PREVOTELLA MELANINOGENICA, AN ORAL ANAEROBIC BACTERIUM, PREVALENT IN CYSTIC FIBROSIS CHRONIC LUNG INFECTION

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ABSTRACT

SARAH ELIZABETH COUNCIL: *Prevotella melaninogenica*, an oral anaerobic bacterium, prevalent in cystic fibrosis chronic lung infection.
(Under the direction of Dr. Matthew Wolfgang)

Prevotella melaninogenica, an anaerobic Gram-negative bacterium*,* is a member of the normal oral flora and is one of the most abundant anaerobic species found in respiratory specimens from individuals with cystic fibrosis (CF). Because of *P. melaninogenica*'s designation as a commensal, its role in CF disease pathogenesis and host immune response has been largely ignored.

In our study of 61 CF patients at UNC hospitals, *P. melaninogenica* was cultured from 61% of adults and 57% of pediatric CF patients, and represented the most abundant strict anaerobe in both groups. Lung function did not correlate with the presence or abundance of *P. melaninogenica* but there was an increased antibody response against *P. melaninogenica* in both adult and pediatric CF patients compared to non-diseased controls. To explore innate host response, we characterized the structure and inflammatory effect of *P. melaninogenica* LPS. *P. melaninogenica* lipid A structure is heterogeneous, with the most prominent form being diphosphorylated and penta-acylated. In THP-1 cells, *P. melaninogenica* LPS induced significantly less IL-8 and IL-1β cytokine production than *Pseudomonas aeruginosa* LPS. We also showed that *P. melaninogenica* LPS could signal through a TLR4 independent pathway. These results show the presence of *P. melaninogenica* in CF patients and its recognition by the human host.

From the tongue to the lung, *P. melaninogenica* must acquire nutrients to sustain life. The lung environment within chronically infected CF patients contains high levels of host iron proteins and pockets of anaerobic space. *In vitro* growth experiments demonstrated that heme or hemoglobin were sufficient iron sources for *P. melaninogenica* growth. To identify the first step of acquisition, we sequenced the *P. melaninogenica* genome and searched for homologues of known hemoglobin receptors. We identified a comprehensive list of putative *P. melaninogenica* hemoglobin receptors.

Together these studies characterize the prevalence of *P. melaninogenica* in CF infection, evaluate *P. melaninogenica*'s impact on the host and determine nutritional requirements, which will lead to a better understanding about the role of *P. melaninogenica* in CF. Continued research into anaerobic pathogens, in particular *P. melaninogenica*, will lead to improvement in treatment interventions to reduce the severity of CF lung disease.

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Now to him who is able to do far more abundantly than all that we ask or imagine, according to the power at work within us, to him be glory in the church and in Christ Jesus throughout all generations, forever and ever. Amen. Ephesians 3:20- 21.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine-5'-triphosphate
ATSB	Anaerobic Tryptic Soy Broth
BALF	Bronchoalveolar lavage fluid
BLAST	Basic Local Alignment Search Tool
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CHO	Chinese hamster ovary
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
Cyclic AMP	Cyclic adenosine monophosphate
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FEV	Forced expiratory volume
FEV_1%	Forced expiratory volume in 1 second compared to patients of similar characteristics (height, age, sex, and weight)
Fur	Ferric uptake regulator

Ig	Immunoglobulin
IL	Interleukin
LB	Luria broth
LPS	Lipopolysaccharide
m/z	Mass-to-charge ratio
MAP3K	Mitogen-Activated Protein Kinase Kinase Kinase
mmHg	Millimeter of mercury
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear Factor-Kappa B
O_2	Dioxygen
OMP	Outer membrane protein
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PMN	Polymorphonuclear
PPIX	Protoporphyrin IX
qPCR	Quantitative polymerase chain reaction
SMG	*Streptococcus milleri* group
THP-1	Human acute monocytic leukemia cell line
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
TRFLP	Terminal Restriction Fragment Length Polymorphism

TRIF	TIR-domain-containing adapter-inducing interferon-β

Chapter 1

Introduction

P. melaninogenica Classification and Epidemiology

Prevotella melaninogenica, previously *Bacteroides melaninogenicus (*subspecies *melaninogenicus*), is an anaerobic, black pigmented, Gram-negative bacterium belonging to the family *Prevotellaceae* (1). *P. melaninogenica* is a non-motile, catalase negative, saccharolytic bacterium that can produce an uncharacterized capsule (2, 3). *P. melaninogenica* is considered to be a member of the normal human oral flora and can be cultivated from the tongue, gingival crevice, saliva and plaque of healthy individuals (4-7). Initial reports (1950-90s) showed that *P. melaninogenica* only colonized the mouth following tooth eruption (8, 9), but more recently, *P. melaninogenica* has been isolated from the oral cavity of infants as young as two months of age (10, 11).

P. melaninogenica infections

P. melaninogenica has been described as a 'potential pathogen' because of its occurrence in disease sites throughout the body and its capacity to produce a variety of virulence factors (2, 12-14). In particular, *P. melaninogenica* is commonly cultured as

the sole infectious agent in 'extra-oral' abscesses such as vertebral osteomyelitis, pyomyositis, peritonsillar abscesses and vaginal mesh infections (15-19). However, closely related oral bacteria, such as *Porphyromonas gingivalis* and *Prevotella intermedia* have received more attention due to their established association with systemic diseases such as atherosclerosis, pneumonia, preeclampsia, cardiovascular disease, stroke, heart disease, and diabetes mellitus (20-22).

In addition to single species infection, *P. melaninogenica* is frequently cultured in the context of polymicrobial disease, including brain abscesses, pleuropulmonary infections, endocarditis, illicit drug injection sites, intra-abdominal infections, wound infections, necrotizing fasciitis, pyogenic infections, decubitus and diabetic ulcers (16, 23-29). *P. melaninogenica* is also one of the most prevalent and abundant anaerobic species found in respiratory specimens from individuals with cystic fibrosis (CF) (12, 30-37).

Bacterial Synergism

The presence of *P. melaninogenica* within complex bacterial populations raises the possibility that its growth at different body sites requires bacterial synergism, where the presence of other bacterial species could improve the likelihood of colonization by this otherwise fastidious species. Pathogenic bacterial synergism occurs when a polymicrobial community is more destructive to the host than any single member of the community. This type of synergism is often the result of microbial interactions such as nutritional sharing or interspecies quorum sensing, that can alter bacterial gene expression, growth and virulence capabilities.

In its simplest form, bacterial synergism has been studied in two species model systems. For example, it has been shown that virulence genes of the opportunistic pathogen *Pseudomonas aeruginosa* are upregulated in the presence of oropharyngeal isolates of either *Streptococcus* or *Staphylococcus* species. This modulation of virulence factors was found to be partially due to an increase in autoinducer-2 signaling and competition for iron (38). Furthermore, experiments using an abscess model showed a greater host inflammatory response to mixed species infection compared to single species infection (39, 40). Additional studies have shown that there are growth benefits to *Bacteroides* species (*Bacteroides fragilis* and *Bacteroides asaccharolyticus*) when present in a polymicrobial abscess model of infection (41). It is believed that *Bacteroides* species specifically benefit from nutrients produced by the community. Similarly, it has been shown that *P. melaninogenica*, can acquire vitamin K, an essential growth factor, when grown in the presence of *Staphylococcus aureus* (42). Because of its ability to survive and grow in polymicrobial infections, *P. melaninogenica* likely contributes to the pathogenic potential of these communities. Ultimately, bacterial virulence potential in the context of polymicrobial conditions depends on the virulence characteristics of the individual species, the host response, and environmental conditions within the infected niche (20, 43, 44).

Virulence Characteristics of *P. melaninogenica*

In the human host, initial colonization by *P. melaninogenica* is aided by fimbrial hemagglutinin, which serves as an epithelial cell adhesion (45). Host nutrients required for *P. melaninogenica* growth are liberated by hemolysin and fibrinolysin (46). To protect

against host detection and antimicrobial factors, *P. melaninogenica* isolates can produce both IgA and IgG proteases (47-49), a β-lactamase (24), and a polysaccharide capsule. To further modulate the immune system, *P. melaninogenica* produces a neuraminidase, which has been shown to cleave sialic acid from host surface glycoproteins and leukocytes to disrupt immune recognition and trigger degradation of host proteins (50). Once colonization is established, *P. melaninogenica* can further damage host tissue through the production of collagenase (11, 51) and a lipase, both of which contribute to abscess formation (11).

Host Immune Response

P. melaninogenica is primarily regarded as a member of the oral commensal flora; consequently its role in disease pathogenesis and host immune response has been largely ignored. However, with the use of molecular-based detection methods, *P. melaninogenica* is frequently identified in abscesses and infected tissues throughout the body. Despite its association with a wide-variety of infections, little is known about its contribution to disease progression.

Innate Immunity

Bacterial lipopolysaccharide (LPS) is a key bacterial factor that impacts host immune response. Pathogen associated molecular patterns (PAMPS), including bacterial LPS, alert the immune system to the presence of bacterial invaders through pattern recognition receptors (PRRs), on immune cells such as macrophages and

dendritic cells. LPS is composed of three distinct components: lipid A, core polysaccharide, and O antigen. The covalently bound lipid component of LPS, lipid A, constitutes the hydrophobic outer leaflet of the Gram-negative outer membrane and is responsible for LPS toxicity. It is traditionally composed of a glucosamine disaccharide backbone with acyl chains of varying length and number and the presence or absence of phosphate groups. Length of the fatty acid chains and the addition of phosphate groups can greatly impact the toxicity of lipid A. The core polysaccharide, attached to lipid A, is composed of heptose and 2-keto-deoxyoctulosonic acid (KDO). The O antigen component, which is the outermost portion and hydrophilic part of the LPS, is made up of repeating subunits of 3 to 5 sugars that vary between species and even strains. The presence of O antigen is responsible for the 'smoothness' characteristics of the bacterial colonies and helps to confer resistance to phagocytosis. LPS, which is released during growth and death of a bacterium, has been shown to induce macrophages to produce reactive oxygen species and to activate antigen presentation and cytokine response pathways. Traditionally, LPS activates the alternative complement pathway.

Historically, *P. melaninogenica* was a member of a group of species collectively designated as *Bacteroides melaninogenicus*. Other members of this closely related group included *Porphyromonas gingivalis, Prevotella intermedia a*nd *Prevotella nigrescens*. *P. gingivalis* has been the most studied species of this group because of its role in periodontal disease. LPS from *P. gingivalis* has been demonstrated to stimulate bone resorption, adhere to erythrocytes and attachment to oral cavity surfaces (52). *P. gingivalis* lipid A is penta-acylated and monophosphorylated and has been consistently

shown to exhibit weak pro-inflammatory properties (endotoxicity) (53). Most reports have shown that *P. gingivalis* LPS is significantly less inflammatory than *E. coli* LPS (54), while some studies suggest *P. gingivalis* LPS is equal or even more proinflammatory than that of *E. coli* (55, 56). It has been suggested that the differences reported in these studies are due to alteration in LPS structure that result from different growth conditions and extraction techniques (57). *P. gingivalis* LPS has been shown to stimulate IL-1β, IL-6, IL-8, IL-10, IL-12, TNFα, IGF1 and NFκB expression and production in THP1 cells (58, 59) as well as, IL-1β, TNFα and IL-8 in polymorphonuclear neutrophils (54, 60, 61). Because controversy still remains about the magnitude of *P. gingivalis* LPS cytokine response, it is difficult to predict the impact of *P. melaninogenica* LPS on the host inflammatory response. Foundational studies need to be completed to thoroughly characterize the structure and inflammatory effect of *P. melaninogenica*.

Toll like receptors:

An important aspect of the inflammatory cytokine response to bacterial infection is due to a cascade of signaling pathways initiated by Toll like receptors (TLRs), which are transmembrane receptors that respond to PAMPS and have a primary role in innate immunity initiation (62). The TLR response to bacterial products occurs by both transcriptional and post translational mechanisms. TLR activation initiates MyD88 and TRIF signaling cascades that activate mitogen-activated protein kinase kinase kinases (MAP3Ks) and lead to NFκB activation (63). NFκB induces transcription of inflammatory cytokines genes such as IL-8, IL-6, IL-1β and TNFα. Once produced, these cytokines alert other cellular components of the immune system. The post-translational signals

stem from cytosolic pattern receptors that stimulate inflammasome components that in turn activate caspase 1 to cleave IL-1β and IL-18 into their active forms (58).

Traditionally, bacterial LPS binds to an accessory protein, lipopolysaccharide-binding protein, which then triggers the CD14-MD-2-TLR 4 complex and activates TLR 4 LPS dependent responses (62, 64, 65). Other TLRs, specifically TLR 2, are known to recognize bacterial products such as peptidoglycan, lipoteichoic acid and lipoproteins. Number and length of lipid A acyl chains in addition to their branched structure can lead to differential TLR signaling. Confusion in the field of LPS dependent TLR signaling has been clouded by lipoprotein contamination of LPS preparation causing TLR 2 activity leading researchers to conclude TLR 2 dependence (66).

Bacteroides species, such as *P. gingivalis*, demonstrate unique structure changes dependent on environmental cues (57, 67-69). In particular, environmental heme concentration modifies the activity of lipid A 1-dephosphorylatases that leads to an alteration in phosphate group attachment to the glucosamine portion of lipid A. This change in structure modulates the ability of *P. gingivalis* to stimulate TLR 4 (53, 57, 62, 69). In these reports, *P. gingivalis* LPS signals through TLR 4 either acting as an agonist in low heme (1 mg/ml) or an antagonist in high heme (10 μg/ml) (68-70).

P. gingivalis LPS response is controversial due to the questions that still remain about the variability of *P. gingivalis* cytokine response compared to other more traditional agonists (i.e. *E. coli* LPS) and the inconsistent reports deciphering *P. gingivalis* TLR signaling pathways. Despite the similarities and phylogenetic relationship between *P. melaninogenica* and *P. gingivalis*, the controversies surrounding *P.*

gingivalis LPS structure and properties make it difficult to make predictions about *P. melaninogenica* TLR signaling.

P. melaninogenica innate immune response

Characterization of host immune response to *P. melaninogenica* has been limited in the past because of its inclusion under the grouping of *B. melaninogenicus*, which is now recognized to have encompassed multiple species including *P. gingivalis*. Consequently, reports prior to 1990 have been considered in this review only if a specific *P. melaninogenica* strain was clearly specified.

P. melaninogenica LPS studies are incomplete; and have only focused on O antigen and the hemagglutination properties of total LPS preparations. *P. melaninogenica* O antigen appears to have similarities to O antigen from *Prevotella levi* (71) which is involved in evasion of phagocytosis. Total LPS preparations from *P. melaninogenica* have been shown to have less hemagglutination activity compared to other *Prevotella* species such as *P. intermedia* and *P. denticola* (52). Because of the role of LPS in initiating bacterial infection and host immune recognition a more thorough characterization of *P. melaninogenica* LPS is needed.

To investigate the role of *P. melaninogenica* in infection, total cellular lysates were shown to stimulate a low level cytokine response (IL-1α, IL-6 and TNF α in human monocytes and human gingival fibroblasts) through a TLR 2 and not TLR 4 signaling pathway (72, 73). Consistent with this report, whole formalin-fixed *P. melaninogenica* have also been shown to signal through TLR 2 when tested in cell lines over expressing either TLR 2 or TLR 4 (73). In addition to its apparent TLR 2 agonist properties, *P.*

melaninogenica could be a TLR 4 antagonist. In a study designed to model the polymicrobial environment of COPD, *P. melaninogenica* lysates dampened *Haemophilus influenzae* TLR 4 signaling in dendritic cells leading to a decrease in IL-12 response (74). Additionally, experiments investigating the effect of 'normal flora' to pathogenic bacteria immune evasion found that *P. melaninogenica* supernatants, representing normal flora, impaired the phagocytosis of the pathogen, *Proteus mirabilis*, by polymorphonuclear leukocytes (75). These studies represent the effect of a complex mixture on cellular response, but do show how *P. melaninogenica* could have a potential impact on innate immune response and the immune stimulation by other pathogens.

Adaptive Immunity

Among the five classes of immunoglobulins, immunoglobulin G (IgG) makes up the majority of serum antibodies and is commonly used as a predictive marker for infection (76). IgG aids phagocytosis through opsonization and complement activation. In the case of bacterial infections, the development of a specific acquired immune response is characteristic of pathogen exposure and bacterial burden (77-79). Elevated antibody titer, (specifically IgG titer) for specific pathogens, has been used to diagnose infections such as syphilis (80), and human papilloma virus (81).

Infections by oral bacteria have been shown to stimulate a humoral response, specifically an increase in serum IgG to bacterial antigens (76, 82). Increased serum IgG titers for periodontal pathogens are used as diagnostic markers of periodontitis (76, 83) and are used to direct prophylactic therapy. In addition, treatment outcomes,

including the reduction of bacterial load, correlate with a reduction of serum IgG titer to specific pathogens (76).

IgG levels to *P. melaninogenica* have been measured in a small number of studies. *P. melaninogenica* IgG titer has been shown to be increased in rheumatoid arthritis patients compared to healthy controls (2). Also, *P. melaninogenica* IgM (an early response antibody) is increased in ventilator-associated pneumonia patients compared to controls (78). Based on these findings, immunoglobulin titer can be an effective tool in evaluating infection by *Prevotella* species (78).

Immune response to commensal bacteria

The presence of commensal bacteria in the body is a constant stimulus to the immune system and requires the host to produce an immune response, both cytokine and antibody, to keep the commensal contained. The host response against endogenous bacteria becomes increased when the bacteria spread to non-traditional locations in the body.

In the oral cavity, commensal bacteria stimulate low-level inflammation that contributes to oral health. Specifically, low levels of IL-8 induce the chemotaxis of neutrophils into the gingivalis crevice to patrol for bacterial pathogens (13). In other parts of the body, the low level stimulation by commensal bacteria aids in the development and maintenance of the immune innate system as seen in experiments with germ-free mice (84). In addition, endogenous bacteria, like *Staphylococcus aureus* or *Pseudomonas aeruginosa*, can cause opportunistic infections when the host immune defenses are impaired or overwhelmed (85, 86).

Nutritional Requirements of *P. melaninogenica*

Iron

Iron, commonly observed in one of two states (Fe^{2+}, Fe^{3+}), has a extensive redox potential, making it a critical enzyme cofactor in the metabolism of amino acid and biosynthesis of nucleotides, vital processes in all organisms (87, 88). For aerobic bacteria, iron is critical for respiration, where it serves as a cofactor for cytochromes involved in electron transport (89). In contrast, anaerobes use iron-sulfur compounds and fumarase, in addition to other iron containing molecules such as catalase and peroxidase, for protection against superoxide and in the production of ATP through pyruvate aided fermentation (90, 91).

Iron in the host:

Iron is a necessary nutrient for basic cellular metabolism; however, because of its reactivity it can be harmful if not complexed. Under reducing (anaerobic) conditions or at low pH, ferrous iron (Fe^{2+}) is the dominant iron form. Ferrous iron is more soluble than ferric iron (Fe^{3+}) making it more toxic and able to pass through semi permeable membranes. Iron can catalyze the Fenton Reaction which leads to the production of reactive oxygen species that damage cellular components (92). Because of the necessity for iron and its intrinsic toxic potential, there is a delicate homeostasis needed to balance iron abundance and scarcity in the host (93). Free iron in a mammalian host is as low as 10^{-24} M (87, 93, 94) due to the presence of high affinity iron binding proteins such as ferritin (inside cells), lactoferrin (mucus secretions), hemoglobin (blood) and

transferrin (bodily fluid such as blood). The majority of iron in the human body is sequestered in hemoglobin in the form of heme (94).

Bacterial Iron Regulation

To survive in the host where iron is scarce, bacteria have developed tightly controlled mechanisms to alter the expression of iron acquisition proteins such as siderophores, degradative enzymes, hemolysins, and hemagglutination (46, 89, 95-97) in response to iron abundance and scarcity. In many bacteria, iron acquisition is regulated by the ferric uptake regulator (Fur), which represses transcription of iron transport and scavenging genes when intracellular iron concentrations are sufficient. Available intracellular iron binds to Fur, facilitating the formation of Fur dimers. The dimer complex binds to the promoter region of iron regulated gene through recognition of specific (Fur box) sequences (98-100). The DNA bound complex hinders access of RNA polymerase. Under iron limiting conditions, Fur controlled genes involved in iron acquisition and storage become derepressed (94, 99, 101-104).

***Porphyromonas* and *Prevotella* heme requirements**

Bacterial organisms require iron to sustain vital cellular processes but some microbes, in addition to iron, require iron in the form of heme. Heme, which represents a stable and highly usable form of iron, consists of iron complexed inside a porphyrin ring structure. Heme can be used directly as an enzyme cofactor or it can be broken down to release molecular iron.

In bacteria, the heme molecule is essential for electron transport and the activity of several metabolic enzymes (100, 105). In *P. gingivalis*, heme is a cofactor of the cytochrome b subunit of fumarate reductase, which plays a role in metabolic energy production (94, 102, 106, 107). Also a heme derivative, μ-oxo bishaem, is stored on the outer surface of many Bacteroides species and produces the characteristic black pigment associated with *P. gingivalis* (108) and other black pigmented *Bacteroides* such as *P. melaninogenica* and *P. intermedia* when exposed to oxygen. The surface localized heme derivative is used for protection against oxygen radicals, and is thought to promote local environmental anaerobiosis (108-110).

Many microbes can produce heme through a complex *in vivo* heme biosynthesis pathway; however, all bacteria in the genera *Bacteroides* lack the enzymes to synthesize their own heme. Specifically, *P. gingivalis* lacks genes encoding 5-aminolevulinic acid synthase and porphobilinogen deaminase (111) and must rely on exogenous sources of this molecule.

P. gingivalis can acquire heme from host heme binding proteins such as hemoglobin and myoglobin or enzymes that use heme as a cofactor such as catalase and myoglobin. In addition, *P. gingivalis* can use non heme based iron sources such as inorganic iron, and iron stored in transferrin and lactoferrin (98, 112, 113). *Prevotella* spp., like *P. intermedia* cannot survive without protoporphyrin IX (PPIX) based iron such as heme, hemoglobin and myoglobin, cytochrome c and catalase (114, 115). Inorganic forms of iron including ferric chloride, ferric citrate, ferric nitrate, and ferric ammonium citrate do not support growth of *P. intermedia* (110, 114).

P. melaninogenica, like other *Bacteroides* species including *P. intermedia*, requires heme for growth (42). *P. intermedia* and *P. gingivalis* represent closely related species that have differing abilities to use iron and heme based sources. Further research is necessary to determine the iron requirements for *P. melaninogenica* and its capacity to utilize host-based iron sources.

In vivo heme acquisition

The majority of useable iron in the human body comes in the form of heme, complexed inside of hemoglobin, myoglobin or haptoglobin. Bacteria have successfully overcome this limitation by using two mechanisms that remove heme from host heme-containing proteins: 1) production of hemophores, which are secreted proteins that bind heme and are subsequently recognized by a cognate bacterial surface receptor, and 2) direct extraction of heme via high affinity heme- or hemoglobin-binding bacterial surface proteins (116). Some bacteria encode only high affinity hemoglobin receptors whereas others use a combination of receptors and hemophores. In addition, there are proteolytic enzymes thought to play a role in releasing heme from heme-containing host proteins (94). In polymicrobial communities, bacterial iron acquisition mechanisms, such as hemolysins that rupture red blood cells, can be a source of interspecies cross feeding. (89, 117-120). High affinity hemoglobin receptors are important for host colonization and have been implemented as a vaccine target for *Haemophilus ducreyi* to prevent bacterial diseases such as chanroid and against *E. coli* as treatment for urinary tract infections (121, 122).

Heme acquisition by Gram-negative bacteria is an orchestrated process that involves heme binding to an outer membrane receptor (in some cases aided by hemophore) followed by a series of steps that transfer heme through the outer membrane and periplasm and into the cytoplasm where it is used as a cofactor itself (e.g. in cytochromes or catalases) or is broken down for its iron component (123). Bacterial hemoglobin receptors form a beta barrel confirmation in the outer membrane and have characteristic domains (FRAP and NPNL signatures) and specific histidine residues that aid in the removal of heme from hemoglobin (116, 124-127). Once bound, heme is transported through the beta barrel channel of the receptor by the energy derived from the proton motive force associated with the binding of the TonB complex to the receptor. Binding of the two proteins is mediated through a 'TonB box', which is a conserved sequence present in the N-terminal periplasmic portion of the outer membrane receptor (128, 129). Once heme enters the periplasm, it is bound and transported by a heme permease to the inner membrane, where an ABC transporter can then transport it to the cytoplasm (130).

