was able to grow in an iron limited environment. Growth occurred similarly in all 3 passages of *A. actinomycetemcomitans* without an iron source. These results lead us to a couple of conclusions, either *A. actinomycetemcomitans* was capable of growing very well in an iron limited environment due to large internal iron storage system that lasted the bacteria well into the third passage or there were trace amounts of iron in media CDM No Iron, even though no exogenous iron source was added to the media. This lead us to to our second experiement of obtaining an iron-chelated environment.

An iron-chelated environment reduces the growth of *A.*

actinomycetemcomitans. Passages in an iron chelated environment were achieved as described above in Figure 4. However, the growth of *A. actinomycetemcomitans* was analyzed in an iron-chelated environment at different concentrations of dipyridyl (DIP). Growth was analyzed for 12 hours by measuring the Optical Density at 600 nm every hour, as above. Figure 5a. DIP was added to the 1st and 2nd passages at 100 μ M. Figure 5b. DIP was added to the 1st and 2nd passages at 150 μ M. and Figure 5c. DIP was added to the 1st and 2nd passages at 200 μ M. Results are averages O.D. at 600 nm \pm standard deviations.

Overlay of 652 Growth in Regular CDM and 1st and 2nd Passages In No Iron CDM with 100 uM DIP

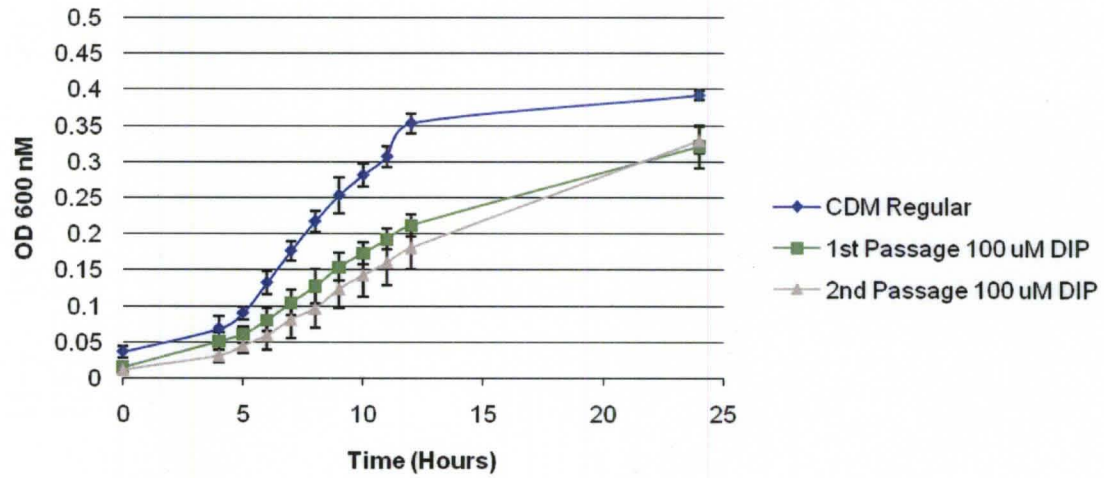


Figure 5a. Achievement of an Iron Chelated Environment with 100 uM DIP.

Growth of *A. actinomycetemcomitans* measured by spectrometry O.D. 600 nm. Growth was measured in triplicates, results of growth were averages \pm standard deviations.