The mechanism of heme/hemoglobin acquisition has been investigated to some degree in oral anaerobes; however, the molecular details await further study. Both *P. gingivalis* and *P. intermedia* have heme acquisition systems that involve proteolytic degradation of erythrocytes and heme-binding receptors (94). Specifically, *P. intermedia* has been shown to lyse erythrocytes, degrade hemoglobin and bind both heme and hemoglobin through an undefined receptor (110, 115, 131, 132). Additionally, several *P. gingivalis* hemoglobin receptor complexes including HmuR, Tla and HemR (102, 133-135) have been identified. For *P. melaninogenica,* no outer membrane receptor has

been characterized but a key to its *in vivo* growth could be its ability to promote aggregation and lysis of red blood cells (46), which suggests that it is capable of freeing hemoglobin for bacterial binding and eventual heme uptake. The proteins involved in the subsequent steps in heme acquisition have yet to be identified.

CF Lung Infection

Mechanism

The most common fatal genetic disease in the Caucasian population is cystic fibrosis (CF). CF is inherited in an autosomal recessive pattern and occurs in approximately 1 in 2,500 live births with about 30,000 recognized CF patients in the United States (136). CF disease is due to dysfunction of the epithelial membrane protein cystic fibrosis transmembrane regulator (CFTR). The most common CFTR mutation associated with CF results in a deletion of phenylalanine at amino acid position 508 resulting in protein misfolding (137-139). CFTR is a cyclic AMP-dependent chloride channel and a negative regulator of the epithelial sodium channel ENaC (138-140). Dysfunctional CFTR causes an imbalance of sodium absorption and chloride ion secretion on mucosal surfaces, affecting many mucosal organs including the lungs, pancreas, skin and the reproductive system (141). In CF, liquid dysregulation in the lungs is of considerable importance because it compromises mechanical clearance and host immune homoeostasis. Hyperabsorption of sodium and water at the apical surface of bronchial epithelium reduces periciliary liquid (PCL) volume resulting in increased mucus viscosity and impaired ciliary function (142-145). This clearance defect combined

with mucin hyper-secretion by goblet cells leads to thickened mucus in the conducting airways of CF patients.

Inefficient clearance of mucus in the CF lung provides an optimal colonization niche for a diverse assembly of bacteria (146-148). From infancy to adulthood, CF patients experience a decline in lung function caused by persistent bacterial infection and unrelenting pulmonary inflammation (149). Over a period of years, an accumulation of bacterial products and cellular debris produces irreversible airway damage and inflammation that ultimately leads to respiratory failure and death, with an average life expectancy of 37 years.

CF Treatment:

Treatment of CF starts early in life through therapeutic bronchodilators, anti-inflammation treatments and antibiotics. Antibiotic treatment is based on aerobic culture of bronchoalveolar lavage fluid (BALF) and sputum. BALF is captured through an invasive procedure where sterile saline is released into the conducting airways through a bronchoscope then collected. Induced and spontaneous sputum samples are collected after the patient coughs up thick mucus, believed to be from the bronchial airway surface (150). Contamination of these methods is evaluated by comparison of organisms recovered from saliva samples. These aerobic culture methods are used to guide clinicians in appropriate antibiotic treatment for individual patients. However, aerobic culture methods are inadequate for representing all members of the bacterial community, in particular fastidious organisms and anaerobic bacteria (12). Antibiotics aimed at classical aerobic pathogens including *P. aeruginosa,* are largely ineffective in

clearing bacterial infection (142, 151, 152). For example, there is no significant change in aerobic bacterial load comparing episodes of disease exacerbation and subsequent recovery periods (38) or testing pre and post antibiotic treatment (153). These discrepancies suggest that aerobic culture does not provide the full picture of CF pathogenesis. There is now growing evidence that pulmonary infections in CF should be treated as a polymicrobial infection with aerobic, anaerobic and fungal components (146).

Key aerobic bacteria in the CF microbiome

By as early as 3 months, nearly 40% of infants diagnosed through neonatal CF screening have a lower respiratory bacterial infection (154). Aerobic culture based techniques and quantitative PCR (qPCR) for the highly conserved bacterial 16S rRNA gene from CF samples (BALF, induced sputum, spontaneous sputum) illustrate that CF affected children are colonized by *Staphylococcus aureus*, *Streptococcus* spp, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (38, 155, 156). As CF patients age, the diversity of the bacterial community decreases and the majority of CF patients become chronically colonized with *P. aeruginosa* (153, 157, 158). During the transition between acute and chronic infection, *P. aeruginosa* acquires mutations in *lasR* leading to antibiotic resistance and a metabolic transition to use host-associated amino acids (159). Another hallmark of chronic *P. aeruginosa* colonization in the CF lung is the cultivation of isolates encased in an alginate exopolysaccharide (160, 161). This state is commonly called mucoidy and correlates to a decline in clinical outcome for individuals with CF (142, 162). Mucoid *P. aeruginosa* are thought to exist in a biofilm-like state

within mucus plugs, allowing the bacteria to be highly resistant to phagocytosis, antibodies and antibiotic treatment (143, 151, 163). There is direct evidence of *P. aeruginosa* enmeshed in alginate biofilm aggregates in CF lung samples (147). In adults, the bacterial community within the biofilm may also include other aerobes such as *Streptococcus milleri* species (164), *Burkholderia cepaci, Stenotrophomonas maltophilia* and multiple fungal species (165). As CF lung disease progresses the successful growth of *P. aeruginosa* is aided by the ability of *P. aeruginosa* to adapt to other bacteria and environmental changes including the reduction of oxygen in CF mucus plugs (166).

CF anaerobic niche

Direct measurements of the oxygen gradient in CF mucus plugs range from 180 mmHg outside to 2.5 mmHg inside the plug, demonstrating a significant drop in oxygen and near anaerobic conditions within the airway mucus (166). The exact mechanism of oxygen depletion within CF mucus plugs has not been confirmed but there are multiple hypotheses involving accelerated O_2 consumption either by the lung epithelium, immune cell respiratory bursts or elevated bacterial respiration (141). *P. aeruginosa* and other facultative anaerobes that are present in the lungs of CF patients can live anaerobically in the presence of an appropriate terminal electron acceptor, such as arginine (163, 166-170).

Because anaerobic bacteria have been documented in other polymicrobial infections of the lung such as pneumonia, lung abscesses and empyema, these bacteria could have significant relevance in pulmonary infection in CF (37, 148). CF

samples processed using anaerobic culture techniques and other unbiased molecular methods such as 16s rRNA gene profiling by microarray, pyrosequencing and reverse transcription terminal restriction fragments length polymorphisms (TRFLP), have revealed the presence of strict anaerobes (29-32, 34-37, 148, 156, 164, 165, 171-173). Because of this, many have hypothesized that anaerobes may play a role in the pathogenesis of CF airway disease (12, 31, 37, 38, 142, 148, 171). Tunney *et al*. (2008) noted that the most prevalent strict anaerobes isolated from sputum were *Prevotella* species, including *P. melaninogenica*. Anaerobic bacteria in this study were isolated in high numbers (10^4-10^9 CFU/g sputum) in 64% of adults and, in some cases, were present in higher numbers than *P. aeruginosa* (37). The prominence of *Prevotella* is consistent with past studies involving anaerobic culture techniques and molecular based approaches (12, 29-32, 34-37, 148, 156, 164, 165, 172-175). It has been suggested that anaerobes, in particular *Bacteroides* spp. (now *Prevotella* spp., *Porphyromonas* spp. etc), could be of clinical importance in CF (148) and potentially pathogenic (30, 32, 37). Of the *Prevotella* species enumerated in CF samples, *P. melaninogenica* is the most common anaerobe reported using culture independent and anaerobic culture techniques (37), and it is frequently present in high numbers (12, 30-32, 34-36). Despite the cumulative evidence for the presence of anaerobic bacteria in the CF lung based on analysis of sputum and BALF, two studies of explanted lung specimens suggested that oral anaerobes are not present in lower airway but instead are found in upper airway specimens such as the trachea due to 'oral contaminants' (174, 176).

CF immune response

In CF, when bacteria colonize the lung, the host responds with an influx of polymorphonuclear leukocytes followed by cytokine and antibody production (138, 140, 141, 146, 149, 177-179). Studies have shown that this CF characteristic response of PMNs is not a CFTR dependent response as some had speculated (177, 180). The immune response of CF patients is defined by ineffective killing of bacteria colonizing the lung, and the development of chronic inflammation that leads to lung dysfunction and respiratory failure. These processes are responsible for the majority of CF deaths (37, 141, 169). The exact initiation events leading to CF airway inflammation are the subject of debate, in part, because of the complexity of CF pathogenesis and inconsistent experimental results. Further studies investigating cytokine response shows that there is significantly more IL-8 and neutrophils in BALF from CF children than children with other non-CF respiratory disease (179). Additionally, studies using sputum and BALF samples from CF adults show an increased number of neutrophils, increased levels of the pro-inflammatory cytokines TNF α, IL-1β, IL-6, IL-8, and reduced levels of the anti-inflammatory cytokine IL-10 (181-183); this response is thought to be mediated by TLR-dependent pathways (137, 184).

In addition to innate inflammatory responses, the adaptive immune system in CF patients also produces a response against bacterial pathogens enmeshed in CF mucus plugs. In CF, elevated levels of *P. aeruginosa*-specific IgG antibody have been noted and been used for diagnostic purposes (185). *P. aeruginosa* infection occurring in subjects as young as 6 months have been diagnosed using *P. aeruginosa* specific IgG serological testing (185-187). Consistent with this finding, a high level of *P. aeruginosa* specific IgG antibodies correlate with negative clinical outcome (185).

Iron in the CF lung

The lung environment within chronically infected CF patients contains higher levels of iron compared to the lungs of healthy patients (89, 150, 158). Significant amounts of iron have been detected in sputum from CF patients in the form of ferritin, lactoferrin, transferrin and small amounts of hemoglobin (89). In an unpublished study, there was more than twice the amount of hemoglobin and heme in CF BALF than in asthmatic patients; ten times more than healthy individuals (188). The underlying cause of elevated iron in the CF lung is thought to be from micro-hemorrhage, inflammation (189), transferrin/lactoferrin proteolysis and release of intracellular iron stores from CF airway epithelial cells (93, 190). Additionally, the iron present in the CF lung is predicted to be more soluble (and therefore better available for bacterial uptake) because the pH of the lung is more acidic (5.8) in CF patients compared to healthy controls (6.1) (191).

The role of *P. melaninogenica* in CF lung infection

Despite evidence that anaerobic bacteria are part of the polymicrobial community in the CF lung, the question of their clinical relevance remains unanswered. The role of anaerobes in CF pathogenesis can be thought of in two ways: 1) the anaerobes themselves could be pathogenic, and/or 2) the presence of anaerobes could influence the pathogenicity of the bacterial community. For the latter, the presence of additional bacteria could impact host inflammation initiated by the pathogenic agent (e.g. *P. aeruginosa*) (192) or provide a source of antibiotic resistance genes that could alter the resistance properties of the community. There has been suggested synergism between

P. aeruginosa and anaerobes in several cases (28, 39, 41). *Bacteroides* species including *Prevotella* are known to produce quorum-sensing signaling molecules and thus have the potential to affect the virulence of *P. aeruginosa*. Further work needs to be done in this area comparing *P. aeruginosa* to prominent anaerobic species.

Here, we will explore the contribution of *P. melaninogenica*, which is the most commonly cultured anaerobic bacterium in the CF lung, to CF pathogenesis. In chapter 2, we investigate the prevalence and abundance of *P. melaninogenica* in a cohort of UNC hospital CF patents and test *P. melaninogenica* reactive antibody response as a measurement of bacterial burden and exposure. This study is aimed at determining whether host response to a nontraditional CF associated bacterium is different in CF patients compared to non CF individuals. In chapter 3, we determine the structure and inflammatory properties of *P. melaninogenica* LPS, the most toxic part of gram-negative outer membrane. In this chapter, we will examine the effect of *P. melaninogenica* LPS on a human monocytic cell line and determine TLR signaling pathways. Chapter 4 focuses on how *P. melaninogenica* survives in the CF lung, specifically determining its ability to acquire iron from host sources and the mechanism for heme acquisition.

My goal is not only to report and confirm the presence of anaerobes in CF but to provide understanding to the clinical relevance of *P. melaninogenica.* My findings may provide the justification for new treatment options to help patients with severe CF lung disease. New avenues for research in anaerobic bacterial pathogenesis will lead to knowledge about treatment interventions to reduce the severity of CF lung disease. My dissertation has immediate clinical significance in terms of applying new understanding to developing novel therapeutic approaches.

References

1. Shah HN, Collins DM. Notes: *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int J Syst Bacteriol. 1990;40(2):205-8.

2. Ogrendik M, Kokino S, Ozdemir F, Bird PS, Hamlet S. Serum antibodies to oral anaerobic bacteria in patients with rheumatoid arthritis. Medscape General Medicine. 2005;7(2):2.

3. Wu CC, Johnson J, Moore W, Moore L. Emended descriptions of *Prevotella denticola, Prevotella loescheii, Prevotella veroralis,* and *Prevotella melaninogenica*. Int J Syst Bacteriol. 1992;42(4):536-41.

4. Duerden B. The isolation and identification of *Bacteroides* spp. from the normal human gingival flora. J Med Microbiol. 1980;13(1):89-101.

5. Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodin EF, et al. Bacterial diversity in the oral cavity of 10 healthy individuals. The ISME journal. 2010;4(8):962-74.

6. Papaioannou W, Gizani S, Haffajee A, Quirynen M, Mamai-Homata E, Papagiannoulis L. The microbiota on different oral surfaces in healthy children. Oral Microbiol Immunol. 2009;24(3):183-9.

7. Könönen E. Pigmented *Prevotella* species in the periodontally healthy oral cavity. FEMS Immunol Med Microbiol. 1993;6(2):201-5.

8. Bailit H, Baldwin D, Hunt E. The increasing prevalence of gingival *Bacteroides melaninogenicus* with age in children. Arch Oral Biol. 1964;9(4):435-8.

9. Hentges DJ. The anaerobic microflora of the human body. Clinical infectious diseases. 1993;16(Supplement 4):S175-80.

10. Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H. Establishment of oral anaerobes during the first year of life. J Dent Res. 1999;78(10):1634-9.

11. Alauzet C, Marchandin H, Lozniewski A. New insights into *Prevotella* diversity and medical microbiology. Future microbiology. 2010;5(11):1695-718.

12. Rogers G, Carroll M, Serisier D, Hockey P, Jones G, Bruce K. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2004;42(11):5176-83.

13. Robert R, Grollier G, Frat JP, Godet C, Adoun M, Fauchère JL, et al. Colonization of lower respiratory tract with anaerobic bacteria in mechanically ventilated patients. Intensive Care Med. 2003;29(7):1062-8.

14. Falagas ME, Siakavellas E. *Bacteroides, Prevotella,* and *Porphyromonas* species: A review of antibiotic resistance and therapeutic options. Int J Antimicrob Agents. 2000;15(1):1-9.

15. Mukhopadhyay S, Rose F, Frechette V. Vertebral osteomyelitis caused by *Prevotella (Bacteroides) melaninogenicus*. South Med J. 2005;98(2):226.

16. Bowler P, Duerden B, Armstrong D. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001;14(2):244-69.

17. Odeh M, Oliven A, Potasman I, Solomon H, Srugo I. Pyomyositis of the thigh due to *Prevotella melaninogenica*. Infection. 2000;28(1):49-50.

18. Athanasiou S, Matthaiou DK, Falagas ME. Vaginal mesh infection due to *Bacteroides melaninogenicus:* A case report of another emerging foreign body related infection. Scand J Infect Dis. 2006;38(11-12):1108-10.

19. Jousimies-Somer H, Savolainen S, Mäkitie A, Ylikoski J. Bacteriologic findings in peritonsillar abscesses in young adults. Clinical infectious diseases. 1993;16(Supplement 4):S292-8.

20. Li X, Kolltveit KM, Tronstad L, Olsen I. Systemic diseases caused by oral infection. Clin Microbiol Rev. 2000;13(4):547-58.

21. Moutsopoulos NM, Madianos PN. Low-Grade inflammation in chronic infectious diseases. Ann N Y Acad Sci. 2006;1088(1):251-64.

22. Paju S, Scannapieco F. Oral biofilms, periodontitis, and pulmonary infections. Oral Dis. 2007;13(6):508-12.

23. Hsiao WWL, Li KL, Liu Z, Jones C, Fraser-Liggett CM, Fouad AF. Microbial transformation from normal oral microbiota to acute endodontic infections. BMC Genomics. 2012;13(1):345.

24. Talan DA, Abrahamian FM, Moran GJ, Citron DM, Tan JO, Goldstein EJC. Clinical presentation and bacteriologic analysis of infected human bites in patients presenting to emergency departments. Clinical infectious diseases. 2003;37(11):1481-9.

25. Hosek WT, Gullett J. Adult female with shoulder pain. Ann Emerg Med. 2010;55(1):e1-2.

26. Harding G, Sutter V, Finegold S, Bricknell K. Characterization of *Bacteroides melaninogenicus.*. J Clin Microbiol. 1976;4(4):354-9.

27. De A, Varaiya A, Mathur M. Anaerobes in pleuropulmonary infections. Indian Journal of Medical Microbiology. 2002;20(3):150.

28. Brook I. *Prevotella* and *Porphyromonas* infections in children. J Med Microbiol. 1995;42(5):340-7.

29. Sibley CD, Grinwis ME, Field TR, Eshaghurshan CS, Faria MM, Dowd SE, et al. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. PloS one. 2011;6(7):e22702.

30. Field TR, Sibley CD, Parkins MD, Rabin HR, Surette MG. The genus *Prevotella* in cystic fibrosis airways. Anaerobe. 2010;16(4):337-44.

31. Worlitzsch D, Rintelen C, Böhm K, Wollschläger B, Merkel N, Borneff-Lipp M, et al. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. Clinical Microbiology and Infection. 2009;15(5):454-60.

32. Ulrich M, Beer I, Braitmaier P, Dierkes M, Kummer F, Krismer B, et al. Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis. Thorax. 2010;65(11):978-84.

33. Bittar F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, et al. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One. 2008;3(8):e2908.

34. Stressmann FA, Rogers GB, Klem ER, Lilley AK, Donaldson SH, Daniels TW, et al. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. J Clin Microbiol. 2011;49(1):281-9.

35. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proceedings of the National Academy of Sciences. 2007;104(51):20529-33.

36. van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME journal. 2010;5(5):780-91.

37. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. American journal of respiratory and critical care medicine. 2008;177(9):995-1001.

38. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol Microbiol. 2003;50(5):1477-91.

39. Brook I. Pathogenicity of capsulate and non-capsulate members of *Bacteroides fragilis* and *B. melaninogenicus* groups in mixed infection with *Escherichia coli* and S*treptococcus pyogenes*. J Med Microbiol. 1988;27(3):191-8.

40. Brook I. Encapsulated anaerobic bacteria in synergistic infections. Microbiol Rev. 1986;50(4):452.

41. Brook I. Enhancement of growth of aerobic and facultative bacteria in mixed infections with *Bacteroides* species. Infect Immun. 1985;50(3):929-31.

42. Gibbons RJ, Macdonald JB. Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J Bacteriol. 1960;80(2):164-70.

43. Dalwai F, Spratt D, Pratten J. Modeling shifts in microbial populations associated with health or disease. Appl Environ Microbiol. 2006;72(5):3678-84.

44. Peters BM, Jabra-Rizk MA, Graeme A, Costerton JW, Shirtliff ME. Polymicrobial interactions: Impact on pathogenesis and human disease. Clin Microbiol Rev. 2012;25(1):193-21.

45. Haraldsson G, Meurman JH, Könönen E, Holbrook WP. Properties of hemagglutination by *Prevotella melaninogenica*. Anaerobe. 2005;11(5):285-9.

46. Allison HE, Hillman JD. Cloning and characterization of a *Prevotella melaninogenica* hemolysin. Infect Immun. 1997;65(7):2765-71.

47. Kilian M, Thomsen B, Petersen T, Bleeg H. Occurrence and nature of bacterial IgA proteases. Ann N Y Acad Sci. 2006;409(1):612-24.

48. Botta GA, Arzese A, Minisini R, Trani G. Role of structural and extracellular virulence factors in gram-negative anaerobic bacteria. Clinical infectious diseases. 1994;18(Supplement 4):S260-4.

49. Grenier D, Mayrand D, McBride BC. Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides*. Oral Microbiol Immunol. 1989;4(1):12-8.

50. Moncla B, Braham P, Hillier S. Sialidase (neuraminidase) activity among gram-negative anaerobic and capnophilic bacteria. J Clin Microbiol. 1990;28(3):422-5.

51. Jin KC, Barua PK, Zambon J, Neiders ME. Proteolytic activity in black-pigmented *Bacteroides* species. J Endod. 1989;15(10):463-7.

52. Okuda K, Kato T. Hemagglutinating activity of lipopolysaccharides from subgingival plaque bacteria. Infect Immun. 1987;55(12):3192-6.

53. Berezow AB, Ernst RK, Coats SR, Braham PH, Karimi-Naser LM, Darveau RP. The structurally similar, penta-acylated lipopolysaccharides of *Porphyromonas gingivalis and Bacteroides* elicit strikingly different innate immune responses. Microb Pathog. 2009;47(2):68-77.

54. Yoshimura A, Hara Y, Kaneko T, Kato I. Secretion of IL-1β, TNF-α, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. J Periodont Res. 2006;32(3):279-86.

55. Koga T, Nishihara T, Fujiwara T, Nisizawa T, Okahashi N, Noguchi T, et al. Biochemical and immunobiological properties of lipopolysaccharide (LPS) from *Bacteroides gingivalis* and comparison with LPS from *Escherichia coli.*. Infect Immun. 1985;47(3):638-47.

56. Zhang D, Chen L, Li S, Gu Z, Yan J. Lipopolysaccharide (LPS) of *Porphyromonas gingivalis* induces IL-1β, TNF-α and IL-6 production by THP-1 cells in a way different from that of *Escherichia coli* LPS. Innate immunity. 2008;14(2):99-107.

57. Al-Qutub MN, Braham PH, Karimi-Naser LM, Liu X, Genco CA, Darveau RP. Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. Infect Immun. 2006;74(8):4474-85.

58. Taxman DJ, Zhang J, Champagne C, Bergstralh DT, Iocca HA, Lich JD, et al. Cutting edge: ASC mediates the induction of multiple cytokines by *Porphyromonas gingivalis* via caspase-1-dependent and-independent pathways. The Journal of Immunology. 2006;177(7):4252-6.

59. Park SY, Park DJ, Kim YH, Kim YH, Choi YW, Lee SJ. *Schisandra chinensis* α-iso-cubebenol induces heme oxygenase-1 expression through PI3K/akt and Nrf2 signaling and has anti-inflammatory activity in *Porphyromonas gingivalis* lipopolysaccharide-stimulated macrophages. Int Immunopharmacol. 2011;11(11):1907-15.

60. Ogawa T, Uchida H, Amino K. Immunobiological activities of chemically defined lipid A from lipopolysaccharides of *Porphyromonas gingivalis*. Microbiology. 1994;140(5):1209-16.

61. Martin M, Katz J, Vogel SN, Michalek SM. Differential induction of endotoxin tolerance by lipopolysaccharides derived from *Porphyromonas gingivalis* and *Escherichia coli*. The Journal of Immunology. 2001;167(9):5278-85.

62. Jain S, Darveau RP. Contribution of *Porphyromonas gingivalis* lipopolysaccharide to periodontitis. Periodontol 2000. 2010;54(1):53-70.

63. Banerjee A, Gerondakis S. Coordinating TLR-activated signaling pathways in cells of the immune system. Immunol Cell Biol. 2007;85(6):420-4.

64. Dziarski R, Wang Q, Miyake K, Kirschning CJ, Gupta D. MD-2 enables toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to gram-positive and gram-negative bacteria and their cell wall components. The Journal of Immunology. 2001;166(3):1938-44.

65. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42(2):145-51.

66. Zähringer U, Lindner B, Inamura S, Heine H, Alexander C. TLR2–promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology. 2008;213(3):205-24.

67. Cutler CW, Eke PI, Genco CA, Van Dyke TE, Arnold RR. Hemin-induced modifications of the antigenicity and hemin-binding capacity of *Porphyromonas gingivalis* lipopolysaccharide. Infect Immun. 1996;64(6):2282-7.

68. Darveau RP, Arbabi S, Garcia I, Bainbridge B, Maier RV. *Porphyromonas gingivalis* lipopolysaccharide is both agonist and antagonist for p38 mitogen-activated protein kinase activation. Infect Immun. 2002;70(4):1867-73.

69. Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, et al. Human toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1-and 4′-phosphatase activities. Cell Microbiol. 2009;11(11):1587-99.

70. Coats SR, Reife RA, Bainbridge BW, Pham TTT, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide antagonizes *Escherichia coli* lipopolysaccharide at toll-like receptor 4 in human endothelial cells. Infect Immun. 2003;71(12):6799-807.

71. Firoozkoohi J, Zandi H, Olsen I. Comparison of lipopolysaccharides from *Bacteroides, Porphyromonas, Prevotella, Campylobacter* and *Wolinella* spp. by Tricine-SDS-PAGE. Dental Traumatology. 1997;13(1):13-8.

72. Rossano F, Rizzo A, Sanges MR, Cipollaro de L'Ero G, Tufano MA. Human monocytes and gingival fibroblasts release tumor necrosis factor-α, interleukin-1α and interleukin-6 in response to particulate and soluble fractions of *Prevotella melaninogenica* and *Fusobacterium nucleatum*. Int J Clin Lab Res. 1993;23(1):165-8.

73. Ahmed N, Hayashi T, Hasegawa A, Furukawa H, Okamura N, Chida T, et al. Suppression of human immunodeficiency virus type 1 replication in macrophages by

commensal bacteria preferentially stimulating toll-like receptor 4. J Gen Virol. 2010;91(11):2804-13.

74. Larsen JM, Steen-Jensen DB, Laursen JM, Søndergaard JN, Musavian HS, Butt TM, et al. Divergent pro-inflammatory profile of human dendritic cells in response to commensal and pathogenic bacteria associated with the airway microbiota. PLoS One. 2012;7(2):e31976.

75. Jones G, Gemmell C. Impairment by *Bacteroides* species of opsonisation and phagocytosis of enterobacteria. J Med Microbiol. 1982;15(3):351-6.

76. Sugi N, Naruishi K, Kudo C, Hisaeda-Kako A, Kono T, Maeda H, et al. Prognosis of periodontitis recurrence after intensive periodontal treatment using examination of serum IgG antibody titer against periodontal bacteria. J Clin Lab Anal. 2011;25(1):25-32.

77. Cole LE, Toffer KL, Fulcher RA, San Mateo LR, Orndorff PE, Kawula TH. A humoral immune response confers protection against *Haemophilus ducrey*i infection. Infect Immun. 2003;71(12):6971-7.

78. Grollier G, Doré P, Robert R, Ingrand P, Gréjon C, Fauchere JL. Antibody response to *Prevotella* spp. in patients with ventilator-associated pneumonia. Clin Diagn Lab Immunol. 1996;3(1):61-5.

79. West Seh, Zeng L, Lee BL, Kosorok MR, Laxova A, Rock MJ, et al. Respiratory infections with *Pseudomonas aeruginosa*in children with cystic fibrosis. JAMA: the journal of the American Medical Association. 2002;287(22):2958-67.

80. Tsang RSW, Martin IE, Lau A, Sawatzky P. Serological diagnosis of syphilis: Comparison of the Trep-Chek IgG enzyme immunoassay with other screening and confirmatory tests. FEMS Immunology & Medical Microbiology. 2007;51(1):118-24.

81. Lundstig A, Dillner J. Serological diagnosis of human polyomavirus infection. Polyomaviruses and Human Diseases. 2006:96-101.

82. Colhoun H, Slaney J, Rubens M, Fuller J, Sheiham A, Curtis M. Antibodies to periodontal pathogens and coronary artery calcification in type 1 diabetic and nondiabetic subjects. J Periodont Res. 2008;43(1):103-10.

83. Dye BA, Herrera-Abreu M, Lerche-Sehm J, Vlachojannis C, Pikdoken L, Pretzl B, et al. Serum antibodies to periodontal bacteria as diagnostic markers of periodontitis. J Periodontol. 2009;80(4):634-47.

84. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology. 2004;4(6):478-85.

85. van Belkum A, Melles DC, Nouwen J, van Leeuwen WB, van Wamel W, Vos MC, et al. Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. Infection, Genetics and Evolution. 2009;9(1):32-47.

86. Gómez MI, Prince A. Opportunistic infections in lung disease: *Pseudomonas* infections in cystic fibrosis. Current opinion in pharmacology. 2007;7(3):244-51.

87. Butler A. Iron acquisition: Straight up and on the rocks? Nature Structural & Molecular Biology. 2003;10(4):240-1.

88. Andrews SC, Robinson AK, Rodríguez-Quiñones F. Bacterial iron homeostasis. FEMS Microbiol Rev. 2006;27(2-3):215-37.

89. Lamont IL, Konings AF, Reid DW. Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. Biometals. 2009;22(1):53-60.

90. Green J, Crack JC, Thomson AJ, LeBrun NE. Bacterial sensors of oxygen. Curr Opin Microbiol. 2009;12(2):145-51.

91. Nakano MM, Zuber PA. Strict and facultative anaerobes: Medical and environmental aspects. CRC Press; 2004.

92. Kell D. Iron behaving badly: Inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC medical genomics. 2009;2(1):2.

93. Reid DW, Anderson GJ, Lamont IL. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;297(5):L795-802.

94. Lewis JP. Metal uptake in host–pathogen interactions: Role of iron in *Porphyromonas gingivalis* interactions with host organisms. Periodontol 2000. 2009;52(1):94-116.

95. Vasil ML, Ochsner UA. The response of *Pseudomonas aeruginosa* to iron: Genetics, biochemistry and virulence. Mol Microbiol. 2002;34(3):399-413.

96. Genco C. Regulation of hemin and iron transport in *Porphyromonas gingivalis*. Adv Dent Res. 1995;9(1):41-7.

97. Kim EJ, Sabra W, Zeng AP. Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa* PAO1. Microbiology. 2003;149(9):2627-34.

98. Olczak T, Simpson W, Liu X, Genco CA. Iron and heme utilization in *Porphyromonas gingivalis*. FEMS Microbiol Rev. 2005;29(1):119-44.

99. Carpenter BM, Whitmire JM, Merrell DS. This is not your mother's repressor: The complex role of fur in pathogenesis. Infect Immun. 2009;77(7):2590-601.

100. Otto B, Verweij-van Vught A, MacLaren D. Transferrins and heme-compounds as iron sources for pathogenic bacteria. Crit Rev Microbiol. 1992;18(3):217-33.

101. Mills M, Payne SM. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an analogous system in *Escherichia coli*O157: H7. J Bacteriol. 1995;177(11):3004-9.

102. Simpson W, Olczak T, Genco CA. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of *Porphyromonas gingivalis*. J Bacteriol. 2000;182(20):5737-48.

103. Bagg A, Neilands J. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry (N Y). 1987;26(17):5471-7.

104. McHugh JP, Rodríguez-Quiñones F, Abdul-Tehrani H, Svistunenko DA, Poole RK, Cooper CE, et al. Global iron-dependent gene regulation in *Escherichia coli*. J Biol Chem. 2003;278(32):29478-86.

105. Loew G. Structure, spectra, and function of heme sites. International Journal of Quantum Chemistry. 2000;77(1):54-70.

106. Meuric V, Rouillon A, Chandad F, Bonnaure-Mallet M. Putative respiratory chain of *Porphyromonas gingivalis*. Future Microbiology. 2010;5(5):717-34.

107. Rizza V, Sinclair PR, White DC, Cuorant PR. Electron transport system of the protoheme-requiring anaerobe *Bacteroides melaninogenicus*. J Bacteriol. 1968;96(3):665-71.

108. Smalley JW, Birss AJ, Silver J. The periodontal pathogen *Porphyromonas gingivalis* harnesses the chemistry of the μ-oxo bishaem of iron protoporphyrin IX to protect against hydrogen peroxide. FEMS Microbiol Lett. 2000;183(1):159-64.

109. Smalley JW, Silver J, Birss AJ, Withnall R, Titler PJ. The haem pigment of the oral anaerobes *Prevotella nigrescens* and *Prevotella intermedia*is composed of iron (III) protoporphyrin IX in the monomeric form. Microbiology. 2003;149(7):1711-8.

110. Byrne DP, Wawrzonek K, Jaworska A, Birss AJ, Potempa J, Smalley JW. Role of the cysteine protease interpain A of *Prevotella intermedia* in breakdown and release of haem from haemoglobin. Biochem J. 2009;425(1):257.

111. Roper JM, Raux E, Brindley AA, Schubert HL, Gharbia SE, Shah HN, et al. The enigma of cobalamin (vitamin B12) biosynthesis in *Porphyromonas gingivalis*. J Biol Chem. 2000;275(51):40316-23.

112. Paramaesvaran M, Nguyen KA, Caldon E, McDonald JA, Najdi S, Gonzaga G, et al. Porphyrin-mediated cell surface heme capture from hemoglobin by *Porphyromonas gingivalis*. J Bacteriol. 2003;185(8):2528-37.

113. Bramanti TE, Holt SC. Roles of porphyrins and host iron transport proteins in regulation of growth of *Porphyromonas gingivalis* W50. J Bacteriol. 1991;173(22):7330-9.

114. Leung KP, Folk SP. Effects of porphyrins and inorganic iron on the growth of *Prevotella intermedia*. FEMS Microbiol Lett. 2002;209(1):15-21.

115. Leung KP, Subramaniam P, Okamoto M, Fukushima H, Lai CH. The binding and utilization of hemoglobin by *Prevotella intermedia*. FEMS Microbiol Lett. 2006;162(2):227-33.

116. Elkins C. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi.* Infect Immun. 1995;63(4):1241-5.

117. Weaver VB, Kolter R. *Burkholderia spp.* alter *Pseudomonas aeruginosa* physiology through iron sequestration. J Bacteriol. 2004;186(8):2376-84.

118. Mashburn LM, Jett AM, Akins DR, Whiteley M. *Staphylococcus aureus* serves as an iron source for *pseudomonas aeruginosa* during*in vivo* coculture. J Bacteriol. 2005;187(2):554-66.

119. Hansen SK, Rainey PB, Haagensen JAJ, Molin S. Evolution of species interactions in a biofilm community. Nature. 2007;445(7127):533-6.

120. Ramsey MM, Rumbaugh KP, Whiteley M. Metabolite cross-feeding enhances virulence in a model polymicrobial infection. PLoS Pathogens. 2011;7(3):e1002012.

121. Afonina G, Leduc I, Nepluev I, Jeter C, Routh P, Almond G, et al. Immunization with the *Haemophilus ducreyi* hemoglobin receptor HgbA protects against infection in the swine model of chancroid. Infect Immun. 2006;74(4):2224-32.

122. Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HLT. Mucosal immunization with iron receptor antigens protects against urinary tract infection. PLoS pathogens. 2009;5(9):e1000586.

123. Stojiljkovic I, Perkins-Balding D. Processing of heme and heme-containing proteins by bacteria. DNA Cell Biol. 2002;21(4):281-95.

124. Burkhard KA, Wilks A. Characterization of the outer membrane receptor ShuA from the heme uptake system of *Shigella dysenteriae* substrate specificty and identification of the heme proteins ligands. J Biol Chem. 2007;282(20):15126-3.

125. Cobessi D, Meksem A, Brillet K. Structure of the heme/hemoglobin outer membrane receptor ShuA from *Shigella dysenteriae*: Heme binding by an induced fit mechanism. Proteins: Structure, Function, and Bioinformatics. 2009;78(2):286-94.

126. Gaudin CFM, Grigg JC, Arrieta AL, Murphy MEP. Unique heme-iron coordination by the hemoglobin receptor IsdB of *Staphylococcus aureus*. Biochemistry (N Y). 2011;50(24):5443-52.

127. Nepluev I, Afonina G, Fusco WG, Leduc I, Olsen B, Temple B, et al. An immunogenic, surface-exposed domain of *Haemophilus ducreyi* outer membrane protein HgbA is involved in hemoglobin binding. Infect Immun. 2009;77(7):3065-74.

128. Postle K. TonB system, *in vivo* Assays and characterization. Meth Enzymol. 2007;422:245-69.

129. Gumbart J, Wiener MC, Tajkhorshid E. Mechanics of force propagation in TonB-dependent outer membrane transport. Biophys J. 2007;93(2):496-504.

130. Létoffé S, Delepelaire P, Wandersman C. The housekeeping dipeptide permease is the *Escherichia coli* heme transporter and functions with two optional peptide binding proteins. Proceedings of the National Academy of Sciences. 2006;103(34):12891-6.

131. Yu F, Anaya C, Lewis JP. Outer membrane proteome of *Prevotella intermedia*17: Identification of thioredoxin and iron-repressible hemin uptake loci. Proteomics. 2007;7(3):403-12.

132. Guan SM, Nagata H, Maeda K, Kuboniwa M, Minamino N, Shizukuishi S. Purification and characterization of a hemoglobin-binding outer membrane protein of *Prevotella intermedia*. FEMS Microbiol Lett. 2004;235(2):333-9.

133. Karunakaran T, Madden T, Kuramitsu H. Isolation and characterization of a hemin-regulated gene,*hemR,* from *Porphyromonas gingivalis.*. J Bacteriol. 1997;179(6):1898-90.

134. Aduse-Opoku J, Slaney JM, Rangarajan M, Muir J, Young KA, Curtis MA. The tla protein of *Porphyromonas gingivalis* W50: A homolog of the RI protease precursor (PrpRI) is an outer membrane receptor required for growth on low levels of hemin. J Bacteriol. 1997;179(15):4778-8.

135. Genco CA, Dixon DW. Emerging strategies in microbial haem capture. Mol Microbiol. 2001;39(1):1-11.

136. Ratjen F, Döring G. Cystic fibrosis. The Lancet. 2003 2/22;361(9358):681-9.

137. Greene CM, Carroll TP, Smith SGJ, Taggart CC, Devaney J, Griffin S, et al. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. The Journal of Immunology. 2005;174(3):1638-46.

138. Jacquot J, Tabary O, Le Rouzic P, Clement A. Airway epithelial cell inflammatory signalling in cystic fibrosis. Int J Biochem Cell Biol. 2008;40(9):1703-15.

139. Pier GB, Grout M, Zaidi TS, Olsen JC, Johnson LG, Yankaskas JR, et al. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science. 1996;271(5245):64-7.

140. Banner KH, De Jonge H, Elborn S, Growcott E, Gulbins E, Konstan M, et al. Highlights of a workshop to discuss targeting inflammation in cystic fibrosis. Journal of Cystic Fibrosis. 2009;8(1):1-8.

141. Boucher R. New concepts of the pathogenesis of cystic fibrosis lung disease. European Respiratory Journal. 2004;23(1):146-58.

142. Davies JC, Bilton D. Bugs, biofilms, and resistance in cystic fibrosis. Respir Care. 2009;54(5):628-40.

143. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science. 1999;284(5418):1318-22.

144. Trout L, King M, Feng W, Inglis SK, Ballard ST. Inhibition of airway liquid secretion and its effect on the physical properties of airway mucus. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1998;274(2):L258-63.

145. Matsui H, Wagner VE, Hill DB, Schwab UE, Rogers TD, Button B, et al. A physical linkage between cystic fibrosis airway surface dehydration and *Pseudomonas aeruginosa* biofilms. Proceedings of the National Academy of Sciences. 2006;103(48):18131-6.

146. Sibley CD, Rabin H, Surette MG. Cystic fibrosis: A polymicrobial infectious disease. Future microbiology. 2006;1(1):53-61.

147. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. 2009;44(6):547-58.

148. Jewes LA, Spencer RC. The incidence of anaerobes in the sputum of patients with cystic fibrosis. J Med Microbiol. 1990 Apr;31(4):271-4.

149. De Rose V. Mechanisms and markers of airway inflammation in cystic fibrosis. European Respiratory Journal. 2002;19(2):333-40.

150. Alexis NE, Richards JH, Carter JD, Ghio AJ. Iron-binding and storage proteins in sputum. Inhal Toxicol. 2002;14(4):387-400.

151. Landry RM, An D, Hupp JT, Singh PK, Parsek MR. Mucin–*Pseudomonas aeruginosa* interactions promote biofilm formation and antibiotic resistance. Mol Microbiol. 2005;59(1):142-51.

152. Smith AL, Fiel SB, Mayer-Hamblett N, Ramsey B, Burns JL. Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration lack of association in cystic fibrosis. CHEST Journal. 2003;123(5):1495-502.

153. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLOS ONE. 2012;7(9):e45001.

154. Armstrong DS, Grimwood K, Carzino R, Carlin JB, Olinsky A, Phelan PD. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. BMJ. 1995;310(6994):1571-2.

155. Razvi S, Quittell L, Sewall A, Quinton H, Marshall B, Saiman L. Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005. CHEST Journal. 2009;136(6):1554-60.

156. Madan J, Koestler D, Stanton B, Davidson L, Moulton L, Housman M, et al. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: Interaction between intestinal and respiratory tracts and impact of nutritional exposures. mBio. 2012;3(4).

157. Foweraker J. Recent advances in the microbiology of respiratory tract infection in cystic fibrosis. Br Med Bull. 2009;89(1):93-110.

158. Reid D, Carroll V, O'May C, Champion A, Kirov S. Increased airway iron as a potential factor in the persistence of *Pseudomonas aeruginosa* infection in cystic fibrosis. European Respiratory Journal. 2007;30(2):286-92.

159. Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, et al. *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease progression. Journal of Cystic Fibrosis. 2009;8(1):66-70.

160. Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS pathogens. 2009;5(3):e1000354.

161. Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, Molin S, et al. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. J Med Microbiol. 2004;53(7):679-90.

162. Klepac-Ceraj V, Lemon KP, Martin TR, Allgaier M, Kembel SW, Knapp AA, et al. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. Environ Microbiol. 2010;12(5):1293-30.

163. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, et al. *Pseudomonas aeruginosa a*naerobic respiration in biofilms: Relationships to cystic fibrosis pathogenesis. Developmental cell. 2002;3(4):593-60.

164. Filkins L, Hampton T, Gifford A, Gross M, Hogan D, Sogin M, et al. Prevalence of *Streptococci* and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol. 2012;194(17):4709-17.

165. Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: A complex fungal and bacterial community—implications for therapeutic management. PloS one. 2012;7(4):e36313.

166. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest. 2002;109(3):317-25.

167. Hassett DJ. Anaerobic production of alginate by *Pseudomonas aeruginosa*: Alginate restricts diffusion of oxygen. J Bacteriol. 1996;178(24):7322-5.

168. Hassett DJ, Sutton MD, Schurr MJ, Herr AB, Caldwell CC, Matu JO. *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. Trends Microbiol. 2009;17(3):130-8.

169. Platt MD, Schurr MJ, Sauer K, Vazquez G, Kukavica-Ibrulj I, Potvin E, et al. Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. J Bacteriol. 2008;190(8):2739-58.

170. Filiatrault MJ, Picardo KF, Ngai H, Passador L, Iglewski BH. Identification of *Pseudomonas aeruginosa* genes involved in virulence and anaerobic growth. Infect Immun. 2006;74(7):4237-45.

171. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Kehagia V, Jones GR, et al. Bacterial activity in cystic fibrosis lung infections. Respir Res. 2005;6(1):49.

172. Rogers G, Hart C, Mason J, Hughes M, Walshaw M, Bruce K. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003;41(8):3548-5.

173. Rogers GB, Skelton S, Serisier DJ, Van Der Gast CJ, Bruce KD. Determining cystic fibrosis-affected lung microbiology: Comparison of spontaneous and serially induced sputum samples by use of terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2010;48(1):78-86.

174. Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, et al. Spatial distribution of microbial communities in the cystic fibrosis lung. The ISME Journal. 2011.

175. Thomassen M, Klinger J, Badger S, Van Heeckeren D, Stern R. Cultures of thoracotomy specimens confirm usefulness of sputum cultures in cystic fibrosis. J Pediatr. 1984;104(3):352-6.

176. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proceedings of the National Academy of Sciences. 2012;109(34):13769-74.

177. Aldallal N, McNaughton EE, Manzel LJ, Richards AM, Zabner J, Ferkol TW, et al. Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. American journal of respiratory and critical care medicine. 2002;166(9):1248-56.

178. Terheggen-Lagro S, Rijkers G, Van der Ent C. The role of airway epithelium and blood neutrophils in the inflammatory response in cystic fibrosis. Journal of Cystic Fibrosis. 2005;4:15-23.

179. Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. American journal of respiratory and critical care medicine. 1999;160(1):186-91.

180. Black HR, Yankaskas JR, Johnson LG, Noah TL. Interleukin-8 production by cystic fibrosis nasal epithelial cells after tumor necrosis factor-alpha and respiratory syncytial virus stimulation. Am J Respir Cell Mol Biol. 1998 Aug;19(2):210-5.

181. Bonfield TL, Konstan MW, Berger M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. J Allergy Clin Immunol. 1999;104(1):72-8.

182. Levy H, Kalish LA, Huntington I, Weller N, Gerard C, Silverman EK, et al. Inflammatory markers of lung disease in adult patients with cystic fibrosis. Pediatr Pulmonol. 2007;42(3):256-62.

183. van de Weert-van Leeuwen, PB, Slieker M, Hulzebos H, Kruitwagen C, van der Ent C, Arets H. Chronic infection and inflammation affect exercise capacity in cystic fibrosis. European Respiratory Journal. 2012;39(4):893-8.

184. Lafferty EI, Qureshi ST, Schnare M. The role of toll-like receptors in acute and chronic lung inflammation. J Inflamm (Lond). 2010;7:57-69.

185. Brett M, Simmonds E, Ghoneim A, Littlewood J. The value of serum IgG titres against pseudomonas aeruginosa in the management of early *Pseudomonal* infection in cystic fibrosis. Arch Dis Child. 1992;67(9):1086-8.

186. Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, et al. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J Infect Dis. 2001;183(3):444-52.

187. Wheeler WB, Williams M, Matthews WJ, Colten HR. Progression of cystic fibrosis lung disease as a function of serum immunoglobulin G levels: A 5-year longitudinal study. J Pediatr. 1984;104(5):695-9.

188. Ghio AJ, Roggli VL, Soukup JM, Richards JH, Randell SH, Muhlebach MS. Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients. Journal of Cystic Fibrosis. 2012.

189. Cosgrove S, Chotirmall SH, Greene CM, McElvaney NG. Pulmonary proteases in the cystic fibrosis lung induce interleukin 8 expression from bronchial epithelial cells via a heme/meprin/epidermal growth factor receptor/toll-like receptor pathway. J Biol Chem. 2011;286(9):7692-704.

190. Moreau-Marquis S, Bomberger JM, Anderson GG, Swiatecka-Urban A, Ye S, O'Toole GA, et al. The DeltaF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. Am J Physiol Lung Cell Mol Physiol. 2008 Jul;295(1):L25-37.

191. Tate S, MacGregor G, Davis M, Innes J, Greening A. Airways in cystic fibrosis are acidified: Detection by exhaled breath condensate. Thorax. 2002;57(11):926-9.

192. Sibley CDSCD, Surette MGSMG. The polymicrobial nature of airway infections in cystic fibrosis: Cangene gold medal lecture. Can J Microbiol. 2011;57(2):69-77

Chapter 2:

Prevotella melaninogenica, an oral anaerobe, in chronic cystic fibrosis lung disease

Abstract

Recent microbiome studies suggest that the airways of individuals with cystic fibrosis (CF) are colonized by a complex microbiota, including strict anaerobes that traditionally inhabit the oral cavity. *Prevotella* species, including *P. melaninogenica*, are consistently the one of the most prevalent members of the CF microbiome; however, their clinical significance outside the oral cavity is unclear. Our study aims to elucidate whether *P. melaninogenica* contributes to CF disease pathogenesis by comparing *P. melaninogenica* culture status in adult and pediatric respiratory specimens with clinical measures of disease (FEV_1%, CRP) and *P. melaninogenica* reactive antibody titer. Anaerobic, microaerophilic and aerobic culture techniques were used to culture sputum and BALF samples from 28 adult and 33 pediatric CF patients. Bacterial 16S rRNA gene sequence alignments were used for identification. FEV1% measurements were collected from CF patients. ELISA based methods were used to determine CRP levels, *P. melaninogenica* reactive antibody titer and total IgG response in our CF patient cohort, healthy volunteers and disease controls. *P. melaninogenica* was a prevalent and abundant member of the CF airway microbiota in CF adults and children. Using clinical

data collected and serological measurements there was no correlation in FEV_1% and CRP values of adults. There was a significantly higher *P. melaninogenica* reactive antibody response in CF adult and child patients compared to controls.

Introduction

Cystic Fibrosis (CF) is the most frequent lethal genetic disease in the Caucasian population. CF is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene that ablate or alter CFTR function. CFTR dysfunction leads to airway surface liquid hyperabsorption and defective mucociliary clearance (1). Mucus stasis creates a favorable environment for bacterial colonization, which leads to chronic pulmonary inflammation and ultimately lung failure (1). In addition to a gradual decline in lung function over time, most CF patients also experience periodic acute pulmonary exacerbation, which is characterized by increased disease symptoms and a rapid decline in lung function requiring hospitalization and intravenous antibiotic treatment (2). In CF, antibiotic maintenance therapy and treatment of exacerbations is guided by aerobic bacterial culture of expectorated sputum or bronchoalveolar lavage fluid (BALF) and bacterial susceptibility testing (3, 4). Aerobic culture-based studies indicate CF airways infection follows a predictable succession, with *Haemophilus influenzae* and *Staphylococcus aureus* dominating early in life and *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex becoming the most prevalent pathogens later in life (5-7).