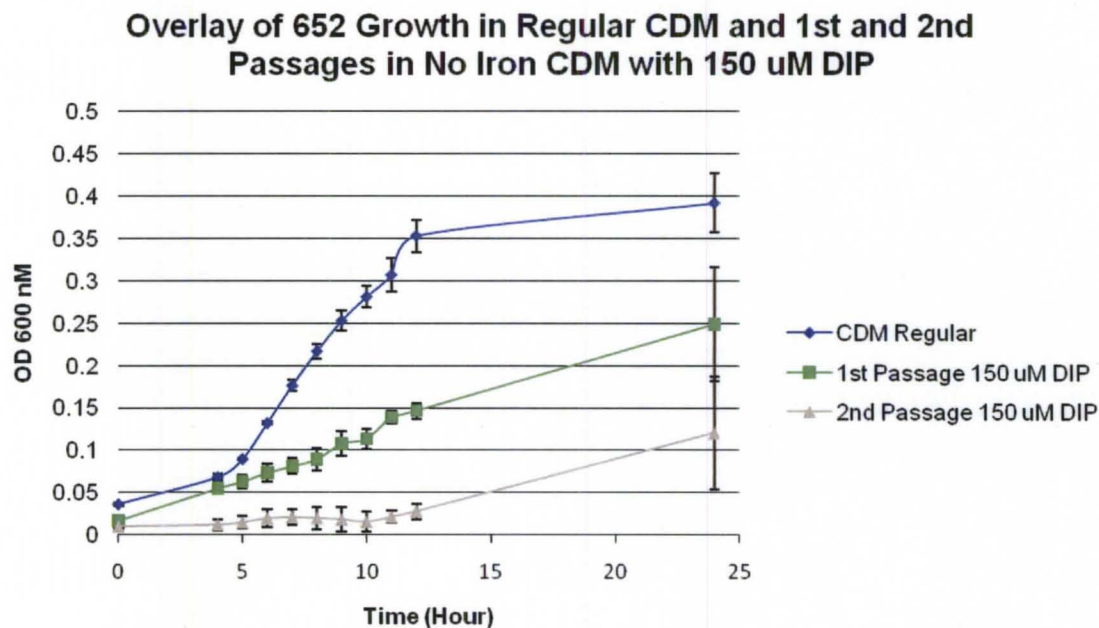


Figure 5b. Achievement of an Iron Chelated Environment with 150 μ M DIP.

Growth of *A. actinomycetemcomitans* measured by spectrometry O.D. 600 nm. Growth was measured in triplicates, results of growth were averages \pm standard deviations.

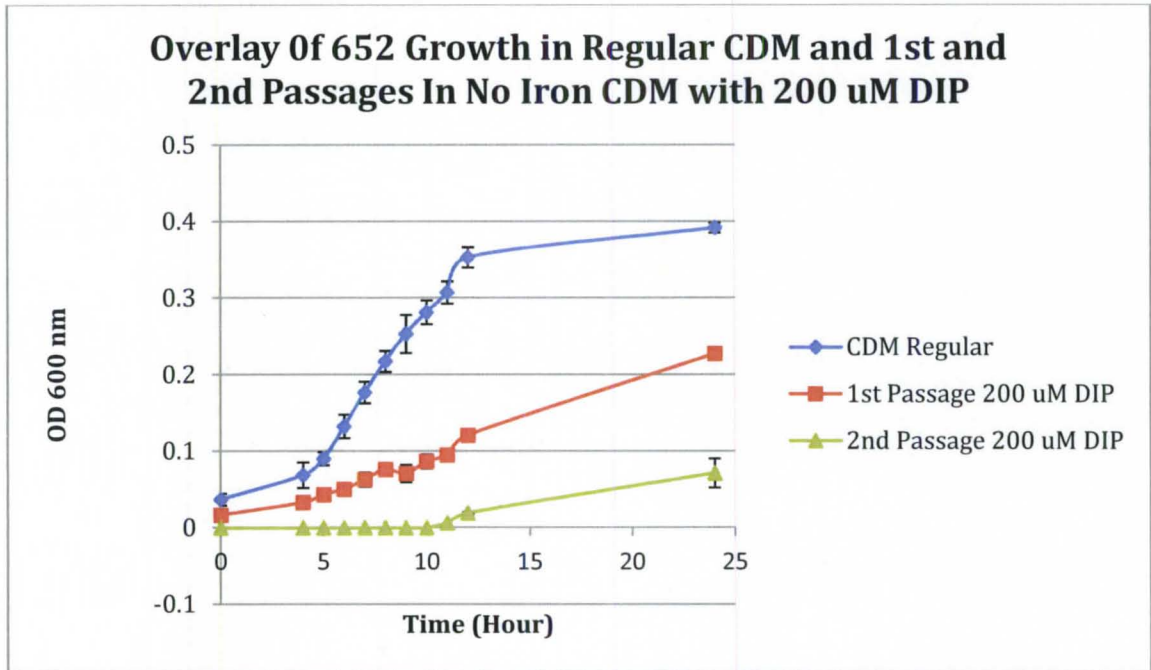


Figure 5c. Achievement of an Iron Chelated Environment with 200 uM DIP.

Growth of *A. actinomycetemcomitans* measured by spectrometry O.D. 600 nm. Growth was measured in triplicates, results of growth were averages \pm standard deviations.

Results from these growth analyses displayed that an iron-chelated environment reduces the growth of *A. actinomycetemcomitans*. It also proved that *A. actinomycetemcomitans* has an internal iron storage system that allows growth in an iron chelated environment well into the 1st passage. However by the 2nd passage of *A. actinomycetemcomitans* in an iron chelated environment the internal iron storage system had been depleted and *A. actinomycetemcomitans* growth was drastically reduced. It also proved that there were trace elements of iron in the No Iron CDM media that *A. actinomycetemcomitans* was able to utilize for growth. Now that an environment was created in which *A. actinomycetemcomitans* was depleted of all sources, we then decided

to add different sources of iron in order to see what types of iron that *A. actinomycetemcomitans* preferred to utilize for growth.

Growth of *A. actinomycetemcomitans* utilizing different iron sources. To determine *A. actinomycetemcomitans*' ability to utilize iron sources for growth, the 1st passage CDM No Iron with 150 μ M DIP was inoculated into fresh CDM No Iron with 150 μ M DIP medium supplemented with 250 μ M of hemin, ferric chloride, ferric citrate, or ferrous sulfate. To prevent ferrous sulfate from oxidizing to ferric sulfate, the growth was analyzed under anaerobic conditions. The 1st passage was achieved as in Figure 3. Growth was analyzed for 12 hours by measuring the Optical Density at 600 nm every hour. Results are averages of O.D. at 600 nm \pm standard deviations.

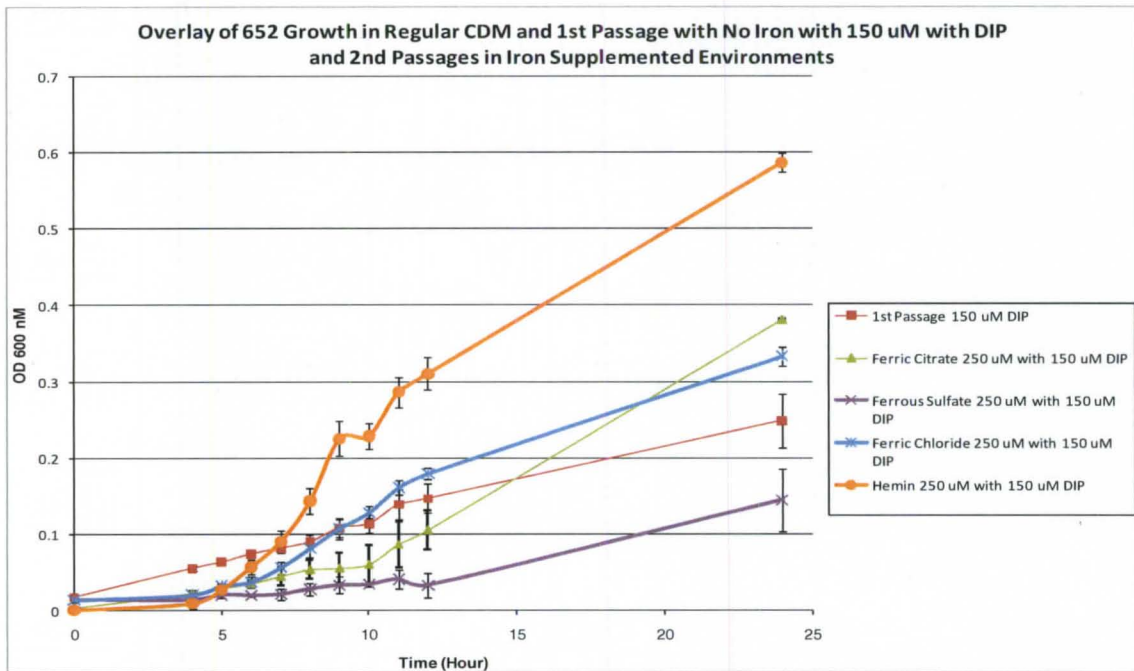


Figure 6. Growth of *A. actinomycetemcomitans* utilizing different iron sources with 150 uM DIP and 250 uM of appropriate iron sources. Growth of *A. actinomycetemcomitans* measured by spectrometry O.D. 600 nm. Growth was measured in triplicates, results of growth were averages \pm standard deviations.