Recent studies by several groups, enumerated strict anaerobes when processing CF samples (sputum, BALF) anaerobically and using unbiased molecular based tools

such as quantitative PCR (qPCR) of 16S rRNA gene (5, 8-20). In Tunney *et. al* 2008, anaerobic bacteria have been isolated in high numbers (10^4-10^9 CFU/g sputum) in adult sputa samples and in occurred at higher levels than *P. aeruginosa.* Anaerobic bacteria, like in other polymicrobial infections such as pneumonia, lung abscesses and empyema, could have relevance in CF (19, 21, 22). Also the occurrence of anaerobes has been found to correlate with the occurrence of *P. aeruginosa* (5, 19). It has been suggested that anaerobes, in particular *Bacteroides* spp. (now *Prevotella* spp., *Porphyromonas* spp. etc), could be potentially pathogenic and of clinical importance in CF (4, 14, 17, 19, 21).

Prevotella melaninogenica is one of the most common strict anaerobes reported in recent molecular-based CF airway microbiome studies. The presence of *P. melaninogenica* in CF airway specimens has been confirmed by culture independent and anaerobic culture techniques of CF samples (13, 15, 17, 19, 23). *P. melaninogenica* is a black pigmented Gram-negative bacterium known to produce several virulence factors such as a capsule, hemolysin and a variety of secreted proteases (24-26). While *P. melaninogenica* is typically considered a member of the normal human oral flora, it is frequently cultured from polymicrobial infections sites including brain abscesses, pleuropulmonary infections, intra-abdominal infections, wound infections and necrotizing fasciitis (24, 27-31). The association of *P. melaninogenica* with clinical outcomes in CF or the pathogenesis of CF lung disease remains to be determined.

In order to establish the role of *P. melaninogenica* in the pathogenesis of CF, we used quantitative aerobic and anaerobic culture to investigate whether the abundance and prevalence of *P. melaninogenica* in a cohort of CF patients at UNC-CH hospitals

was associated with clinical measurements of inflammation and lung function. Further, we assessed whether CF patients developed a *P. melaninogenica* associated adaptive immune response indicative of pathogenic exposure by measuring *P. melaninogenica* reactive antibody in serum. We found that although *P. melaninogenica* was present in the lungs of CF patients that bacterial isolation did not correlate with lung function (forced expiratory volume in 1 second, FEV_1%) or acute inflammation (C-reactive protein, CRP). Additionally, we found a significance difference in *P. melaninogenica* reactive antibody response in pediatric CF patients and CF adults compared to disease controls.

Material and Methods:

Patient Selection and Design: All patients and volunteers included in this study were recruited under University of North Carolina Institutional Review Board approved protocols and patients or parents signed written consent, or assent for children old enough to read, for the primary study and for use of samples in this study. Serum was collected from 4 groups: Adults (n=28) and Children (n=33) with cystic fibrosis; disease control children (n=16) adult Healthy volunteers (n=21), and adults with mild asthma (n=9); SCCOR patients (n=11). For healthy adults only age and gender was collected. In patients with CF detailed clinical information was collected as part of the primary study from the patients' chart. In the current investigation sex, age, FEV_1% predicted, genotype and disease status (stable or exacerbation of CF). Spirometry in patients was measured at UNC clinics per ATS criteria. To allow for comparison across all age ranges results are expressed as % predicted [NHANES].

Bronchoscopy sample collection: Respiratory secretions: All bronchoalveolar lavage (BALF) samples were obtained as part of clinically indicated bronchoscopy at the UNC Children's Hospital as previously described (32). Briefly, children received general anesthesia, and topical lidocaine was instilled at the level of the larynx and the tracheal bifurcation. To avoid aspiration or contamination of the specimen most CF patients had a laryngeal mask airway placed that allows advancing the bronchoscope protected from nasal or oral secretions to the level of the vocal cords. In non-CF patients exam of the upper airway was indicated and the bronchoscope was inserted through the left nare. In all cases suctioning through the bronchoscope was only performed below the vocal cords to avoid contamination. The location of bronchoalveolar lavage was at the discretion of the bronchoscopist, but was generally done in the lung segment most affected by disease, as evidenced by radiographic changes or by visual appearance at bronchoscopy. The bronchoscope was wedged in a bronchus and two to three aliquots of buffered normal saline solution were instilled and immediately aspirated through the bronchoscope. The total volume of instilled lavage fluid was 1-3 ml/kg body weight. An aliquot of BALF for the research project was taken in a sterile manner. The remainder of the BALF was sent to the hospital laboratory for cytology and cultures, which were ordered by the physician responsible for the patient.

Blood collection: Venous blood was collected by standard phlebotomy, allowed to clot for 30 minutes and spun at 3500 x g for 5 minutes prior to storage at -80° C. In children phlebotomy was typically combined with clinically indicated phlebotomy.

Clinical Culture Procedures: Sputum and BALF samples were collected and placed under anaerobic conditions (5% $C0_2$, 10% H_2, 85% N_2 atmosphere, Coy Anaerobic

Chamber or AnaeroGen Pouch, Oxoid) within 30 minutes of collection. Sputum samples were plug-selected and the plugs treated with Sputolysin (Calbiochem) according to manufacturer's instructions. 10-fold serial dilutions of the samples were prepared in 25% strength Ringer's Solution (Sigma) and 100µL aliquots from undiluted through $1:10^5$ were spread plated onto 3 sets of Anaerobic Blood Agar (Remel) and 1 set each of Kanamycin-vancomycin laked-blood agar (Remel), Chocolate agar with Bacitracin (Remel), and McKay agar. One set of Anaerobic Blood agar plates was incubated at 37°C in ambient atmosphere for 2 days; one set of Anaerobic Blood agar, the Chocolate agar with Bacitracin, and the McKay agar were incubated at 37°C in 5% CO_2 for 2 days; and the remaining Anaerobic Blood agar set and Kanamycin-vancomycin laked-blood agar were incubated for at 37°C anaerobically for 5-7days. Plates were examined and unique isolates were enumerated and subcultured. The threshold of bacterial detection was approximately 100 CFU/g. Fungal isolates and isolates recovered primarily from anaerobic plates which grew under 5% CO_2 upon subculture were already collected through primary CO_2 incubation and were no longer followed.

Molecular Identification of bacterial species: Isolates were Gram-stained and their DNA extracted (Qiagen Human Blood and Tissue kit). The 16s ribosomal RNA gene of the isolates was PCR amplified using primers UniBac_0008F (AGAGTTTGATCMTGGCTCAG) and UniBac_1492R (TACGGYTACCTTGTTACGACTT) with MyTaq Red 2x mix (Bioline). Thermocycler Conditions: 95°C- 3min; 30x {95-20sec 50-20sec 72-90sec}; 72-10min. PCR cleanup was performed with ExoSAP-IT (Affymetrix). Sequencing was performed by Genewiz, Inc using primers UniBac_0008F, UniBac_0926R (CCGTCAATTCCTTTRAGTTT) and

UniBac_1492r. Sequence reads were assembled using Sequencher v4.8 (Gene Codes Corp.) using default settings. Assembled contigs were aligned and assigned taxonomic identification to the closest typed species using the SeqMatch tool of the Ribosomal Database Project (Michigan State University http://rdp.cme.msu.edu/).

Bacteria growth and outer membrane preparation (OMP): *P. melaninogenica* and *Porphyromonas gingivalis* was plated from frozen stock onto Columbian Blood Agar then inoculated anaerobically in Anaerobic Broth (Becton Dickson Difco) at 37°C in Coy Anaerobic Chamber and grown until stationary phase. PAO1, a laboratory strain of *P. aeruginosa*, was plated from frozen stock on to Luria Broth (Becton Dickson) and was grown anaerobically in LB supplemented with 15mM KNO_3 until stationary phase. Bacteria were then harvested by centrifugation 10,000 x g for 25 minutes at 4°C. Supernatant was removed then the pellet was resuspended in lysis buffer (50mM NaH_2PO_4, adjust to pH 7, 300mM NaCl) with 1mg/1ml lysozyme and incubated on ice for 30 minutes. The material was French Pressed twice. Intact cells and cell debris were removed by centrifugation at 10,000 x g for 20 minutes at 4°C. Supernatant was centrifuged at 100,000 x g for 1 hour at 4°C. The pellet was then solubilized in 1% Sarkosyl and resuspended with a syringe 4 times and incubated for 30 minutes at room temperature with gentle shaking. The Sarkosyl solution was subjected to centrifugation at 100,000 x g for 1 hour at 4°C. Supernatant was removed and pellet was resuspended pellet in 100 µl 1x PBS and was considered the outer membrane preparation. One large batch of each antigen was made and used for all ELISAs.

Bacterial OMP ELISA: Total serum immunoglobulin G was measured according to manufacturer's protocol (Jackson). Bacterial reactive IgG antibody titer was measured

as follows. Briefly, 96-well microtitration plates (Nunc Maxisorp) were coated and stored overnight at room temperature with 10 µg/ml bacterial OMP antigenic preparation in 100mM Na_2CO_3 pH 9.6. Nonspecific binding sites were blocked for 1 hour with 1% bovine serum albumin and after washing with PBST 0.05%, patient sera in three well replicates were serially diluted five fold into 1% BSAT and incubated for one hour. Plates were then washed with PBST and incubated with Jackson goat anti-human IgG polyclonal antibody conjugated with Horseradish peroxidase (HRP) for 1 hour. Tetramethyl benzidine (TMB) was added to wells to detect HRP then the reaction was stopped by 2N H_2SO_4 and the color change was be measured by spectrophotometer at 450nm minus plate background of 570nm. Antiserum against *P. melaninogenica* OMP was raised in rabbits and obtained from Cocalico Biologicals, Inc., (Reamstown, PA) using standard procedures. Anti-OprF was used as a positive control for PAO1 to ELISA plate (laboratory stock). *P. melaninogenica* antibody developed against OMPs and *P. aeruginosa* OprF antibody was used on each plate to confirm equal amount of antigen coated per plate. A Jackson Normal Human serum (Jackson labs) was used on every plate to normalize plate to plate differences.

CRP ELISA*:* As a clinical measurement of acute inflammation, C-reactive protein (CRP) was measured by an ELISA based method using CRP Ultra Sensitive (ILab™ Chemistry Systems) and analyzed by the automated biochemistry analyzer Ilab600 (Instrumentation Laboratories Ltd).

Absorption ELISA: Plates were coated with 10ug/ml of OMP preparation in 100mM Na_2CO_3 pH 9.6 overnight. Nonspecific binding sites were blocked for 15 minutes with 1% bovine serum albumin then washed with PBST 0.05%. Pooled serum sample from

20 healthy and 20 CF adult patients were both diluted 1:1000 in BSA/PBS/Tw and 100µl was placed in triplicate into wells for 15 minutes at room temperature with gentle rocking. 100µl of the absorbed sera was then placed in fresh wells that had been coated with OMP preparation. The serum sample was absorbed at least 4 times. Aliquots from each absorbance in triplicate were collected and tested against each OMP preparation for changes in antibody level following the ELISA protocol above.

Titer calculations and statistics: Linear regression models were used to determine correlation of total CFU/g and *P. melaninogenica* CFU/g and clinical measurements. Relative abundance was calculated imputing ones in those samples without a traceable CFU/g in order to complete linear regression analysis. To compare absorbance readings in supplementary data ANOVA Tukey's Multiple Comparison Test and the 4^{th} absorbance was graphed using Graphpad Prism software. Mann-Whitney non parametric two tailed tests were used to compare IgG titers. *P. melaninogenica, P. aeruginosa* and *P. gingivalis* reactive antibody titers were calculated by subtracting background from absorbance values then taking the Log of absorbance greater than OD 0.1 minus the Log of the absorbance less than OD 0.1. The results are expressed as mean values plus/minus standard deviation, and statistical significance ($P < 0.05$) using Graphpad Prism software.

Results:

Clinical Characteristics of UNC Cohort

Demographic and clinical characteristics of the study subjects are summarized (Table 1). BALF was collected from 29 children ranging in age from 1 to 17 years.

Spontaneous expectorated sputum was collected from 28 CF adults, ranging from 18 to 33 years of age, and four stable CF children from 12 to 16 years old. Control subjects for this study included asthmatic children (n=16), asthmatic and smoking adults (n=20) and healthy non-smoking adults (n=21) that ranged from 1 to 57 years old. The majority of the CF patients were female (57%) and had delta F508 genotype (86%).

The CF microbiota in Adults and Children

Using culture based methods to examine microbes in the CF lung, we found that *Staphylococcus, Streptococcus and Haemophilus* were the most abundant genera in pediatric BALF and sputum samples (Figure1A, Figure1B). From adult sputum samples, *Staphylococcus, Pseudomonas* and *Streptococcus* were the most abundant genera (Figure 1C). Among strict anaerobes cultured *Prevotella* spp. were the most abundant genus cultured from CF adult sputa, pediatric CF sputa and BALF.

***P. melaninogenica* is a frequent strict anaerobe in CF**

In BALF cultures from pediatric patients, *P. melaninogenica* was the most abundant species found in exacerbating patients and was highly abundant in stable patients, second only to *S. aureus* and *Streptococcus mitis* (Figure 2A). *P. melaninogenica* was cultured from all sputa collected from stable CF pediatric patients (Figure 2B). The most prevalent species in adult stable patients and one of the most prevalent species in adult exacerbations is *P. melaninogenica* (Figure 2C). *P. aeruginosa* and *Streptococcus salivarius* both were cultured frequently from stable adult patients. *P. aeruginosa* and *S. aureus* were also recovered at high levels from adult

exacerbations (Figure 2C). *P. melaninogenica* was not only a frequent colonizer of CF patients, but was also isolated in high numbers (as high as $1.8x10^8$ CFU/g in one adult stable CF sputum and $8.0x10^8$ CFU/g in one child stable CF sputum) (Figure 3).

Clinical measurements:

To determine whether bacterial lung colonization correlated with clinical parameters, we compared total amount of cultivable bacteria per gram of sample (CFU/g) with pulmonary function (FEV_1%) and C-reactive protein (CRP). The total amount of bacteria cultivated did not correlate with clinical measurements of the patient population (data not shown). In our cohort, consistent with previous studies (7, 33, 34), there was a significant positive correlation between CF patient age and FEV_1% (n=35, p=0.0115), CRP (n=61, p=0.0220) and total CFU/g (n=61, p=0.0167), not seen in non-CF controls (Figure 4A). We found that adult CF patients (n=28) had significantly higher CRP values than non-CF disease controls (n=20, p<0.0001) and healthy individuals (n=21, p<0.0001) (Figure 4B). Additionally, stable CF adults (n=16) had significantly lower CRP than exacerbating CF adults (n=12, p=0.0005). In children, we detected no significant difference between CF and non-CF diseased controls and between stable and exacerbating CF children (Figure 4C).

Given the high frequency of *P. melaninogenica* in our study (19/28 adults, 19/33 children), we investigated the effect of *P. melaninogenica* culture status on clinical parameters CRP and FEV_1%. There was no significant correlation of abundance or frequency of *P. melaninogenica* in adult CF patients with the FEV_1% and CRP measurements (Data not shown). In summary, *P. melaninogenica* is the most prevalent

strict anaerobic species in adult sputum samples and child BALF and sputum, although *P. melaninogenica* abundance or positive culture appears to not significantly correlate with clinical measurements.

Adaptive immune response to *P. melaninogenica*

To determine whether isolation of *P. melaninogenica* was associated with an adaptive immune response, patient sera were tested by ELISA for reactivity with an outer membrane preparation of *P. melaninogenica* ATCC 25845. In order to assess the specificity of the outer membrane ELISA, we tested pooled sera from 20 adult CF patients and 20 healthy adults. Pooled sera were absorbed against wells coated with *P. melaninogenica, P. aeruginosa* or empty wells. As expected there was a significant decrease in *P. melaninogenica* reactive antibody level ($p<0.05$) when *P. melaninogenica* antibodies were removed from diluted CF and healthy sera (Figure 5A). As a Gram-negative control to show ELISA specificity (and a bacterium that is seen in low numbers in CF patients), we measured *P. gingivalis* reactive antibody level and found that when either *P. melaninogenica* or *P. aeruginosa* antibodies were removed there was not a reduction in *P. gingivalis* reactive antibody levels in CF or healthy adult sera (Figure 5B). In addition the removal of *P. melaninogenica* antibodies did not cause a significant reduction in CF and healthy total IgG level (Figure 5C). In summary, this outer membrane ELISA does represent a method of assessing *P. melaninogenica* reactive antibody response in patient sera.

To investigate individual serum *P. melaninogenica* reactive antibody titer, we tested individual patient sera by a *P. melaninogenica* OMP ELISA. There was a

significant increase in *P. melaninogenica* reactive antibody titers in CF adults (n=28) compared with non-CF individuals (n=20, $p<0.0001$) and healthy volunteers (n=21, $p<0.0001$) (Figure 6A). Investigating exacerbation and stable CF patients there was no significant difference in *P. melaninogenica* reactive antibody titers (data not shown). There was no significant difference in total IgG in the adult CF, non-CF and healthy adults (data not shown). To further investigate reactive antibody titers, pediatric patients with CF and non-CF individuals' response was measured. There was a significant difference in *P. melaninogenica* reactive antibody titers in CF children (n=33) and non-CF individuals (n= 16, $p=0.0196$) (Figure 6B). There was no difference in *P. melaninogenica* reactive antibody titers in stable CF verse exacerbation patients or in total IgG in CF children and non-CF individuals (data not shown). *P. aeruginosa* OMP ELISAs were also completed on adult and pediatric CF and non CF individuals and mirrored the results of *P. melaninogenica* reactive antibody response (data not shown).

Because of the specific anaerobic and nutritional environmental requirements of *P. melaninogenica*, we hypothesized that CF adults would have more opportunity for colonization by *P. melaninogenica* and thus would have a higher immune response against OMPs of *P. melaninogenica* than children. Indeed, CF adults (n=28) have a significantly higher *P. melaninogenica* titer compared to CF children (n=33, $p=0.0002$) (Figure 6C). No significant difference was observed in *P. melaninogenica* reactive antibody titer in non-CF adults compared to non-CF children (Figure 6D) or total IgG in the same groups (data not shown).

While *P. melaninogenica* is prevalent in our study, *P. aeruginosa* is traditionally considered the classic CF pathogen so we investigated *P. aeruginosa* reactive antibody

response and *P. melaninogenica* reactive antibody response. Comparing bacterial reactive antibody titer reveals that there is a correlation of *P. melaninogenica* and *P. aeruginosa* titer in CF adults and children (Figure 7).

Discussion

Bacterial cultivation represented as total CFU/g has been compared to clinical measurements previously (4, 6). The weak correlation of these components earlier has shed light on the complex nature CF lung disease. Detection threshold and culturing limits, along with the cross-sectional nature of this study presents a limited picture of the CF microbiome which plays a role in this inconsistent result. As expected, there was a correlation between patient age and a time dependent development of microflora which has been seen in other studies (11).

The clinical significance of anaerobes, specifically *P. melaninogenica*, remains uncertain. In this study, *P. melaninogenica* is the most frequently cultured strict anaerobe in children and adults with CF relative to other species. This has been corroborated by previous studies investigating the CF microbiome (13, 15, 17, 19, 23). Two studies of explanted lung specimens have still suggested that oral anaerobe cultivation in CF samples due to 'oral contaminants' (35, 36). In our study, *P. melaninogenica* could be cultured from mouthwash samples, but it did not occur at the same abundance as was seen in sputum samples (data not shown). Here, we found that frequency and abundance of *P. melaninogenica* does not correlate with FEV_1% in CF children or adults. This study is the first paper to follow up the cultivation of a strict anaerobe and determine *P. melaninogenica* effect on FEV_1% and CRP in the CF population.

In addition to culturing bacteria, host acquired immune response is indicative of bacterial exposure. Serum antibody titers are frequently used as a readout of bacterial infection (37, 38). IgG antibody production is the most common antibody used because of its abundance in the blood and its specificity to repeat pathogen exposure. In adult CF populations, a high level of *P. aeruginosa* specific IgG antibody has been used for diagnostic purposes (39, 40). Serological diagnosis can be made 6-12 months before culturing the bacteria (38). Consistent with this finding, a high level of *P. aeruginosa* specific IgG antibodies correlate with negative clinical outcome reflecting bacterial colonization (40).

In this study, both CF children and adults had an increased *P. melaninogenica* IgG titer compared to controls. Additionally *P. melaninogenica* IgG titer is significantly increased in CF adults compared with CF children. Diagnostic serological testing in periodontal disease and CF has been a valuable tool for treatment regimens (40, 41). Recently, in a study investigating the contribution of another *Prevotella* species, *Prevotella intermedia*, the authors showed an increased *P. intermedia* antibody titer in teenagers (median age 15) compared with healthy adults (median age 25 years) (15). Our results show that *P. melaninogenica* IgG titer also is increased in adult patients compared to children. This finding is consistent with older CF patients having an increased likelihood of encountering *Prevotella* spp., or other bacteria, as they age.

In summary, *P. melaninogenica* is an abundant member of the CF airway microbiota. Our study supports a CF specific adaptive immune response to *P. melaninogenica* antigens though no clinical measurement correlation with culturing of *P. melaninogenica* or antibody titer.

Figures

		Cystic fibrosis (n=61)		Non-CF (n=36)		Healthy (n=21)
Demographics		**Child (n=33)**	**Adult (n=28)**	**Child (n=16)**	**Adult (n=20)**	**Adult (n=21)**
	Age: Median (Range)	5(1-17)	22(18-33)	5.5(1-11)	27 (19-44)	24(20-57)
	Gender: Male/Female	11/22	15/13	7/9	13/7	14/9
	Genotype: (Delta F508/Other)	28/5	25/3	n/a	n/a	n/a
Disease State						
	Disease Status: Exacerbation/Stable	20/13	12/16	n/a	n/a	n/a
	FEV1% predicted: Mean (Median)	Not reliable	57.7 (60.5)#	n/a	n/a	n/a
	CRP µg/ml: Mean	1.38E+04	2.47E+05	2.43E+04	2.73E+04	3.82E+04

4 not recorded

Table 2.1- Characteristics of UNC study cohort.

CF= cystic fibrosis. FEV1%= forced expiratory volume in one second compared to patients of similar characteristics (height, age, sex, and weight). CRP= C-reactive protein

A.

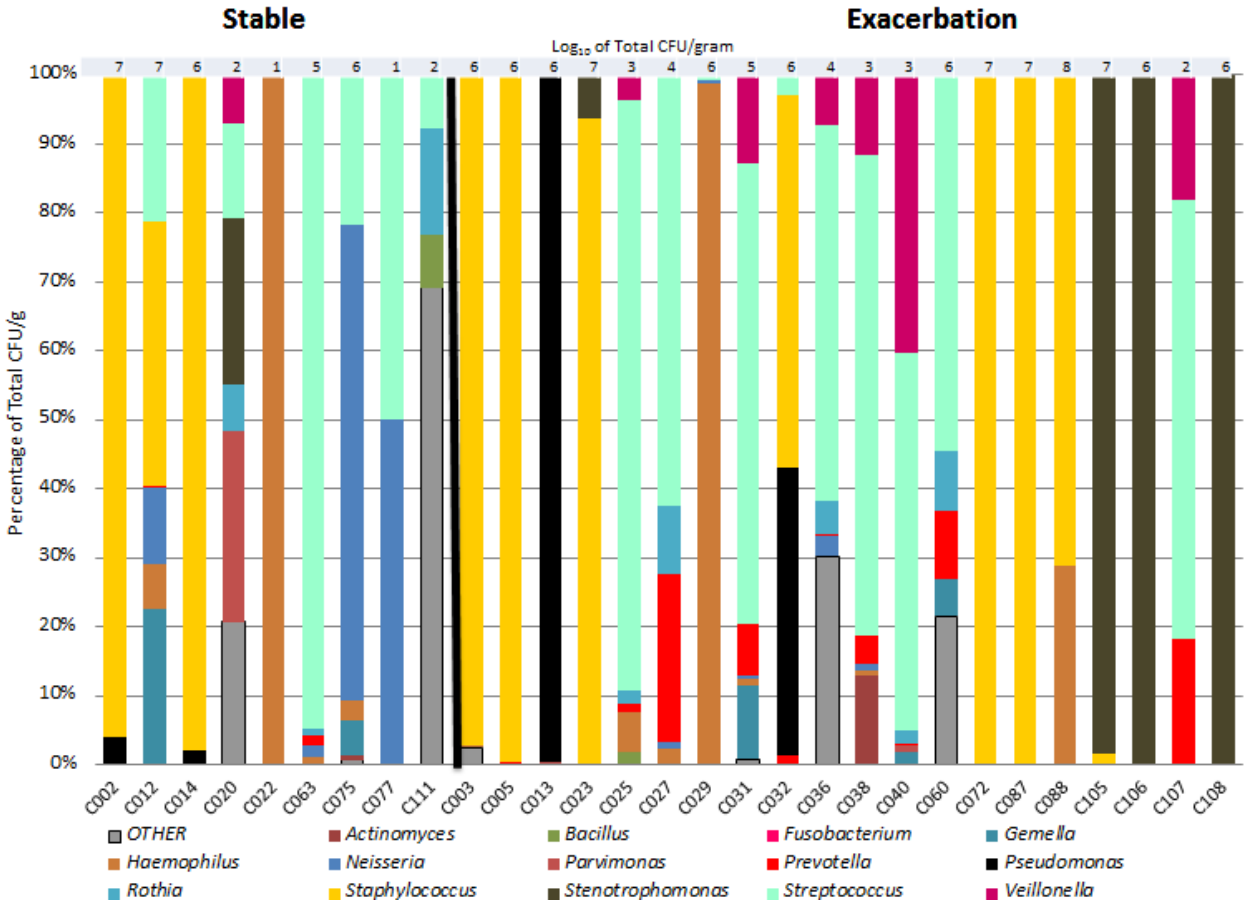
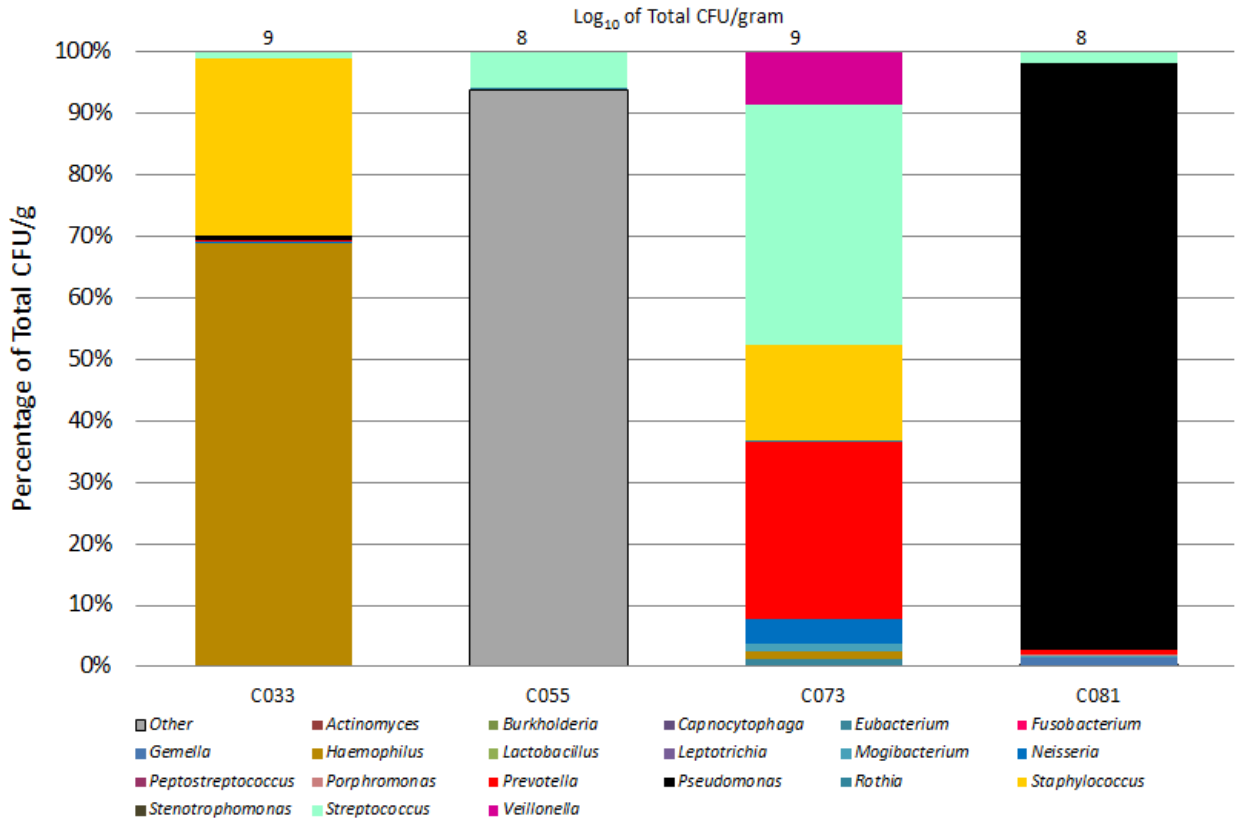
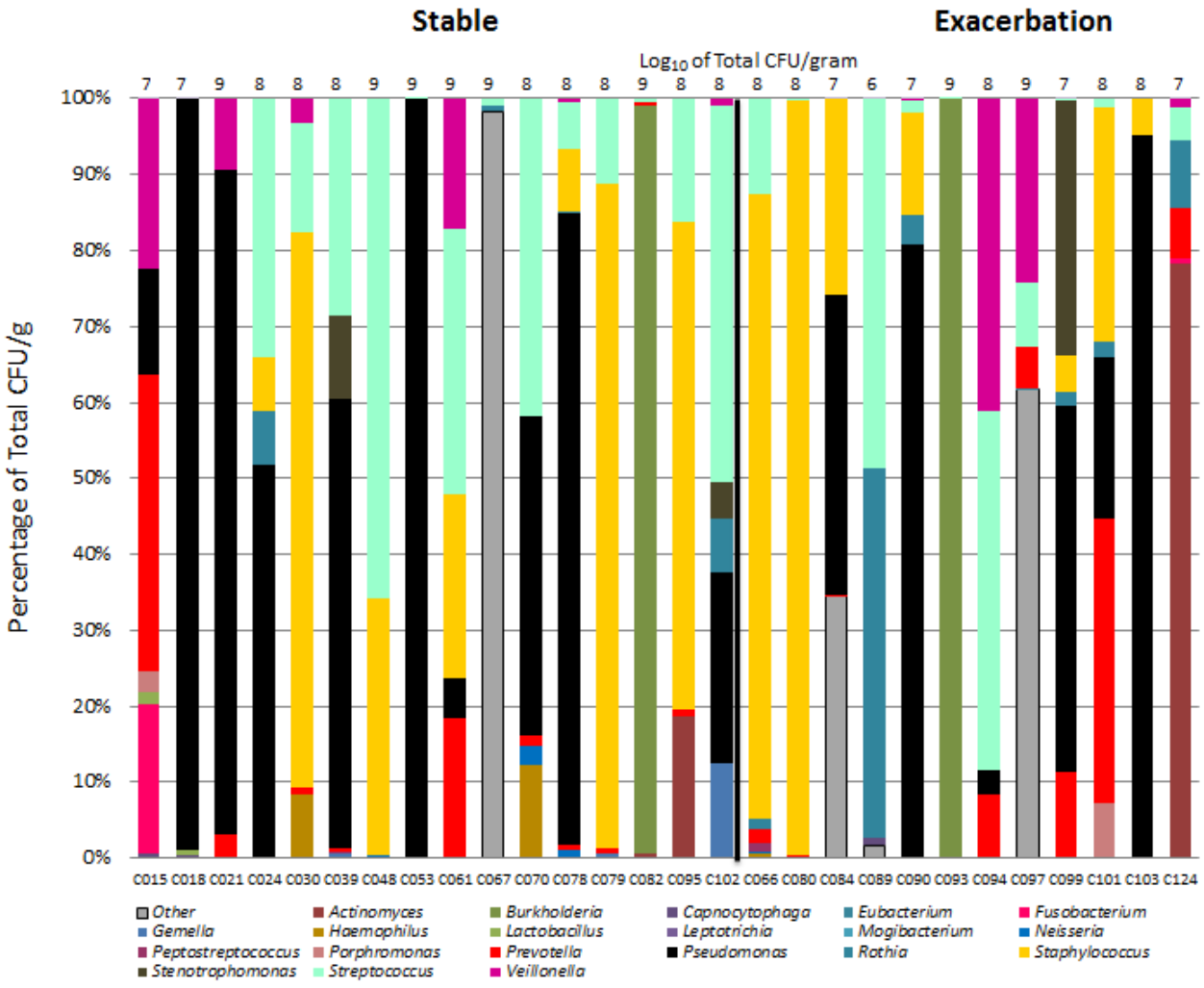


Figure 2.1. Microbiome Composition of Sputum and BALF Samples. Abundance of genera in A) Pediatric BALF samples B) Pediatric Sputum samples. C) Microbiome Composition of Adult sputum samples Data are percent of total CFU/g recovered for each genus. Total Log_{10} CFU/g recovered from each specimen is listed at the top of each bar.

B.


Log10 of Total CFU/gram
9
8
9
8
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Total CFU/g
C033
C055
C073
C081
Other
Actinomyces
Burkholderia
Capnocytophaga
Eubacterium
Fusobacterium
Gemella
Haemophilus
Lactobacillus
Leptotrichia
Mogibacterium
Neisseria
Peptostreptococcus
Porphromonas
Prevotella
Pseudomonas
Rothia
Staphylococcus
Stenotrophomonas
Streptococcus
Veillonella

C.


Stable
Exacerbation
Log10 of Total CFU/gram
7 7 9 8 8 8 9 9 9 9 8 8 8 9 8 8 8 8 7 6 7 9 8 9 7 8 8 7
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Total CFU/g
C015 C018 C021 C024 C030 C039 C048 C053 C061 C067 C070 C078 C079 C082 C095 C102 C066 C080 C084 C089 C090 C093 C094 C097 C099 C101 C103 C124
Other
Actinomyces
Burkholderia
Capnocytophaga
Eubacterium
Fusobacterium
Gemella
Haemophilus
Lactobacillus
Leptotrichia
Mogibacterium
Neisseria
Peptostreptococcus
Porphromonas
Prevotella
Pseudomonas
Rothia
Staphylococcus
Stenotrophomonas
Streptococcus
Veillonella

A

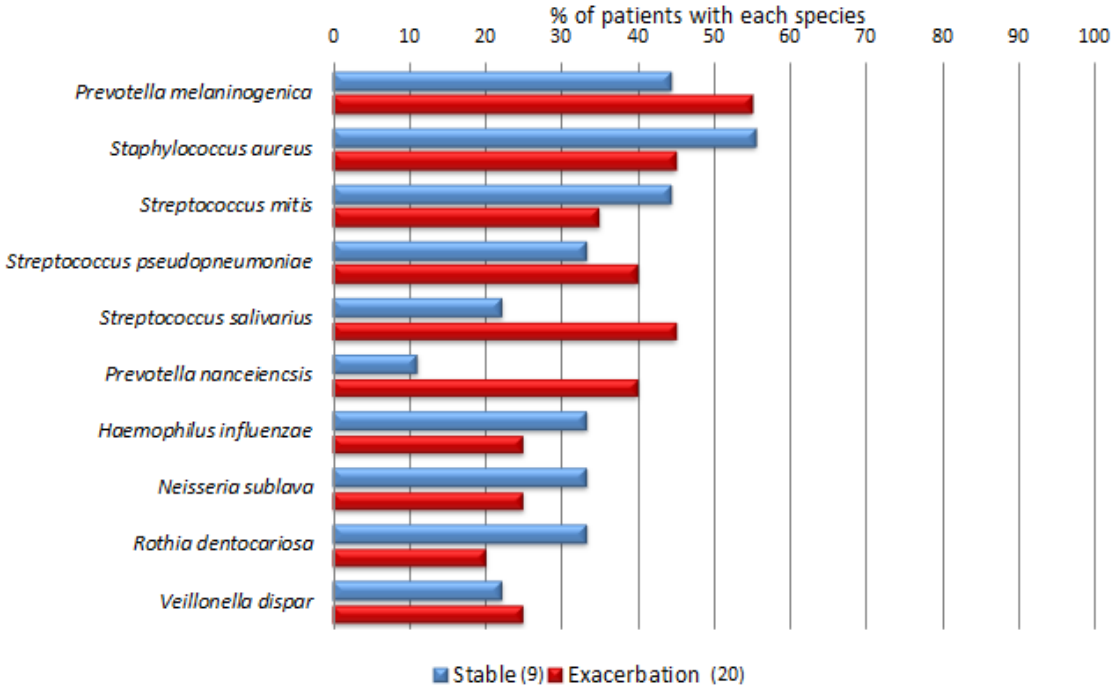
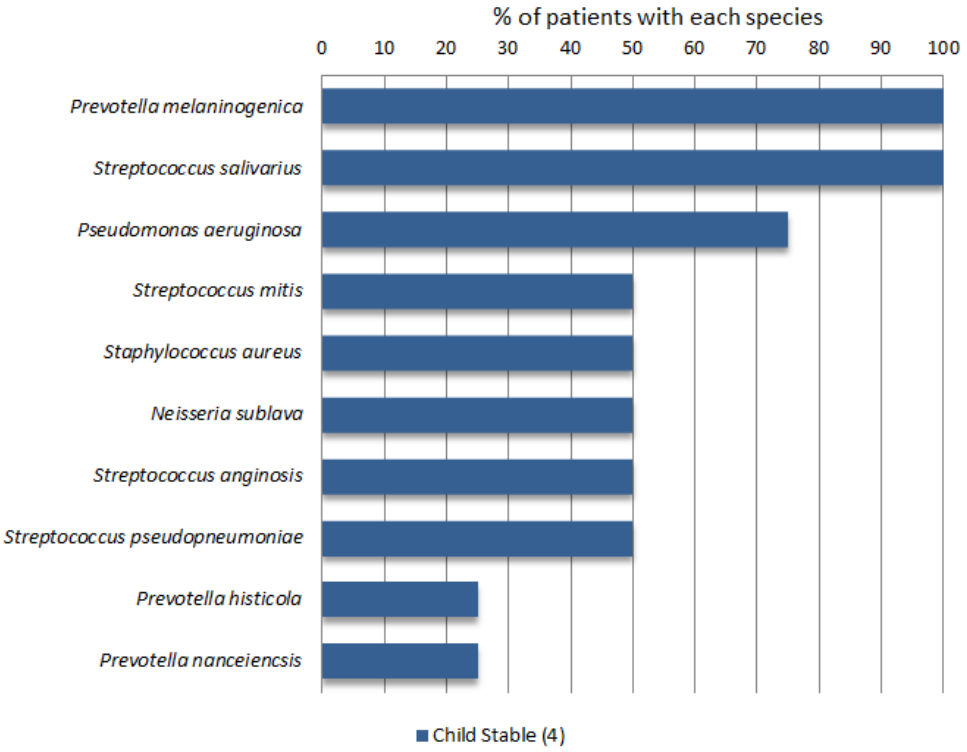
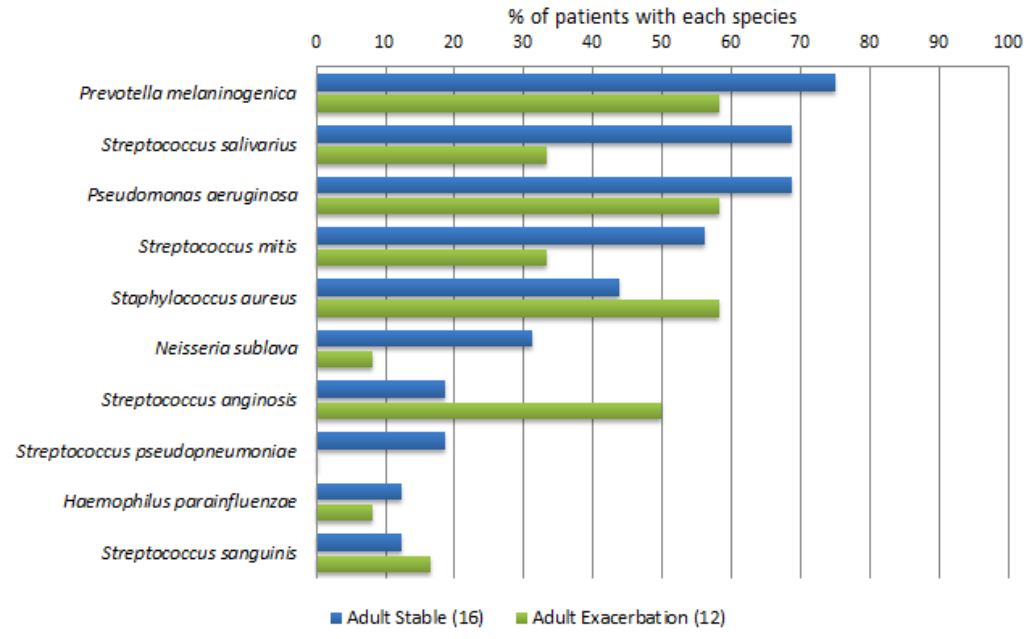


Figure 2.2. Most frequently cultured species in CF samples. A) Pediatric BALF samples B) Pediatric Sputum samples. C) Adult sputum samples

B


% of patients with each species
0
10
20
30
40
50
60
70
80
90
100
Prevotella melaninogenica
Streptococcus salivarius
Pseudomonas aeruginosa
Streptococcus mitis
Staphylococcus aureus
Neisseria sublava
Streptococcus anginosis
Streptococcus pseudopneumoniae
Prevotella histicola
Prevotella nanceiencsis
Child Stable (4)

C.


% of patients with each species
0
10
20
30
40
50
60
70
80
90
100
Prevotella melaninogenica
Streptococcus salivarius
Pseudomonas aeruginosa
Streptococcus mitis
Staphylococcus aureus
Neisseria sublava
Streptococcus anginosis
Streptococcus pseudopneumoniae
Haemophilus parainfluenzae
Streptococcus sanguinis
Adult Stable (16)
Adult Exacerbation (12)

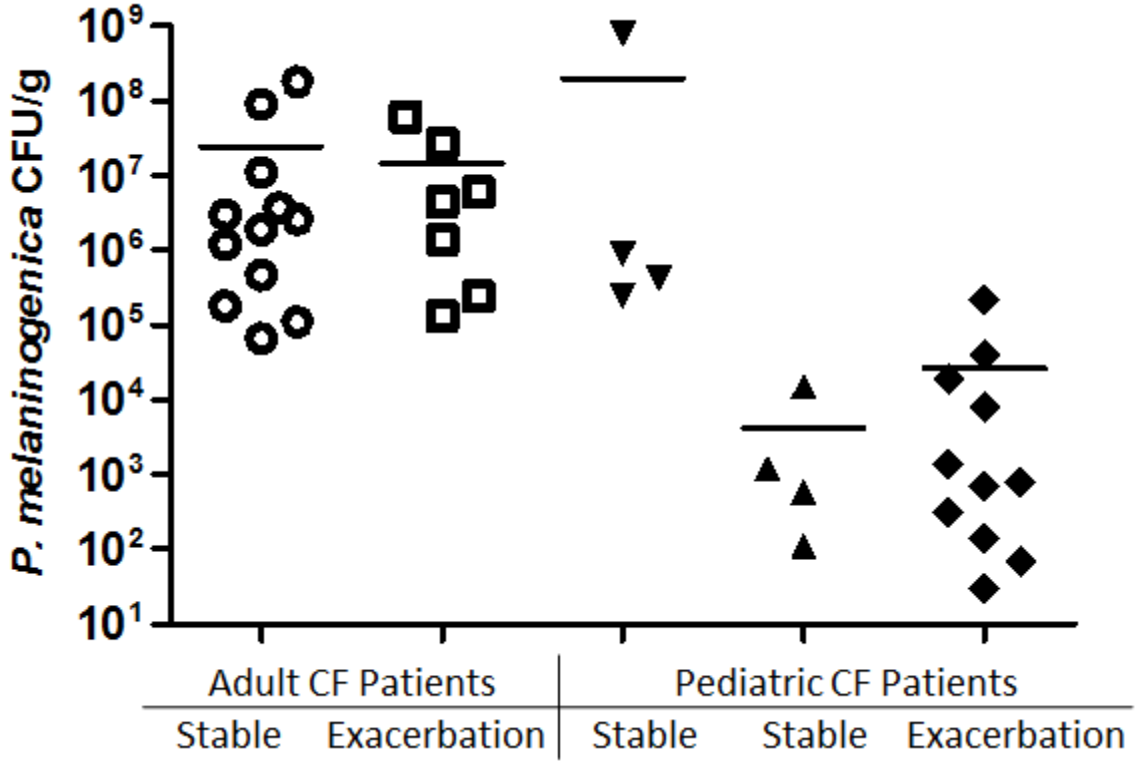


Figure 2.3. *P. melaninogenica* CFU/g in CF patient samples. Vertical Scatter Plot using mean, graphed in Graphpad Prism.

Grayed bars= sputum
White bars= BALF

A.

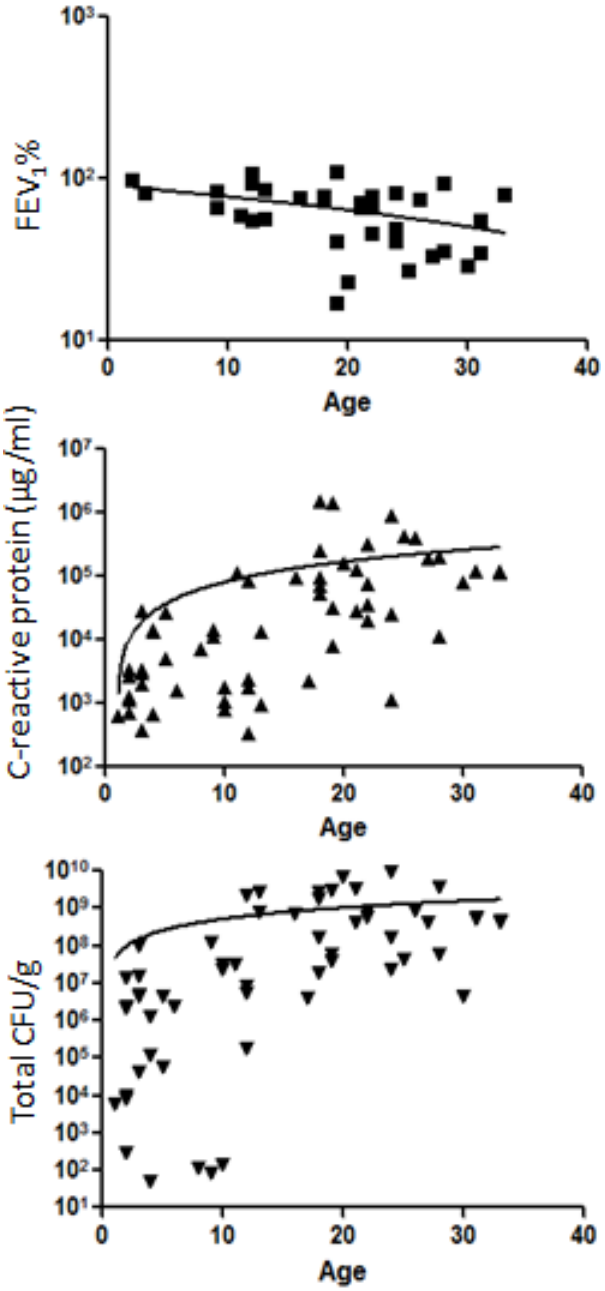
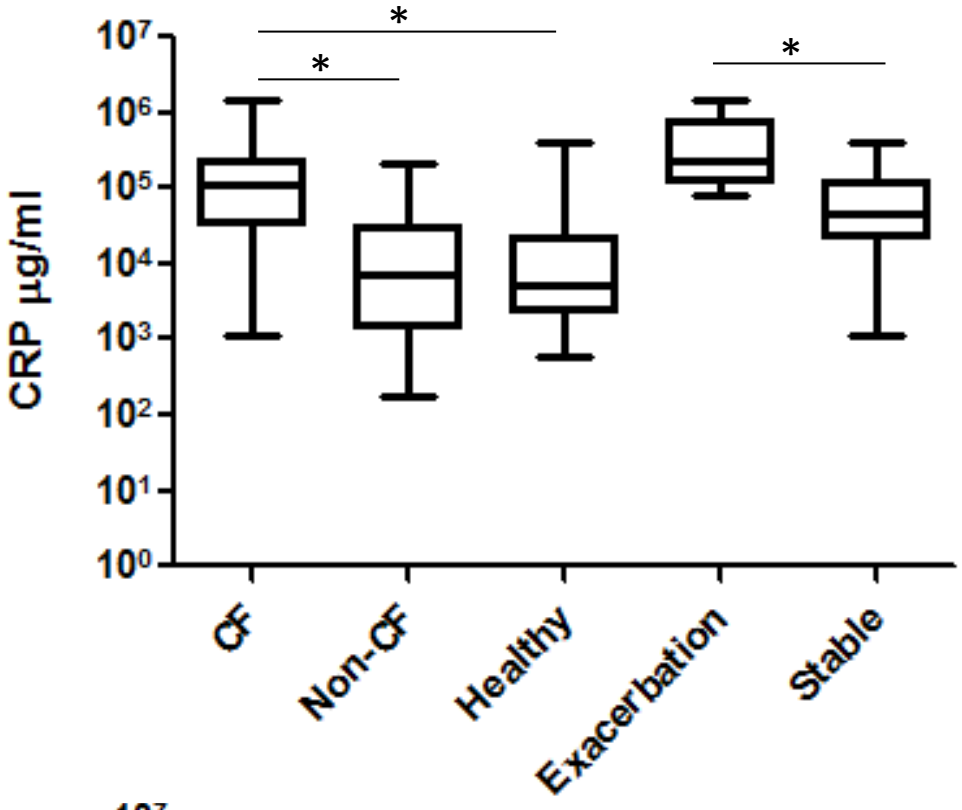
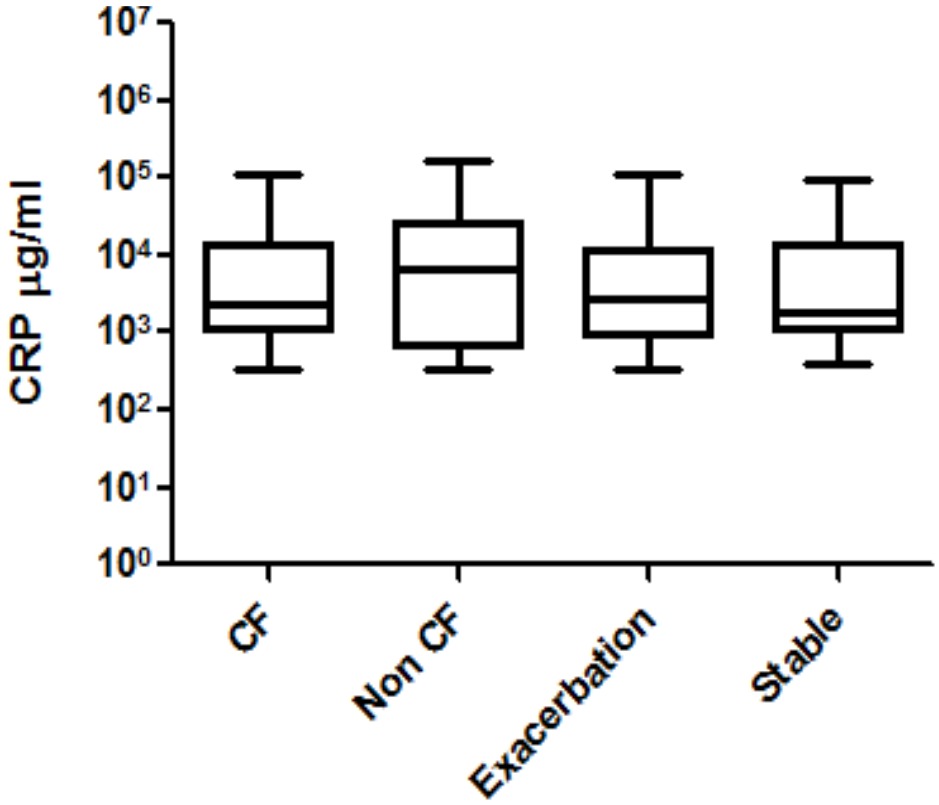


Figure 2.4. Comparisons of age to clinical measurements. A) Correlation of CF patient age to FEV_1% (n=35, r^2=0.1785, p=0.0115), CRP (n=61, r^2=0.08576, p=0.0220) and total CFU/g (n=61, r^2=0.0933, p=0.0167). Values were derived from linear regression model using Graphpad Prism. B) Significance of CRP values in adult patient groupings (p> 0.001). C) CRP levels in pediatric CF patients and non CF individuals. Values were derived using Mann-Whitney non parametric two tailed predictions in Graphpad Prism.