Hemin, Ferric Chloride, and Ferric Citrate are iron sources that *A. actinomycetemcomitans* is capable of utilizing for growth. Growth of *A. actinomycetemcomitans* in ferrous sulfate was similar to the amount of growth that was witnessed in the 2nd passage in an iron chelated environment leading us to the conclusion that *A. actinomycetemcomitans* appears to not to be able to utilize ferrous sulfate as effectively for growth. Now that *A. actinomycetemcomitans* growth was analyzed under different iron conditions, we wanted to see what particular iron genes were up regulated or down regulated during these various iron conditions.

Expression of iron-uptake genes under an iron-chelated environment.

Cultures utilized in this experiment were achieved as described in Figure 2. RNA was extracted mid exponential phase (O.D. of .08 for the first passage in CDM no iron and O.D. of .025 for the second passage in CDM no iron). RNA was isolated and cDNA was synthesized as described in the previous methods. Real-time PCR was performed using primers specific for iron genes. Iron genes utilized in this study were chosen based on the genes association with quorum sensing, due to the fact that previous research has shown that iron acquisition in *A. actinomycetemcomitans* is regulated by quorum sensing (72). Expression of genes are normalized to 1st passage expression. Results are averages of Delta T ± standard deviations. Delta T was calculated by subtracting the cycle threshold (Ct) determined for the each particular iron gene reaction from the Ct of the 5s rRNA control.

Table 4a. *afuA* 1642 (iron (III) ABC transporter

| | delta T | delta (delta T) | Fold Change |
|--|---------------------|-------------------------|----------------------|
| 2nd Passage in CDM No Iron with 150 uM DiP | 9.52 ± 1.008 | 0.0013 ± 0.8 E-3 | 0.134 ± 3.350 |
| 1st Passage in CDM No Iron with 150 uM DIP | 6.63 ± 0.001 | 0.010 ± 0.7 E-3 | 1 |

delta T was calculated by subtracting the cycle threshold (Ct) determined for the *afuA*

01642 reaction from the Ct of the 5s rRNA control. P value = 0.031

Table 4b. *afuA* 0696 (periplasmic iron-compound-binding protein)

| | delta T | delta (delta T) | Fold Change |
|--|----------------------|---------------------------|--------------------|
| 2nd Passage in CDM No Iron with 150 uM DIP | 13.68 ± 0.601 | 7.62E-5 ± 0.03 E-3 | 9.52 ± .081 |
| 1st Passage in CDM No Iron with 150 uM DIP | 15.87 ± 0.467 | 8 E-6 ± 0.002 E-3 | 1 |

delta T was calculated by subtracting the cycle threshold (Ct) determined for the *afuA* 0696 reaction from the Ct of the 5s rRNA control. P value = 0.0150

Table 4c. *hgpA* 0762 (hemoglobin binding protein A)

| | delta T | delta (delta T) | Fold Change |
|--|----------------------|------------------------|--------------------|
| 2nd Passage in CDM No Iron with 150 uM DIP | 9.133 ± 1.100 | 0.002 ± .001 | .319 ± .241 |
| 1st Passage in CDM No Iron with 150 uM DIP | 7.486 ± 0.962 | 0.006 ± .004 | 1 |

delta T was calculated by subtracting the cycle threshold (Ct) determined for the *hgpA* 0762 reaction from the Ct of the 5s rRNA control. P value = 0.0978

Table 4d. fecB 0795 (iron(III) dicitrate-binding protein)

| | delta T | delta (delta T) | Fold Change |
|--|----------------------|------------------------|--------------------|
| 2nd Passage in CDM No Iron with 150 uM DIP | 2.06 ± .064 | 0.240 ± .011 | 2.02 ± .007 |
| 1st Passage in CDM No Iron with 150 uM DIP | 3.076 ± 0.050 | 0.119 ± .004 | 1 |

delta T was calculated by subtracting the cycle threshold (Ct) determined for the *fecB* 0795 reaction from the Ct of the 5s rRNA control. P value = 0.0003

Table 4e. ftnA 2120 (nonheme ferritin)

| | delta T | delta (delta T) | Fold Change |
|--|---------------------|--------------------------|---------------------|
| 2nd Passage in CDM No Iron with 150 uM DIP | 8.009 ± .003 | 0.004 ± 0.004 E-3 | .809 ± 0.286 |
| 1st Passage in CDM No Iron with 150 uM DIP | 7.704 ± .424 | 0.005 ± .001 | 1 |

delta T was calculated by subtracting the cycle threshold (Ct) determined for the *fecB* 0795 reaction from the Ct of the 5s rRNA control. P value = 0.0816

Discussion:

Target genes utilized in this study were chosen due to the fact that previous research has shown that iron acquisition in *A. actinomycetemcomitans* are regulated by quorum sensing (58). The target genes in this study were associated with quorum sensing. Results showed that in an iron chelated environment, iron uptake genes of *A. actinomycetemcomitans* are differentially regulated. Using the 1st passage as a starting point presented as a potential limitation. CDM regular, media containing an iron source, should have been baseline and as we started to starve the bacteria we could have witnessed differential regulation in gene expression. Real time PCR results were normalized to the 1st. *AfuA 1642*, an iron III ABC transporter, was ultimately turned down during the second passage in CDM No Iron with 150 uM DIP. *AfuA 0696* showed an up regulation of the gene during the 2nd passage in CDM No Iron with 150 uM DIP. This was a very interesting result. *AfuA 1642* showed down regulation during the 2nd passage and *AfuA 0696* showed an up regulation during the 2nd passage. This suggest that there may be some distinction between these two ferric iron transporters. It may be possible that under very limiting iron conditions that *A. actinomycetemcomitans* is upregulating ferric iron transport that may be specific for iron coming from a particular source and the other ABC transporter may be down regulated because it is intended to transport iron obtained from a different source.. While cells are starving, there seems to be an upregulation of *afuA 0696* and *fecB 0795*, an iron (III) ABC transporter, and a iron (III) dicitrate binding protein (scavenging system) respectively. The gene regulation of *ftnA 2120* essentially stayed the same in the first and the second passage. This makes sense that *ftnA 2120* is not being regulated at all considering it is essentially an iron

storage molecule. There was down regulation of the hemoglobin binding protein, *hgpA* 0762 and *afuA* 0696. What is interesting is that there are three different *afuA* annotated gene clusters in *A. actinomycetemcomitans*, and all three have different sequences. This may suggest that these genes may differentially expressed, may have different functions, and may be expressed under different conditions.

Due to the fact that *A. actinomycetemcomitans* possess a wide variety of potential mechanisms to obtain iron sources, of the iron acquisition genes encoded in *A. actinomycetemcomitans* genome, *A. actinomycetemcomitans* differentially expresses genes according to what environment it is in and what different iron sources it is exposed to.

CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS

Iron is the most abundant metal in nature, and as such plays a major role in many life processes of all organisms. In the human host, iron is highly regulated, creating a bacteriostatic environment. However in order for organisms to survive they must be able to overcome this iron limitation by encoding in their genome iron acquisition systems. Research to date has revealed that *A. actinomycetemcomitans* has encoded in its genome at least 15 iron acquisition systems to overcome this bacteriostatic environment (67). The iron acquisition systems encoded in *A. actinomycetemcomitans* are tailored towards the specific survival strategies needed to survive in the oral biofilm. Thus *A. actinomycetemcomitans* is expected to be able to utilize inorganic and organic as well as ferric and ferrous iron sources both in planktonic culture and biofilms. These particular findings were expected due to the redundancy of ferric iron transporters in *A. actinomycetemcomitans* genome, and the particular iron sources that *A. actinomycetemcomitans* encounters in the oral cavity. In this study, we investigated the planktonic growth of *A. actinomycetemcomitans* in an iron-limited and iron-chelated environment as well as analyze the ability of *A. actinomycetemcomitans* to grow in an iron-chelated environment supplemented with different iron sources and investigate what particular iron acquisition genes are being upregulated in an iron chelated environment.

We discovered that *A. actinomycetemcomitans* is capable of growing in an iron limited environment, that though there wasn't an external iron source added to the media that *A. actinomycetemcomitans* was able to scavenge trace amounts of iron from other chemicals in the CDM, and efficiently use it for growth. However *A. actinomycetemcomitans* was not capable of growing in an iron chelated environment. By the second passage in an iron chelated environment *A. actinomycetemcomitans* growth was drastically reduced. This led us to the conclusion that *A. actinomycetemcomitans* has an excellent internal iron storage system that the bacteria is capable of using for growth during the first passage of growth but is not large enough to use for growth during the second passage. The reduction of growth of the bacteria in an iron-chelated environment occurred in a dose dependent manner.