B.


CRP µg/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
*
CF
Non-CF
Healthy
Exacerbation
Stable

C.


CRP µg/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CF
Non CF
Exacerbation
Stable

A.

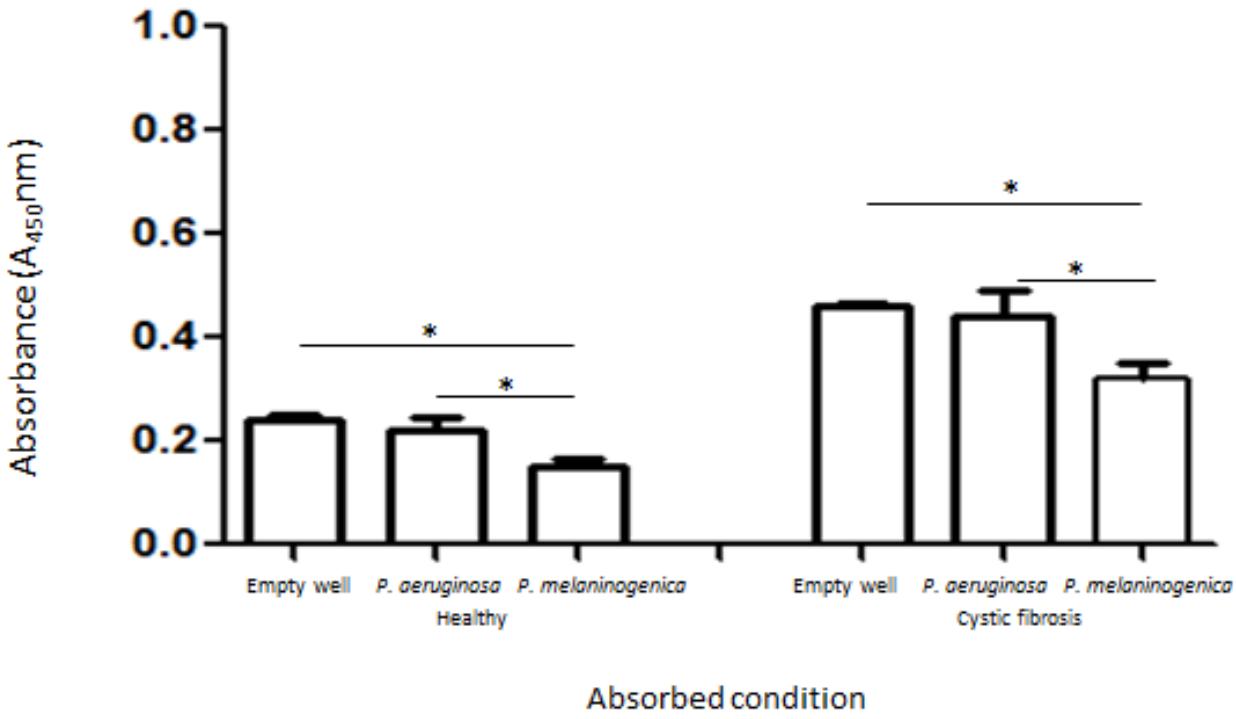
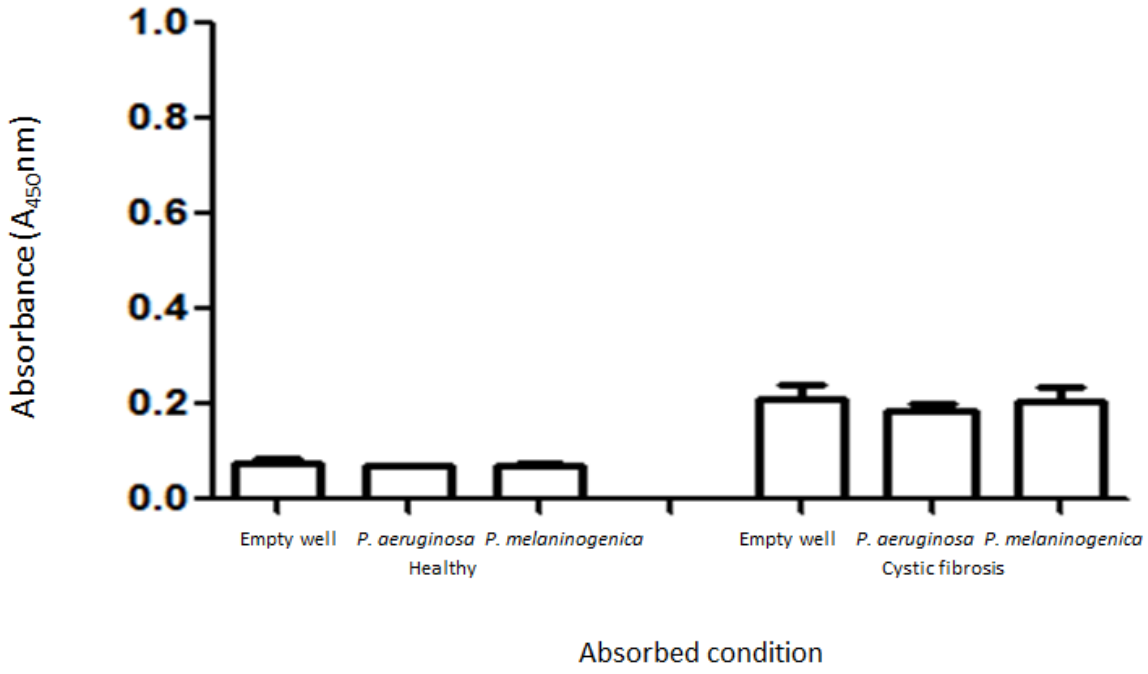
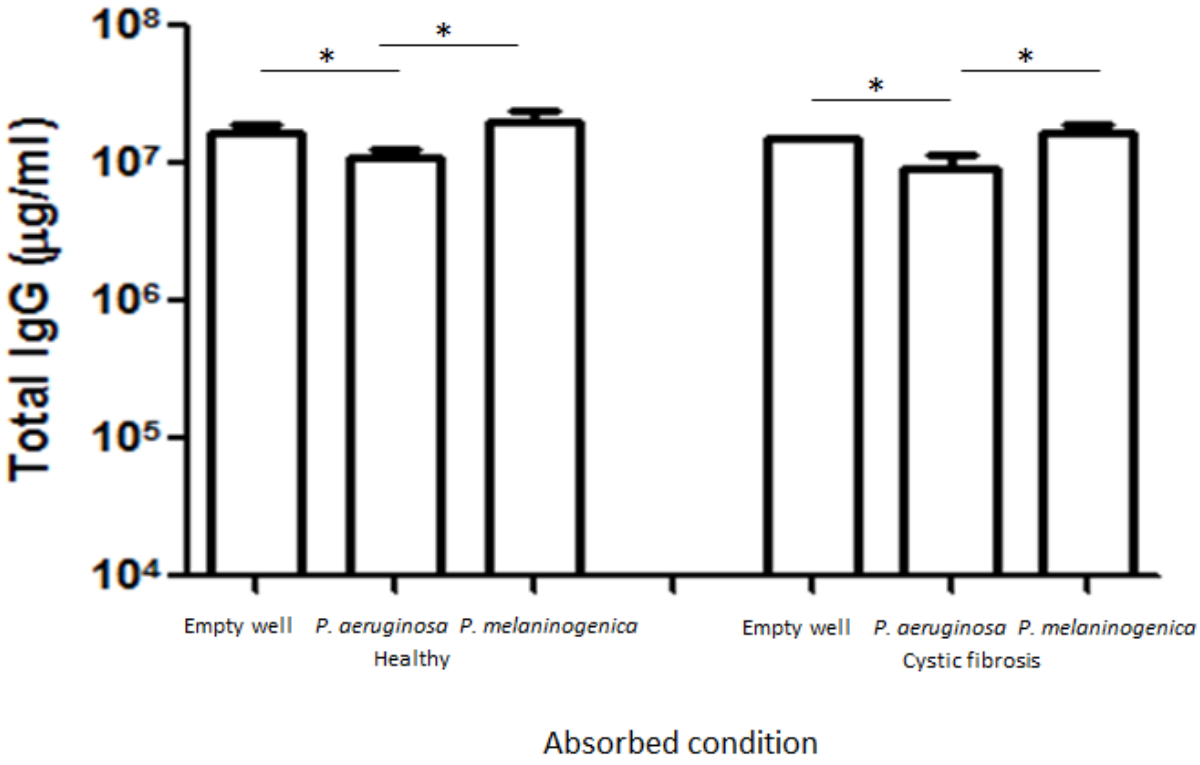


Figure 2.5: Absorbance experiments testing specificity of *P. melaninogenica* ELISA A) *P. melaninogenica* levels when absorbing sera against *P. aeruginosa* antigen or empty wells. B*) P. gingivalis* levels when absorbing against *P. aeruginosa* antigen or *P. melaninogenica* antigen or empty wells. C) Total IgG measurements of absorbed sera. Each measurement was done in triplicate and averaged. Shown is the fourth absorption event. ANOVA- Tukey's Multiple Comparison Test was used and analyzed in Graphpad Prism.

B.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (A_{450}nm)
Empty well
P. aeruginosa
P. melaninogenica
Healthy
Empty well
P. aeruginosa
P. melaninogenica
Cystic fibrosis
Absorbed condition

C.


10^8
10^7
10^6
10^5
10^4
Total IgG (µg/ml)
*
*
*
*
Empty well
P. aeruginosa
P. melaninogenica
Healthy
Empty well
P. aeruginosa
P. melaninogenica
Cystic fibrosis
Absorbed condition

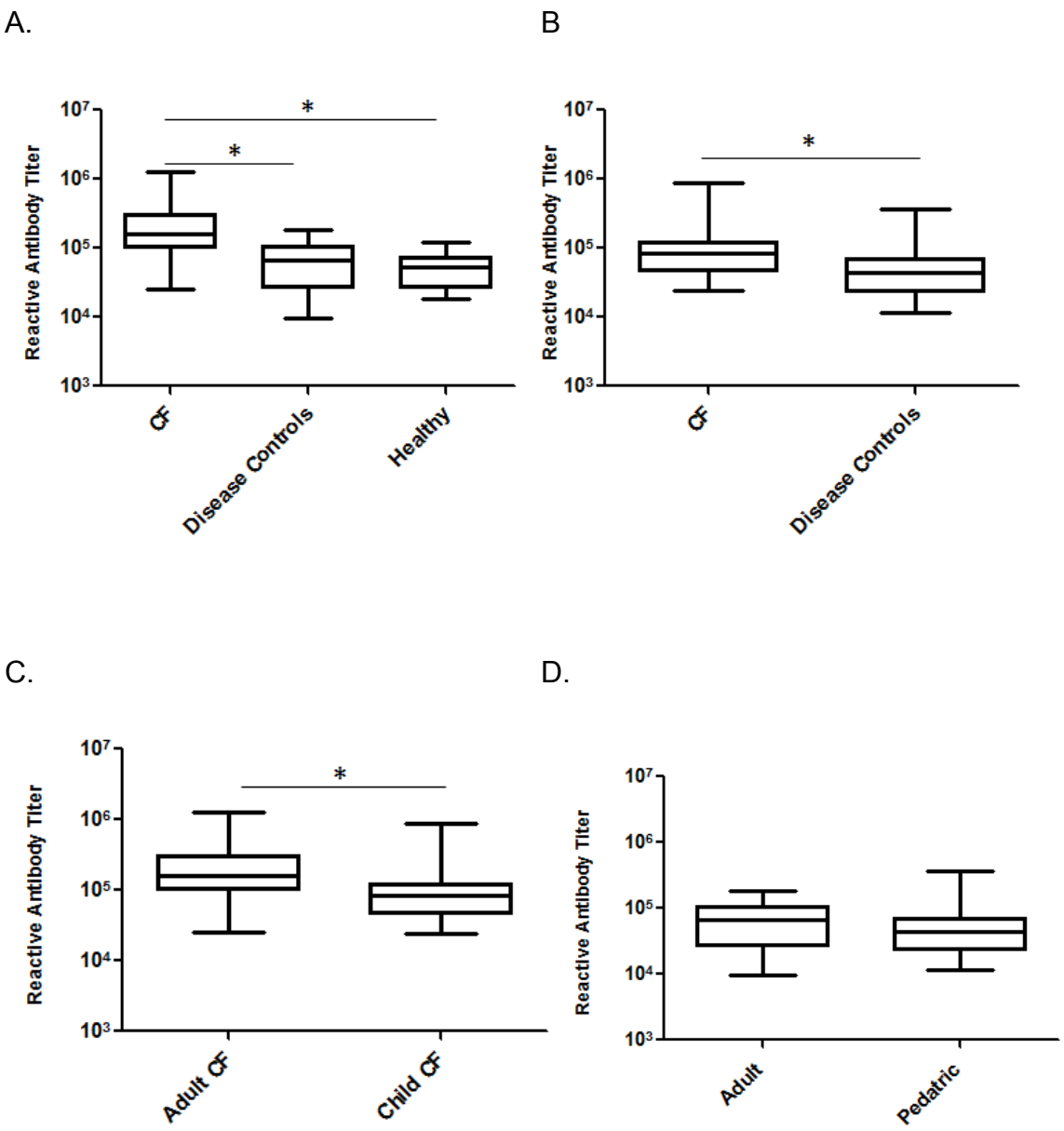


Figure 2.6 *P. melaninogenica* reactive antibody titer in CF Adults, CF children and controls A) *P. melaninogenica* titer measured in individual adult sera ($p<0.0001$). B) *P. melaninogenica* titer measured in CF children compared to Non CF individuals ($p=0.0196$). C) *P. melaninogenica* titers in adult and pediatric CF patients ($p= 0.0002$). D) *P. melaninogenica* titers in adult and pediatric individuals ($p=0.4540$). Box and Whisker Plot was graphed by Graphpad Prism using minimum, maximum, media and 25% and 75% percentile.

A. B.

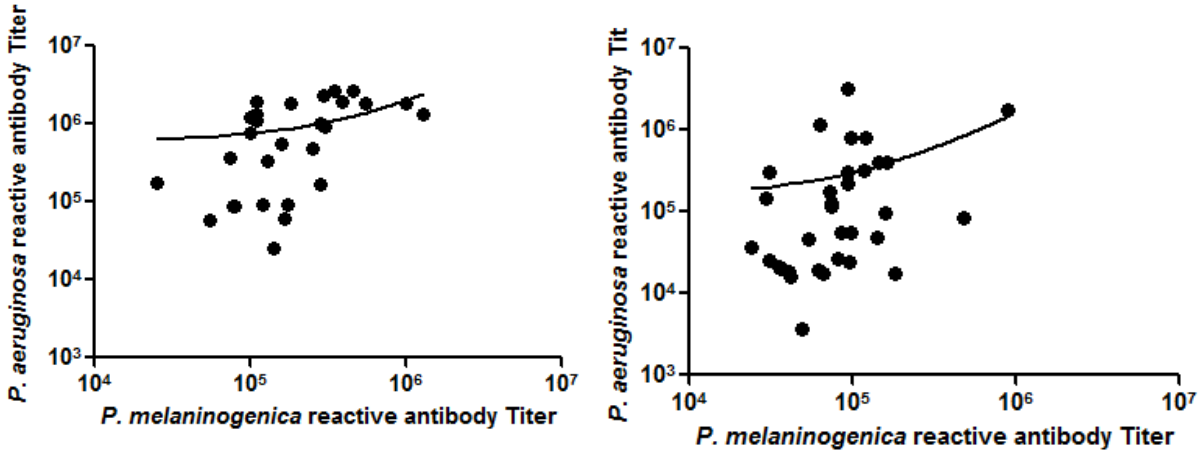


Figure 2.7: *P. melaninogenica* and *P. aeruginosa* reactive antibody comparisons A) *P. melaninogenica* IgG and *P. aeruginosa* IgG titer correlation in Adult CF patients. r^2=0.2038, p=0.0159. B) *P. melaninogenica* and *P. aeruginosa* IgG titer correlation in CF children. r^2=0.1284, p=0.0406. Graphs were derived from linear regression model using Graphpad Prism.

References

1. Trout L, King M, Feng W, Inglis SK, Ballard ST. Inhibition of airway liquid secretion and its effect on the physical properties of airway mucus. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1998;274(2):L258-63.

2. Banner KH, De Jonge H, Elborn S, Growcott E, Gulbins E, Konstan M, et al. Highlights of a workshop to discuss targeting inflammation in cystic fibrosis. Journal of Cystic Fibrosis. 2009;8(1):1-8.

3. Davies JC, Bilton D. Bugs, biofilms, and resistance in cystic fibrosis. Respir Care. 2009;54(5):628-40.

4. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLOS ONE. 2012;7(9):e45001.

5. Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: A complex fungal and bacterial community—implications for therapeutic management. PloS one. 2012;7(4):e36313.

6. Klepac-Ceraj V, Lemon KP, Martin TR, Allgaier M, Kembel SW, Knapp AA, et al. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and pseudomonas aeruginosa. Environ Microbiol. 2010;12(5):1293-30.

7. Razvi S, Quittell L, Sewall A, Quinton H, Marshall B, Saiman L. Respiratory microbiology of patients with cystic fibrosis in the united states, 1995 to 2005. CHEST Journal. 2009;136(6):1554-60.

8. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Kehagia V, Jones GR, et al. Bacterial activity in cystic fibrosis lung infections. Respir Res. 2005;6(1):49.

9. Filkins L, Hampton T, Gifford A, Gross M, Hogan D, Sogin M, et al. Prevalence of *Streptococci* and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol. 2012;194(17):4709-17.

10. Sibley CD, Grinwis ME, Field TR, Eshaghurshan CS, Faria MM, Dowd SE, et al. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. PloS one. 2011;6(7):e22702.

11. Madan J, Koestler D, Stanton B, Davidson L, Moulton L, Housman M, et al. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: Interaction between intestinal and respiratory tracts and impact of nutritional exposures. mBio. 2012;3(4).

12. Rogers GB, Skelton S, Serisier DJ, Van Der Gast CJ, Bruce KD. Determining cystic fibrosis-affected lung microbiology: Comparison of spontaneous and serially induced sputum samples by use of terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2010;48(1):78-86.

13. Field TR, Sibley CD, Parkins MD, Rabin HR, Surette MG. The genus *Prevotella* in cystic fibrosis airways. Anaerobe. 2010;16(4):337-44.

14. Worlitzsch D, Rintelen C, Böhm K, Wollschläger B, Merkel N, Borneff-Lipp M, et al. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. Clinical Microbiology and Infection. 2009;15(5):454-60.

15. Ulrich M, Beer I, Braitmaier P, Dierkes M, Kummer F, Krismer B, et al. Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis. Thorax. 2010;65(11):978-84.

16. Stressmann FA, Rogers GB, Klem ER, Lilley AK, Donaldson SH, Daniels TW, et al. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. J Clin Microbiol. 2011;49(1):281-9.

17. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proceedings of the National Academy of Sciences. 2007;104(51):20529-33.

18. van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME journal. 2010;5(5):780-91.

19. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. American journal of respiratory and critical care medicine. 2008;177(9):995-1001.

20. Tunney M, Klem E, Fodor A, Gilpin D, Moriarty T, McGrath S, et al. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. Thorax. 2011;66(7):579-84.

21. Jewes LA, Spencer R. The incidence of anaerobes in the sputum of patients with cystic fibrosis. J Med Microbiol. 1990;31(4):271-4.

22. Grollier G, Doré P, Robert R, Ingrand P, Gréjon C, Fauchere JL. Antibody response to *Prevotella* spp. in patients with ventilator-associated pneumonia. Clin Diagn Lab Immunol. 1996;3(1):61-5.

23. Rogers G, Carroll M, Serisier D, Hockey P, Jones G, Bruce K. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2004;42(11):5176-83.

24. Harding G, Sutter V, Finegold S, Bricknell K. Characterization of *Bacteroides melaninogenicus*. J Clin Microbiol. 1976;4(4):354-9.

25. Allison HE, Hillman JD. Cloning and characterization of a *Prevotella melaninogenica* hemolysin. Infect Immun. 1997;65(7):2765-71.

26. Grenier D, Mayrand D, McBride BC. Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides*. Oral Microbiol Immunol. 1989;4(1):12-8.

27. Hsiao WWL, Li KL, Liu Z, Jones C, Fraser-Liggett CM, Fouad AF. Microbial transformation from normal oral microbiota to acute endodontic infections. BMC Genomics. 2012;13(1):345.

28. Bowler P, Duerden B, Armstrong D. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001;14(2):244-69.

29. Talan DA, Abrahamian FM, Moran GJ, Citron DM, Tan JO, Goldstein EJC. Clinical presentation and bacteriologic analysis of infected human bites in patients presenting to emergency departments. Clinical infectious diseases. 2003;37(11):1481-9.

30. De A, Varaiya A, Mathur M. Anaerobes in pleuropulmonary infections. Indian Journal of Medical Microbiology. 2002;20(3):150.

31. Brook I. *Prevotella* and *Porphyromonas* infections in children. J Med Microbiol. 1995;42(5):340-7.

32. Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. American journal of respiratory and critical care medicine. 1999;160(1):186-91.