Growth of *A. actinomycetemcomitans* utilizing 250 μM of ferric citrate, ferric chloride, and hemin respectively with 150 μM DIP restored growth to the levels above the 1st passage in an iron chelated environment. Growth of *A. actinomycetemcomitans* was higher in an hemin iron environment, suggesting that *A. actinomycetemcomitans* prefers hemin as an iron source. However *A. actinomycetemcomitans* growth in a ferrous sulfate iron source was very low. It was very similar to the amount of growth that was witnessed in the 2nd passage in an iron-chelated environment leading us to the conclusion that *A. actinomycetemcomitans* appears to not be able to utilize ferrous sulfate as effectively for growth. This was not surprising considering the analysis of *A. actinomycetemcomitans* genome did not reveal iron acquisition genes for ferrous iron sources. However we thought that because *A. actinomycetemcomitans* is an anaerobic

bacteria, that in an anerobic environment that *A. actinomycetemcomitans* may be able to utilize the non oxidized ferrous iron source.

Results from Realtime PCR showed that in an iron chelated environment, iron uptake genes of *A. actinomycetemcomitans* are differentially regulated. Using the 1st passage as a starting point presented as a potential limitation. CDM regular, media containing an iron source, should have been baseline and as we started to starve the bacteria we could have witnessed differential regulation in gene expression. Real time PCR results were normalized to the 2nd passage and what was seen was afuA 1642 showed a 7 fold increase than in the 1st passage compared to the 2nd passage. AfuA 0696 showed a 10 fold higher increase than in the 2nd passage that in the 1st passage. While cells are starving, there seems to be an upregulation of afuA 0696 and fecB 0795, and iron (III) ABC transporter, and a iron (III) dicitrate binding protein (scavenging system) respectively. The gene regulation of ftnA2120 essentially stayed the same in the first and the second passage. This makes sense that ftnA2120 is not being regulated at all considering it is essentially an iron storage molecule. There was a down regulation of the hemoglobin binding protein and afuABC 0696. What is interesting is that there are three different afuA annotated gene clusters in *A. actinomycetemcomitans*, and all three have different sequences. This may suggest that these genes may be differentially expressed and may have different functions, and may be expressed under different conditions.

Though many questions have had some light shed on them during my research, more research still needs to be done. Ultimately the original idea of my research was to first look at the expression of a couple of these iron acquisition systems under the initial iron conditions used, in other words the 1st and 2nd passages in CDM No Iron with 150

µM DIP. After the expression of the iron acquisition genes were established under the iron chelated conditions, our goal then was to add back specific iron sources and see what happened to gene expression with the different iron operons under these different iron conditions. The first part of our goal we accomplished, however the second part is a project that a future student will carry on. I also only focused on a limited numbers of operons, in the future more iron acquisition genes should be included. Also to make my research show more validity, mutant strains of *A. actinomycetemcomitans* should be formulated, knocking out specific iron operons and testing the growth and gene regulation under the different iron conditions. Because past and current research all describe *A. actinomycetemcomitans* as growing in a biofilm, more studies on iron acquisition genes with *A. actinomycetemcomitans* grown in a biofilm formation are needed if there are any hopes in formulating a chelator that is strong enough to chelate the preferred iron source of *A. actinomycetemcomitans*, that the human host is also able to tolerate.

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Academic Appointments:

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|-----------|---|
| 2011-2012 | Biochemistry Academic Tutor University of Louisville School of Dentistry |
| 2011-2012 | Aesthetics Academic Tutor University of Louisville School of Dentistry |
| 2011-2012 | Oral Diagnosis and Oral Pharmacology Academic Tutor University of Louisville School of Dentistry |
| 2009-2012 | Research Student University of Louisville School of Dentistry |
| 2009-2012 | Head and Neck Anatomy Academic Tutor University of Louisville School of Dentistry |
| 2009-2010 | Microbiology Academic Tutor University of Louisville School of Dentistry |
| 2007-2008 | Upward Bound Academic Tutor University of Louisville Louisville, KY |

Honors and Academic Achievements

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| 2012 | Oral Health and Systemic Research Award |
| 2012 | American Academy of Pediatric Dentistry Award |
| 2012 | Pierre Fauchard Award |
| 2012 | Karen Andrus Award |
| 2012 | Kentucky Off-site Clinical Community Service Scholar |
| 2012 | Hispanic Dental Association U of L Chapter Award |
| 2011 | The Hinman Research Symposium- Louisville Representative |
| 2011-2012 | Clinical Outreach Scholarship |

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| 2011 | SNDA National Convention Delegate (Baltimore, Maryland) |
| 2011 | Indiana University Research Participant |
| 2011 | SNDA Leadership Conference Delegate |
| 2011 | 3 rd Place in Research Louisville |
| 2010-2012 | Student Representative on ULSD Admissions Committee |
| 2010 | Student Representative for Outreach Scholar Committee |
| 2010 | Research Louisville Participant |
| 2010 | 2 nd Place in Research Louisville |
| 2010 | 2 nd Place in Research at SNDA National Research Competition |
| 2009 | 4 th Place in Research at Student Convention |
| 2010 | SNDA National Convention Delegate (Hawaii) |
| 2008-2010 | University of Louisville School of Dentistry Dean's List |
| 2008-2012 | General Dentistry Scholarship (Full Tuition) |
| 2007 | MCAT/DAT Summer Program Participant |
| 2006-2008 | Alpha Epsilon Delta Health Honor Society |
| 2006-2008 | University of Louisville Dean's List |
| 2004-2008 | Woodford Porter Senior Scholarship (Full Tuition & Room and Board) |
| 2004 | Salutatorian |
| 2004 | Louisville Book Award |
| 2004 | Advanced Placement Social Studies Award |
| 2004 | Key to the City of Covington for Academic Achievement |
| 2004 | Northern Kentucky Coaches Association Cheerleading Scholarship |

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| 2003 | Advanced Placement Math Award |
| 2003 | Northern Kentucky University Spanish Award |
| 2002-2003 | Governor School for the Arts Participant |
| 2002-2003 | Governor Scholar Program Recipient |

Academic Positions

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| 2011 | Cultural Competency Seminar- Student Panel Member |
| 2010-2011 | Student National Dental Association- President |
| 2010-2011 | American Association of Women Dentist- Secretary |
| 2010-2011 | Dental Class Secretary |
| 2009-2010 | Student National Dental Association- Social Chair |
| 2008-2012 | American Student Dental Association- General Member |
| 2008-2010 | American Dental Education Association- General Member |
| 2008-2012 | Psi Omega Dental Fraternity |
| 2004-2008 | Kappa Delta Sorority General Member |
| 2004-2008 | Alpha Epsilon Delta Pre Health Honor Society |
| 2003-2004 | Holmes High School Class President |
| 2003-2004 | National Honor Society President |

Work Experiences

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|--------------|---|
| 2012-current | Pediatric Dental Resident University of Louisville School of Dentistry Louisville Ky, 40208 |
| 2012-current | Dentist University of Louisville School of Dentistry Emergency Clinic Louisville Ky, 40208 |

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| 2011 | Dental Student Park Duvalle Community Health Clinic Louisville Ky, 40217 |
| 2008 | Library Student Assistant Kornhaeuser Library Louisville Ky, 40208 |
| 2007-2008 | Upward Bound Academic Tutor University of Louisville Louisville Ky, 40208 |
| 2005 | Brand Representative Abercrombie & Fitch Louisville Ky, 40208 |
| 2005 | Sales Associate Charolette Russe Louisville Ky, 40208 |
| 2004-2006 | Lifeguard City of Covington Recreation Parks Covington Ky, 41014 |
| 2003-2006 | Veterinarian Assistant Ft. Wright Pet Care Ft. Wright, Ky 40214 |

Community Service

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| 2009-2011 | Rural America Missions (Pikeville) Volunteer |
| 2008-2012 | Whitney Young Foundation Volunteer |
| 2008-2012 | Black Achievers Volunteer |
| 2008-2012 | Lincoln Foundation Volunteer |
| 2008-2012 | University of Louisville School of Dentistry Impressions Program |
| 2008-2012 | Wayside Christian Services Soup Kitchen Volunteer |
| 2008-2012 | Angel Tree Volunteer |

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| 2008-2012 | SNDA Canned Food Drive Volunteer |
| 2008-2011 | Presbyterian Fall Festival Volunteer |
| 2009-2011 | Beat the Pumpkin Volunteer |
| 2009-2012 | Spanish Health Fair Volunteer |
| 2010 | Presbyterian Health Fair |
| 2011 | Hispanic Health Fair- Volunteer |
| 2011 | Smile of KY Selection Committee |