33. Levy H, Kalish LA, Huntington I, Weller N, Gerard C, Silverman EK, et al. Inflammatory markers of lung disease in adult patients with cystic fibrosis. Pediatr Pulmonol. 2007;42(3):256-62.

34. VandenBranden SL, McMullen A, Schechter MS, Pasta DJ, Michaelis RL, Konstan MW, et al. Lung function decline from adolescence to young adulthood in cystic fibrosis. Pediatr Pulmonol. 2011;47(2):135-43.

35. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-

airway specimens can misrepresent lung microbiota. Proceedings of the National Academy of Sciences. 2012;109(34):13769-74.

36. Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, et al. Spatial distribution of microbial communities in the cystic fibrosis lung. The ISME Journal. 2011.

37. Sugi N, Naruishi K, Kudo C, Hisaeda-Kako A, Kono T, Maeda H, et al. Prognosis of periodontitis recurrence after intensive periodontal treatment using examination of serum IgG antibody titer against periodontal bacteria. J Clin Lab Anal. 2011;25(1):25-32.

38. West SEH, Zeng L, Lee BL, Kosorok MR, Laxova A, Rock MJ, et al. Respiratory infections with *Pseudomonas aeruginosa* in children with cystic fibrosis. JAMA: the journal of the American Medical Association. 2002;287(22):2958-67.

39. Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, et al. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J Infect Dis. 2001;183(3):444-52.

40. Brett M, Simmonds E, Ghoneim A, Littlewood J. The value of serum IgG titres against *Pseudomonas aeruginosa* in the management of early *Pseudomonal* infection in cystic fibrosis. Arch Dis Child. 1992;67(9):1086-8.

41. Dye BA, Herrera-Abreu M, Lerche-Sehm J, Vlachojannis C, Pikdoken L, Pretzl B, et al. Serum antibodies to periodontal bacteria as diagnostic markers of periodontitis. J Periodontol. 2009;80(4):634-47.

Chapter 3

Structure and innate immune response to lipopolysaccharide lipid A of *Prevotella melaninogenica*

Abstract:

Prevotella melaninogenica, regarded as a commensal oral bacterium, is frequently isolated from extra-oral polymicrobial infections, including the airways of individuals with the genetic disease cystic fibrosis (CF). The CF airway environment is known to support a complex polymicrobial community that persists and evolves over time. Aerobic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are considered the primary pathogens in CF; consequently, little is known about the contribution of anaerobic bacteria such as *P. melaninogenica* in CF disease pathogenesis. Lipopolysaccharide (LPS), the primary constituent of the outer membrane of Gram-negative bacteria, has the potential to be an important proinflammatory mediator. We characterized the lipid A structure of *P. melaninogenica* and determined whether *P. melaninogenica* LPS stimulated an inflammatory response in the human monocytic cell line THP1. We found that lipid A of *P. melaninogenica* is highly heterogeneous under *in vitro* growth conditions, and is composed of monophosphorylated and diphosphorylated forms that are penta-acylated with unusually long chain fatty acids. Consistent with the presence of long chain fatty acids the LPS of *P. melaninogenica* LPS was more than 1000-fold less stimulatory than that

of *Pseudomonas aeruginosa*. Further, *P. melaninogenica* LPS activity was not mediated by Toll-like receptor 4 (TLR4), suggesting that residual activity was due to lipoprotein contamination. Our results indicate that *P. melaninogenica* produces a highly unusual non-stimulatory LPS and that it is unlikely to contribute to the unrelenting inflammatory response associated with chronic CF airway infection.

Introduction:

Bacterial lipopolysaccharide (LPS) comprises the outer layer of the outer membrane of Gram-negative bacteria. LPS has three biochemically distinct regions: lipid A, core polysaccharide and O-antigen (1). Lipid A, traditionally considered the most toxic portion, constitutes the hydrophobic component of LPS and is made up of long chain fatty acids covalently attached to a sugar derivative, glucosamine. The core polysaccharide, which is attached to lipid A, is composed of an inner core of short oligosaccharide and 2-keto-3-deoxyoctonoic acid (KDO), and an outer core that is composed of phosphates, amino acids and sugars. Attached to the core is the O-antigen, the most variable part of the LPS, which is made up of repeating saccharide subunits.

Bacterial LPS aids in colonization and survival of bacteria inside the host. LPS is vital to bacterial structure and outer membrane integrity and is involved in bacterial pathogenesis by acting as a physical/permeability barrier to protect bacteria from host immune defenses and by aiding in the attachment to host cells (2). The addition of sialic acid to the core or O-antigen portion of LPS can aid in bacterial immune evasion by mimicking host cell surface molecules, thus preventing host immune detection and clearance by antibody opsonization (3).

The lipid A component of LPS also serves as a microbe-associated molecular pattern (MAMP) that is specifically recognized by host cells through the Toll-like receptor 4 (TLR4) complex (4). In the host, bacterial LPS is recognized by LPS binding protein (LBP). The LPS-LBP complex can subsequently bind CD14 and activate the TLR4-MD-2 receptor complex. Binding of LPS to TLR4 initiates a signaling cascade that ultimately activates NFκB transcription. Upon translocation to the nucleus, NFκB activates the transcription of proinflammatory cytokine genes, such as IL-8, as well as other responses including complement activation and reactive oxygen release from macrophages and neutrophils (5). The host response induced by bacterial LPS plays a role in the pathogenesis of many human diseases including cystic fibrosis (CF) (6).

CF is the most common genetic disorder affecting Caucasians of European descent. In CF, mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene lead to dysregulation of chloride ion transportation across the apical surface of epithelial cells. The resulting ion imbalance causes mucus dehydration and formation of thick luminal mucus plugs (7). Mucus plugs are proposed to provide an ideal niche that protects bacteria from antimicrobial agents and antibody mediated host responses. Bronchoalveolar lavage fluid (BALF) collected from CF patients, shows increased neutrophil counts, elevated concentrations of proinflammatory cytokines (e.g. IL-8, IL-1β, TNFα, IL-6), and inflammatory markers such as C-reactive protein (CRP) compared to BALF from individuals with non-CF respiratory disease or healthy individuals (8-10). Bacterial infection within the thickened luminal mucus of CF airways leads to unrelenting and non-productive inflammation, which contributes to progressive lung damage, and ultimately respiratory failure.

In individuals with CF, polymicrobial colonization occurs as early as two months of age (11). In the context of this underlying bacterial community, typical CF pathogens appear in a succession; with *Staphylococcus aureus* and *Haemophilus influenzae* dominating early in life, followed by chronic colonization by *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex (11, 12). Unbiased molecular detection methods, based on bacterial 16S rRNA gene sequencing or phylogenetic microarrays, have shed new light on the complexity of the CF microbiome and implicate anaerobic bacterial species as dominant members of the CF airway microbiota (11, 13-22). These findings have been confirmed by parallel anaerobic culture based studies (23-33). Among the anaerobic species, *Prevotella melaninogenica* has emerged as a frequent and abundant member of the CF airway microbiota (14, 19-21, 23, 29, 30).

P. melaninogenica is a black pigmented, Gram-negative bacterium and a member of the human oral flora (34). *P. melaninogenica* is found in extra-oral abscess sites such as vertebral osteomyelitis, pyomyositis, peritonsillar abscesses and vaginal mesh infections (35-39). *P. melaninogenica* has the capacity to produce a range of potential virulence factors such as hemolysin, β-lactamases, fibrinolysin, IgA protease, IgG protease and lipase (40-45). Additionally, *P. melaninogenica* has been described as a potential pathogen because of its presence in polymicrobial diseases such as brain abscesses, pleuropulmonary infections, endocarditis, illicit drug injection sites, intra-abdominal infections, wound infections, necrotizing fasciitis, pyogenic infections, decubitus and diabetic ulcers in addition to CF (26, 36, 42, 46-51).

In the context of CF airway disease, the potential role of *P. melaninogenica* in inflammation has not been examined. In particular, the structure and stimulatory

properties of *P. melaninogenica* LPS, a major proinflammatory mediator of Gram-negative infection, have not been characterized. The goal of this study was to determine the *P. melaninogenica* lipid A structure and its inflammatory potential, in order to better understand its contribution to the pathogenesis of CF airway infection and disease pathogenesis. We found that there are two dominant forms of *P. melaninogenica* lipid A composed of monophosphorylated and diphosphorylated forms with five unusually long (C15-C17) acylated chains. Additionally, *P. melaninogenica* LPS was found to be exceptionally non-stimulatory with regard to IL-8 and IL-1β activation compared to LPS from *P. aeruginosa* in THP1 cells.

Materials and Methods:

Bacterial strains and growth conditions: *P. melaninogenica* ATCC 25845 was cultured from frozen stock onto Columbian Blood agar plates (Thermo Scientific) for three days. *P. melaninogenica* was then grown in prereduced Tryptic Soy Broth (Becton Dickinson) supplemented with 5 μg/ml menadione (Sigma) and 5 μg/ml (7.6 μM) heme (anaerobic Tryptic Soy Broth, ATSB) with shaking overnight at 37°C in a Coy anaerobic chamber (52). 20 milliliters of overnight culture was used to inoculate 1L of ATSB and grown for three days. Bacteria were then centrifuged at 10,000xg for 25 minutes at 4°C and the pellet was frozen at -20°C. *P. aeruginosa* strain PAK was plated on LB and grown in N-minimal media supplemented with 38 mM glycerol, 0.1% casamino acids and 8 μM $MgCl_2$, a condition that promotes production of lipid A with modifications typical of CF clinical isolates (53).

LPS and lipid A isolation and purification. LPS was extracted by the hot/phenol/water method (54). Freeze-dried bacteria were resuspended in endotoxin-free water at a

concentration of 10 mg/ml. 12.5 milliliters of 90% phenol was added and the resultant mixture was vortexed and incubated in a hybridization oven at 65°C. The mixture was cooled on ice and centrifuged at 10,000 rpm at room temperature for 30 minutes. The aqueous phase was collected and an equal volume of endotoxin-free water was added to the organic phase. The sample was treated as above and aqueous phases were combined and dialyzed against Milli-Q purified water to remove residual phenol and then freeze-dried. The resultant pellet was resuspended at a concentration of 10 mg/ml in endotoxin-free water and treated with DNase at 100 µg/ml and RNase A at 25 µg/ml and incubated at 37°C for 1 hour in a water bath. Proteinase K was added and incubated for 1 hour in a 37°C water bath. The solution was extracted with an equal volume of water-saturated phenol. The aqueous phase was collected, dialyzed against Milli-Q purified water and freeze-dried as above. The LPS was further purified by adding a 2/1 mixture of chloroform/methanol to remove membrane phospholipids using the Folch procedure (55) and further purified by an additional water-saturated phenol extraction and 75% ethanol precipitation using the Vogel procedure (56) to remove lipoproteins. 1 mg of purified LPS was converted to lipid A by mild-acid hydrolysis (57), which was used for High-Order MS analysis.

Rapid microextraction Lipid A isolation from whole cells. The *P. melaninogenica* Lipid A that was analyzed by MALDI-TOF mass spectrometry was prepared using a published isolation method (58). Briefly, approximately 10 mg of lyophilized material derived from an overnight culture of each strain was resuspended in 400 µl of isobutyric acid and 1 M ammonium hydroxide (5:3 vol/vol) and incubated at 100°C for 1 h. After cooling, individual samples were centrifuged for 15 min at 2,000 x g, and supernatants were

collected and diluted 1:1 (vol/vol) with endotoxin-free water. The samples were subsequently frozen and lyophilized overnight. The resultant powered material was then washed twice with 1 ml of methanol and the insoluble lipid A was extracted in 200 µl of a mixture of chloroform, methanol, and water (3:1:0.25 [vol/vol/vol]). One microliter of this extract was then spotted onto a MALDI plate followed by 1 µl of norharmane matrix (Sigma) and air-dried.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS): Lipid A was analyzed in the negative ion mode on an AutoFlex Speed MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples were dissolved in 10 µL of a mixture of n-harmane (20 mg/mL) in chloroform/methanol/water 4:4:1 (vol/vol/vol), and 0.5 µL of sample was spotted directly onto the MALDI target plate. Data was acquired in reflectron mode with a Smartbeam laser with 1 kHz repetition rate and up to 4000 shots were accumulated for each spectrum. Instrument calibration and all other tuning parameters were optimized using Agilent Tuning mix (Agilent Technologies, Foster City, CA). Data was acquired and processed using Data Analysis (Bruker Daltonics, Billerica, MA).

Matrix-Assisted Laser Desorption Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI FT-ICR MS): Lipid A was analyzed by MALDI in the negative ion mode on a 12-Tesla FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples were dissolved in 10 µL of a mixture of n-harmane (20 mg/mL) in chloroform/methanol/water 4:4:1 (vol/vol/vol), and 0.5 µL of sample was spotted directly onto the MALDI target plate. Data was acquired with a Smartbeam laser with 2 kHz repetition rate and up to 3000 shots were accumulated for each spectrum.

Instrument calibration and tuning parameters were optimized using the recommended Bruker calibration standard mixture (Bruker Daltonics, Bremen, Germany) in negative ion mode. Tandem mass spectrometry experiments involved isolation and collision induced fragmentation of the precursor ion in the front end quadruple. Precursor isolation was set to 5 Da and fragmentation energy was adjusted accordingly to maximize observation of product ions. Data was acquired and processed using Data Analysis (Bruker Daltonics, Billerica, MA).

Cell culture: THP1 XBlue cells (Invivogen, San Diego, CA) were grown according to American Type Culture Collection (ATCC) instructions in RPMI-1640 medium with Zeocin (100 µg/ml) in 5% CO_2. For LPS challenge studies, 5 x10^5 THP1 XBlue cells contained in 500 µl were seeded into 24 well plates, incubated for 30 minutes and challenged for 24 hours with LPS. LPS was diluted in endotoxin free H_2O. *E. coli* LPS (Sigma) and Pam3CSK4 (Invivogen) were used as controls for TLR neutralizing experiments at 50 ng/ml and 10 ng/ml, respectively. TLR2 and TLR4 neutralization experiments were conducted as previously published (59, 60). Specifically, 1x10^5 THP1 cells contained in 100 µl media were plated and 5 µg/ml of anti-TLR2 or anti-TLR4 antibody (TLR2: Anti-human (CD282) Purified TL2.1; TLR4: Anti-Human CD284 (TLR4)); eBioscience) or isotype IgG2a control (Mouse IgG2a K Isotype Control, eBioscience) were added to cell culture, swirled and incubated for two hours at 37°C before LPS was applied and incubated for 24 hrs.

ELISAs: Total IL-1β and IL-8 were measured by enzyme-linked immunosorbent assay (ELISA) in 96 well polystyrene high binding plates (Corning Costar) according to manufacturer's instructions (R&D). For NFκB activation measurements, cells were

maintained with Zeocin and supernatant was collected and frozen at -20°C. 20 µls of cell supernatant was added to 200 ul of QuantiBlue (Invivogen) and incubated for 1 to 2 hours and read at 650nm.

Statistics: Data were analyzed by analysis of variance (ANOVA) and the Tukey multiple-comparison test using the Graphpad program (Graphpad Software, San Diego, Calif.). Statistical differences were considered significant for P values of less than 0.05. All reported experiments were performed at least twice in triplicate and each graph represents standard deviation of averages.

Results:

Characterization of *P. melaninogenica* lipid A structure.

The lipid A structure of *P. melaninogenica* has not been previously described. To determine lipid A structure, the typed *P. melaninogenica* strain ATCC 25845 was grown in ATSB (7.6uM heme) and lipid A was purified by Caroff method and analyzed by negative matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (MS) and Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometry.

The negative ion mode MALDI TOF mass spectrum of lipid A isolated from *P. melaninogenica* LPS is shown in Figure 1A and 1B. The most abundant ions observed corresponded to two different ion series at *m/z* values ranging from 1646-1702 and from 1726-1796. These ion series corresponded to singly deprotonated lipid A structures that contained one phosphate group (monophosphoryl) or two phosphates (diphosphoryl) and five acyl chains (penta-acylated), respectively. Within the ion series, each ion peak differed by 14 Da, which corresponded to an addition/subtraction of a methyl group.

Initial structure characterization using precursor ion *m/z* values were supported by elemental composition based on accurate mass measurements.

Tandem mass spectrometric experiments on penta-acylated lipid A isolated from *P. melaninogenica* LPS were carried out to confirm the location of the five fatty acids. The most abundant monophosphoryl *m/z* value, 1674, was selected for full acyl chain configuration assignment (Figure 1B). Initial characterization based on elemental composition from accurate mass measurements of the mono-phosphoryl penta-acylated lipid A anion, *m/z* 1674, revealed the composition of the five fatty acids comprised of four primary fatty acids [two 3-hydroxypalmitic acid (C16(3-OH)) acyl chains, one 3-hydroxy-methyl-tetradecanoic acid (C15(3-OH)(isomethyl)), and one 3-hydroxy-methyl-hexadecanoic acid (C17(3-OH)(isomethyl)) acyl chains] and one secondary fatty acid [palmitic acid (C16) acyl chain].

In order to determine the positioning of the five fatty acids, we conducted tandem mass spectrometric experiments aimed at highlighting diagnostic cross-ring and glycosidic cleaveage product ions that provided decisive evidence for pinpointing acyl chain positions (Figure 1B). The penta-acylated lipid A structure was determined to have the following configuration: the C-2 position contained a primary amide-linked C16(3-OH), the C-3 position contained a primary ester-linked C16(3-OH), the C-2' position contained a primary amide-linked C17(3-OH)(isomethyl) and a secondary ester-linked C16, and the C-3' position contained a primary ester-linked C15(3-OH)(isomethyl). The acyl chain configuration as outlined above held true for diphosphoryl penta-acylated lipid A extracted from *P. melaninogenica* (Figure 1A).

IL-8 and IL-1β response to *P. melaninogenica* LPS.

To test the ability of *P. melaninogenica* LPS to stimulate the host inflammatory response, we exposed the THP1 human monocytic cell line to purified LPS and evaluated production of IL-1β, a key acute phase inflammatory cytokine, and IL-8, a neutrophil chemokine. LPS from *P. melaninogenica* stimulated low levels of IL-8 (approximately 50 pg/ml), but no detectible level of IL-1β (Figure 2A and 2B). In contrast, LPS from *P. aeruginosa*, a traditional pathogen associated with CF, stimulated IL-8 production 1000 fold more than *P. melaninogenica* LPS.

***P. melaninogenica* LPS is not a TLR4 agonist.**

To determine whether the weak cytokine response to *P. melaninogenica* LPS was mediated through a TLR4-dependent pathway, we used TLR neutralizing antibodies to block LPS signaling. *E. coli* LPS was used as a traditional TLR4 agonist to test the effectiveness of the blocking of TLR4 by antibodies. *E. coli* LPS signal was ablated with the addition of TLR4 neutralizing antibody. In our studies, TLR4 neutralizing antibody had no effect on *P. melaninogenica* LPS dependent NFκB activation or IL-8 production in THP1 cells (Figure 3). As expected the isotype mAb control did not reduce *P. melaninogenica* NFκB activation or IL-8 secretion. We also tested the ability of *P. melaninogenica* to signal through TLR2 because of contrasting studies in a closely related bacterium *Porphyromonas gingivalis*. Pam3CSK4, a lipoprotein, is a traditional TLR2 agonist and its effect was successfully reduced with the addition of TLR2 neutralizing antibody. The TLR2 neutralizing antibody reduced *P. melaninogenica* LPS dependent NFκB activation and IL-8 production three fold each.

The isotype mAb control did not reduce *P. melaninogenica* NFκB activation or IL-8 production (Figure 4).

Discussion

P. melaninogenica, a prominent anaerobic member of the CF microbiome, has an uncharacterized lipid A structure and unknown LPS dependent inflammatory effect that could contribute to CF pathogenesis. This study represents the first investigation into lipid A structure and inflammatory response to *P. melaninogenica* LPS.

Mass spectra of *P. melaninogenica* lipid A displayed penta-acylated diphosphorylated and monophosphorylated form (m/z 1754 and 1674, Figure 1). In studying the structure of lipid A there are four main areas of potential variation: the number of phosphates attached to the glucosamine backbone, the number and length of acyl chains and whether the acyl chains are branched (61). Contrasting *E. coli* lipid A structure to *P. melaninogenica* reveals key differences in phosphate composition, acyl chain number and length. *E. coli* lipid A traditionally is composed of 6 acyl chains with lengths ranging from 12 to 14 carbons and two phosphates attached to the glucosamine backbone (62). These key differences could explain the striking difference in IL-8 production and TLR signaling between the two complete LPS structures. Lipid A molecules that have two phosphates attached to the glucosamine background are known to be more potent activators of TLR4 than lipid A molecules with a single phosphate or no phosphate (62). Additionally, lipid A containing 5 or 7 acyl chains is 100 fold less active compared to structures with 6 acyl chains (63). *P. melaninogenica* lipid A moiety displays a similar structure and acylation pattern to *P. gingivalis*, an oral

pathogen associated with periodontitis (62, 64). *P. gingivalis* lipid A is penta-acylated and monophosphorylated and has a less potent cytokine profile than *E. coli* (62, 65).

In our experiments with purified LPS, we established that *P. melaninogenica* did not initiate high levels of IL-1β and IL-8 inflammatory cytokine production or NFκB activation in THP1 cells; even lower than *P. gingivalis* in similar conditions (66, 67). The low stimulatory effect of *P. melaninogenica* is confirmed by a past report of low IL-1α and TNFα secretion induced by *P. melaninogenica* supernatants on human monocytes and human gingival cells compared with *Salmonella typhimurium* LPS and *Fusobacterium nucleatum* lysate (68). In the oral cavity, where *P. melaninogenica* is considered a commensal, the community of oral bacteria contributes to periodontal health by stimulating low levels of IL-8 to induce the chemotaxis of neutrophils into the gingivalis crevice where they patrol for bacterial pathogens (69). In other parts of the body, low level stimulation by commensal bacteria aids in the development and maintenance of the immune innate system as seen in experiments with germ-free mice (70). In CF lung infections though, the additional low-level stimulation could add to the overall excessive inflammatory phenotype displayed which leads in the destruction of lung tissue and pulmonary dysfunction. *P. melaninogenica* could minimally contribute to increase the inflammation of the CF through increased IL-8 cytokine response.

To investigate the mechanism of THP1 IL-8 production in response to *P. melaninogenica*, TLR signaling pathways were investigated to determine if *P. melaninogenica* was signaling through a traditional TLR pathway. Experiments blocking TLR2 or TLR4 with neutralizing antibodies were done to elucidate TLR signaling pathways of *P. melaninogenica* LPS. Blocking TLR2, in contrast to blocking the

traditional TLR4 pathway, resulted in a significant decrease in *P. melaninogenica* LPS dependent NFκB activation and IL-8 production. Antibody blocking experiments demonstrated that the low-level proinflammatory activity associated with *P. melaninogenica* LPS is detected through a TLR4 independent pathway. This result substantiates a continuing controversy in the field *P. gingivalis*.

The LPS dependent TLR response to *P. gingivalis* is unclear because of reports outlining both TLR2 and TLR4 LPS dependent pathways. Zhang et al., demonstrates in THP-1 cells that TLR2 antibodies block the production of significant amounts of IL-1β, TNFα and IL-6 cytokines (67). This study though also reports robust *P. gingivalis* LPS cytokine response above that of *E. coli*, contradicting previous studies comparing the two (53, 71). This could be because of lipoprotein contamination leading to TLR2 agonism and high levels of cytokine response. Exploring lipid A structure and environmental cues, Coats et al. shows a variable TLR dependent response depending on the amount of heme in the environment (52). Specific lipid A phosphatases are regulated by heme concentration and are responsible for changes in lipid A structure leading to variable TLR4 response either acting as an agonist in low heme (1 mg/ml) or an antagonist in high heme (10 μg/ml) (52).

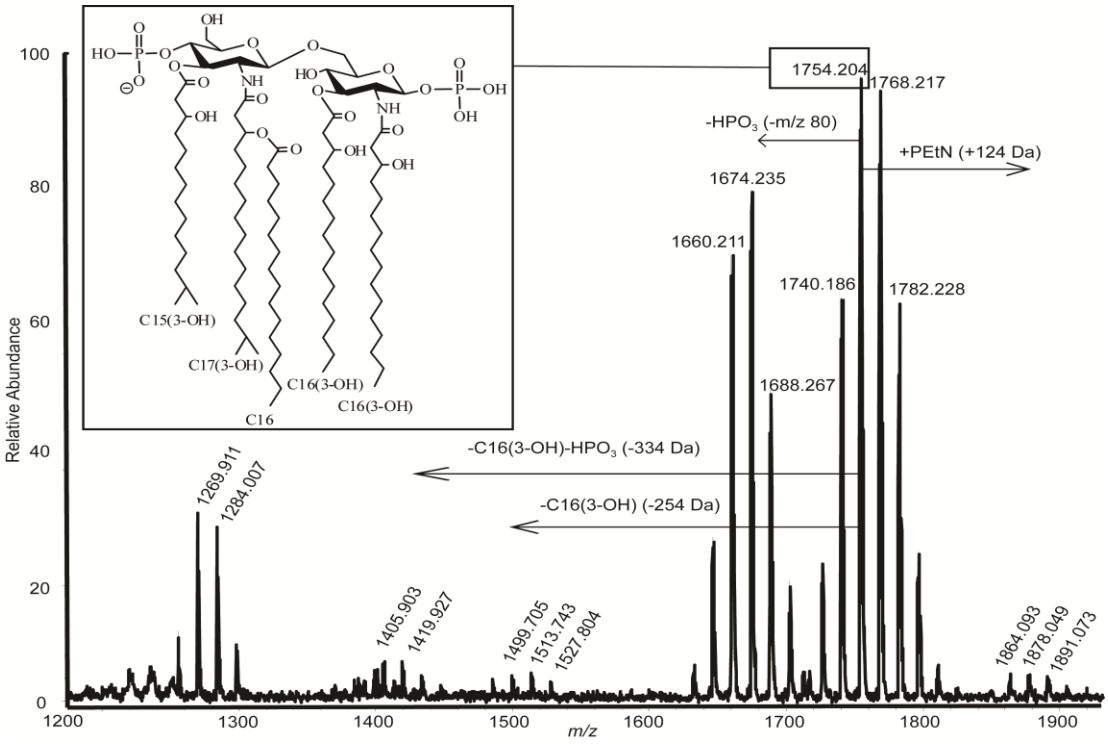
Further experiments will be done with additional *P. melaninogenica* LPS preparations to confirm that *P. melaninogenica* LPS is signaling through a TLR4 independent pathway. TLR2 signaling was seen previously in experiments testing formalin fixed *P. melaninogenica* on 293/hTLR-2 and 293/hTLR-4/CD14/MD2 cell lines (72). Formalin fixed bacteria is a complex mixture of membrane components and does not represent an exclusive LPS dependent response but does show the ability for *P.*

melaninogenica signaling through TLR2 pathways. Additionally, further experimentation will be needed in understanding the role of bacterial environmental growth conditions as it affects *P. melaninogenica* lipid A structure in light of heme dependent variation of lipid A structure leading to changes in TLR activation as seen in *P. gingivalis* (5, 52).

In future studies, concurrent studies with *P. melaninogenica* and *P. aeruginosa* should be investigated because *P. melaninogenica* is more often associated within a group of organisms such as its presence in the oral biofilm (34) than as a single species infection. In experiments mimicking the environment of COPD, *P. melaninogenica* lysates inhibited the ability of *Haemophilus influenzae* lysates to stimulate TLR4 signaling in dendritic cells, resulting in reduced IL-12 production (51). Similarly, LPS from the closely related oral bacterium, *P. gingivalis* can act as a TLR4 antagonist and dampen or eliminate the effect of *E. coli* LPS on activating CHO cells expressing human TLR4, human THP-1 monocytes or murine macrophages (53, 71). Because of the presence of *P. melaninogenica* in polymicrobial environments such as CF, the dampening effect of *P. melaninogenica* lysate and *P. gingivalis* LPS dependent inhibiting properties, *P. melaninogenica* LPS may actually provide protection to other organisms such as *P. aeruginosa* by preventing or dampening the pro-inflammatory response.

Figures:

A.


1754.204
1768.217
-HPO3 (-m/z 80)
+PEtN (+124 Da)
1674.235
1660.211
1740.186
1782.228
1688.267
-C16(3-OH)-HPO3 (-334 Da)
-C16(3-OH) (-254 Da)
1269.911
1284.007
1405.903
1419.927
1499.705
1513.743
1527.804
1864.093
1878.049
1891.073
C15(3-OH)
C17(3-OH)
C16(3-OH)
C16(3-OH)
C16
Relative Abundance
m/z

B.

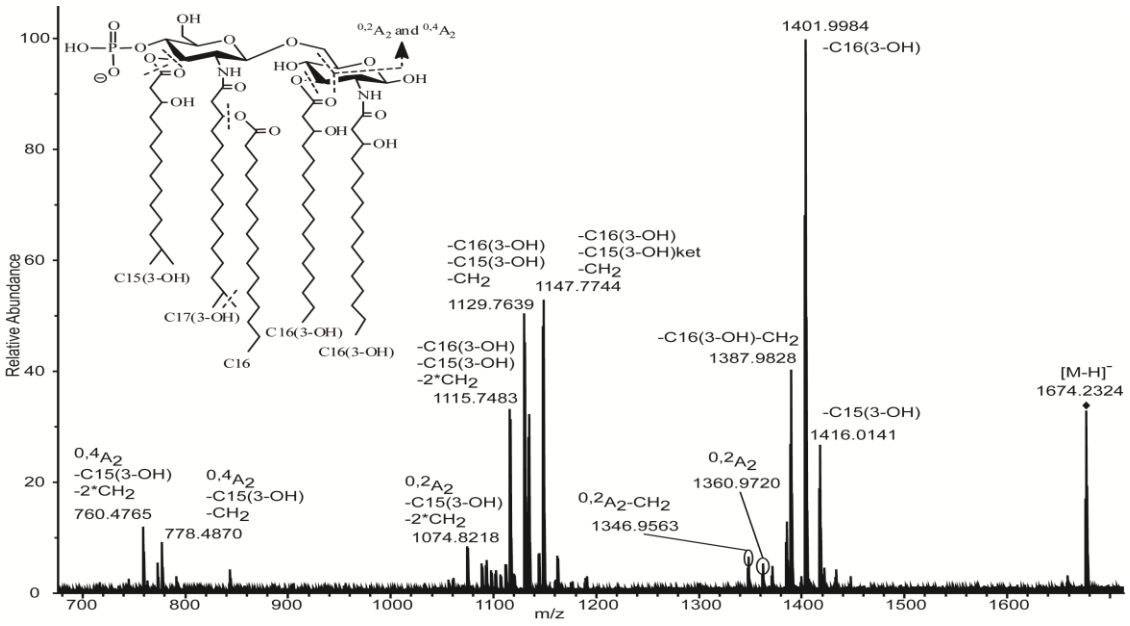
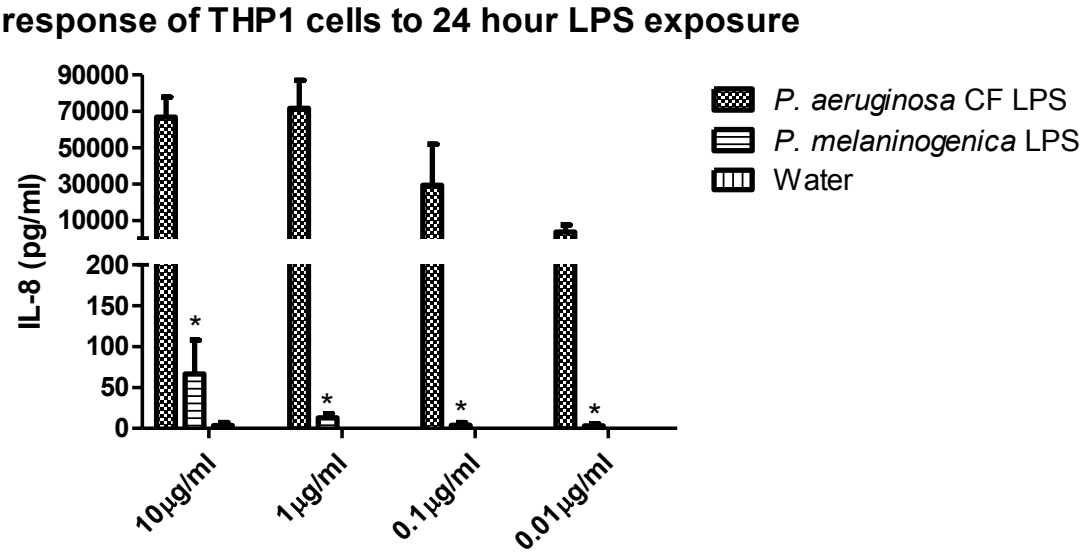


Figure 3.1. Structure of *P. melaninogenica* endotoxin A) Negative ion MALDI TOF mass spectrum of lipid A from Pm. Inset structure is proposed structure for ion at *m/z* 1754 corresponding to a diphosphoryl penta-acylated lipid A structure. B) Negative ion MALDI FTMS MS^2 mass spectrum of precursor ion at *m/z* 1674. Inset structure is proposed structure for ion at *m/z* 1674 corresponding to a monophosphoryl penta-acylated lipid A structure. Acyl chain configuration for the mono- and diphosphorylated lipid species were identical. Diagnostic cross-ring product ions ($^{0,2}A_2$ and $^{0,4}A_2$) are highlighted which allowed confident assignment of acyl chains.

A.



B.

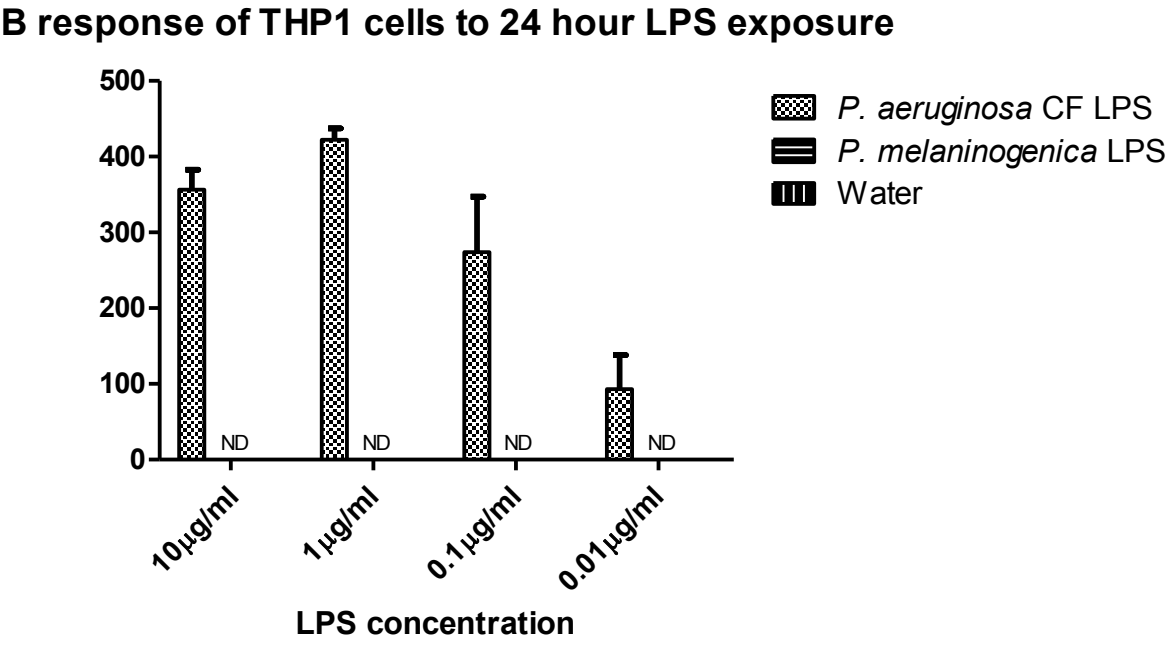
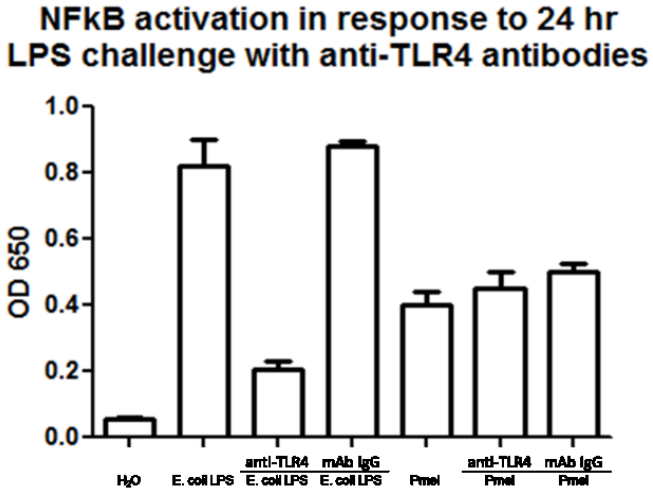


Figure 3.2. *P. melaninogenica* LPS stimulates THP1 cells significantly less than *P. aeruginosa* LPS. A) IL-8 production from THP1 cells stimulated for 24 hours with *P. melaninogenica* LPS or *P. aeruginosa* LPS. B) IL-1β response of THP1. IL-8 and IL-1β measured by ELISA. Graph displays mean and standard deviation of three independent experiments in triplicate $P<0.05$. ND= not detectible.

A.



B.

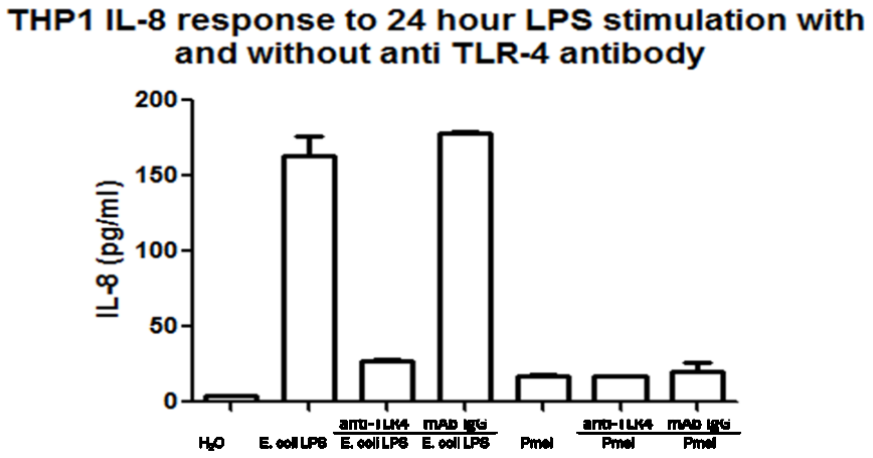
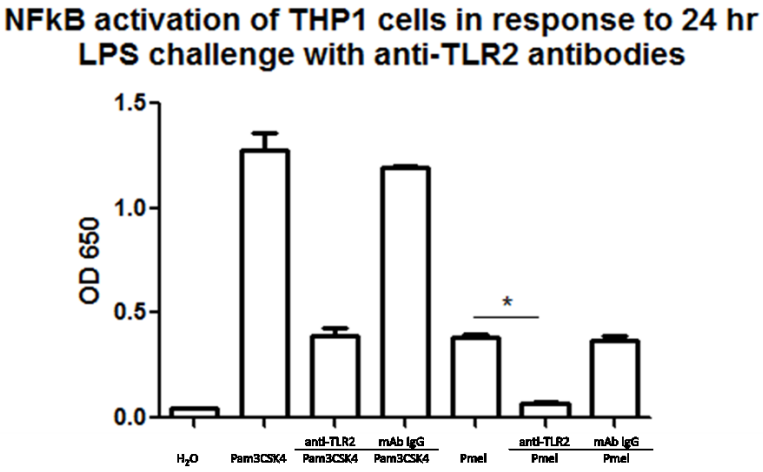


Figure 3.3. *P. melaninogenica* stimulates NFκB and IL8 production in a TLR4 independent mechanism. A) NFκB activation and B) IL-8 production by THP1 cells with TLR4 neutralizing antibody. NFκB measured by Quanti-Blue. IL-8 measured by ELISA. Anti-TLR4- 5µg/ml, mAb IgG- 5µg/ml, Purified E. coli LPS- 50ng/ml, *P. melaninogenica* 10µg/ml. Results are means and standard deviations of triplicate wells and are representative of at least two independent determinations. Pmel = *P. melaninogenica*

A.



B.

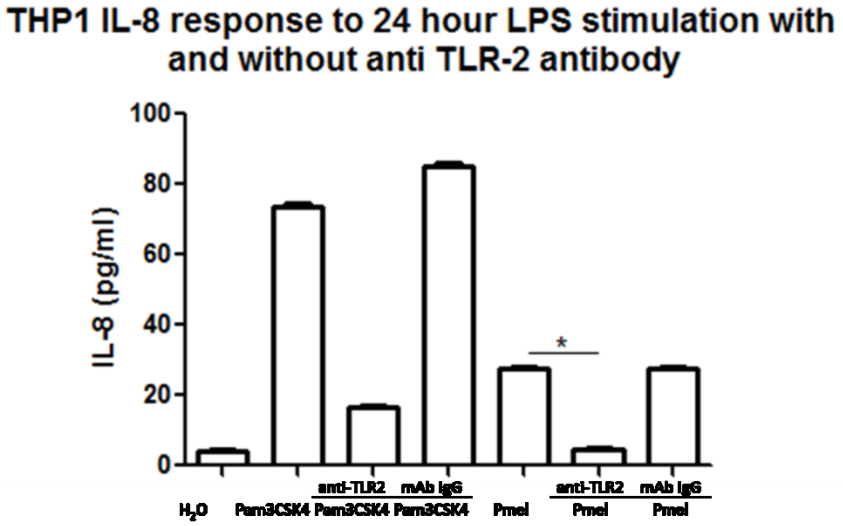


Figure 3.4. *P. melaninogenica* LPS NFκB and IL-8 production response with TLR 2 antibodies. A) NFκB activation and B) IL-8 production by THP1 cells with TLR 2 neutralizing antibody. NFκB measured by Quanti-Blue. IL-8 measured by ELISA. *P. melaninogenica* 10µg/ml. Anti-TLR2- 5µg/ml, mAb IgG- 5µg/ml,Pam3CSK3-10ng/ml, *P. melaninogenica* 10µg/ml. Results are means and standard deviations of triplicate wells and are representative of at least two independent determinations. Pmel = *P. melaninogenica*

References

1. Apicella MA. Isolation and characterization of lipopolysaccharides. Methods in Molecular Biology. 2008;431:3.

2. Pier GB, Grout M, Zaidi TS, Olsen JC, Johnson LG, Yankaskas JR, et al. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science. 1996;271(5245):64-7.

3. Guerry P, Ewing CP, Hickey TE, Prendergast MM, Moran AP. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of campylobacter jejuni. Infect Immun. 2000;68(12):6656-62.

4. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42(2):145-51.

5. Cutler CW, Eke PI, Genco CA, Van Dyke TE, Arnold RR. Hemin-induced modifications of the antigenicity and hemin-binding capacity of *Porphyromonas gingivalis* lipopolysaccharide. Infect Immun. 1996;64(6):2282-7.

6. Lafferty EI, Qureshi ST, Schnare M. The role of toll-like receptors in acute and chronic lung inflammation. J Inflamm (Lond). 2010;7:57-69.

7. Boucher R. New concepts of the pathogenesis of cystic fibrosis lung disease. European Respiratory Journal. 2004;23(1):146-58.

8. Levy H, Kalish LA, Huntington I, Weller N, Gerard C, Silverman EK, et al. Inflammatory markers of lung disease in adult patients with cystic fibrosis. Pediatr Pulmonol. 2007;42(3):256-62.

9. De Rose V. Mechanisms and markers of airway inflammation in cystic fibrosis. European Respiratory Journal. 2002;19(2):333-40.

10. Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. American journal of respiratory and critical care medicine. 1999;160(1):186-91.

11. Madan J, Koestler D, Stanton B, Davidson L, Moulton L, Housman M, et al. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: Interaction between intestinal and respiratory tracts and impact of nutritional exposures. mBio. 2012;3(4).

12. Foweraker J. Recent advances in the microbiology of respiratory tract infection in cystic fibrosis. Br Med Bull. 2009;89(1):93-110.

13. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLOS ONE. 2012;7(9):e45001.

14. Stressmann FA, Rogers GB, Klem ER, Lilley AK, Donaldson SH, Daniels TW, et al. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. J Clin Microbiol. 2011;49(1):281-9.

15. Filkins L, Hampton T, Gifford A, Gross M, Hogan D, Sogin M, et al. Prevalence of *Streptococci* and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol. 2012;194(17):4709-17.

16. Twomey KB, O'Connell OJ, McCarthy Y, Dow JM, O'Toole GA, Plant BJ, et al. Bacterial cis-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of *Pseudomonas aeruginosa*. The ISME Journal. 2011.

17. Rogers GB, Skelton S, Serisier DJ, Van Der Gast CJ, Bruce KD. Determining cystic fibrosis-affected lung microbiology: Comparison of spontaneous and serially induced sputum samples by use of terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2010;48(1):78-86.

18. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proceedings of the National Academy of Sciences. 2012;109(34):13769-74.

19. Rogers G, Carroll M, Serisier D, Hockey P, Jones G, Bruce K. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2004;42(11):5176-83.

20. van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME journal. 2010;5(5):780-91.

21. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proceedings of the National Academy of Sciences. 2007;104(51):20529.

22. Rogers G, Hart C, Mason J, Hughes M, Walshaw M, Bruce K. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003;41(8):3548-5.

23. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic

fibrosis. American journal of respiratory and critical care medicine. 2008;177(9):995-1001.

24. Tunney M, Klem E, Fodor A, Gilpin D, Moriarty T, McGrath S, et al. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. Thorax. 2011;66(7):579-84.

25. Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: A complex fungal and bacterial Community—Implications for therapeutic management. PloS one. 2012;7(4):e36313.

26. Sibley CD, Grinwis ME, Field TR, Eshaghurshan CS, Faria MM, Dowd SE, et al. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. PloS one. 2011;6(7):e22702.

27. Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. Proceedings of the National Academy of Sciences. 2008;105(39):15070-5.

28. Field TR, Sibley CD, Parkins MD, Rabin HR, Surette MG. The genus *Prevotella* in cystic fibrosis airways. Anaerobe. 2010;16(4):337-44.

29. Worlitzsch D, Rintelen C, Böhm K, Wollschläger B, Merkel N, Borneff-Lipp M, et al. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. Clinical Microbiology and Infection. 2009;15(5):454-60.

30. Bittar F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, et al. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One. 2008;3(8):e2908.

31. Ulrich M, Beer I, Braitmaier P, Dierkes M, Kummer F, Krismer B, et al. Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis. Thorax. 2010;65(11):978-84.

32. JEWES LA, Spencer R. The incidence of anaerobes in the sputum of patients with cystic fibrosis. J Med Microbiol. 1990;31(4):271-4.

33. Thomassen M, Klinger J, Badger S, Van Heeckeren D, Stern R. Cultures of thoracotomy specimens confirm usefulness of sputum cultures in cystic fibrosis. J Pediatr. 1984;104(3):352-6.

34. Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H. Establishment of oral anaerobes during the first year of life. J Dent Res. 1999;78(10):1634-9.

35. Mukhopadhyay S, Rose F, Frechette V. Vertebral osteomyelitis caused by *Prevotella (Bacteroides) melaninogenicus.* South Med J. 2005;98(2):226.

36. Bowler P, Duerden B, Armstrong D. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001;14(2):244-69.

37. Odeh M, Oliven A, Potasman I, Solomon H, Srugo I. Pyomyositis of the thigh due to *Prevotella melaninogenica*. Infection. 2000;28(1):49-50.

38. Athanasiou S, Matthaiou DK, Falagas ME. Vaginal mesh infection due to *Bacteroides melaninogenicus:* A case report of another emerging foreign body related infection. Scand J Infect Dis. 2006;38(11-12):1108-10.

39. Jousimies-Somer H, Savolainen S, Mäkitie A, Ylikoski J. Bacteriologic findings in peritonsillar abscesses in young adults. Clinical infectious diseases. 1993;16(Supplement 4):S292-8.

40. Kilian M, Thomsen B, Petersen T, Bleeg H. Occurrence and nature of bacterail IgA proteases. Ann N Y Acad Sci. 2006;409(1):612-24.

41. Allison HE, Hillman JD. Cloning and characterization of a *Prevotella melaninogenica* hemolysin. Infect Immun. 1997;65(7):2765-71.

42. Talan DA, Abrahamian FM, Moran GJ, Citron DM, Tan JO, Goldstein EJC. Clinical presentation and bacteriologic analysis of infected human bites in patients presenting to emergency departments. Clinical infectious diseases. 2003;37(11):1481-9.

43. Botta GA, Arzese A, Minisini R, Trani G. Role of structural and extracellular virulence factors in gram-negative anaerobic bacteria. Clinical infectious diseases. 1994;18(Supplement 4):S260-4.

44. Grenier D, Mayrand D, McBride BC. Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides*. Oral Microbiol Immunol. 1989;4(1):12-8.

45. Alauzet C, Marchandin H, Lozniewski A. New insights into *Prevotella* diversity and medical microbiology. Future microbiology. 2010;5(11):1695-718.

46. Hsiao WWL, Li KL, Liu Z, Jones C, Fraser-Liggett CM, Fouad AF. Microbial transformation from normal oral microbiota to acute endodontic infections. BMC Genomics. 2012;13(1):345.

47. Hosek WT, Gullett J. Adult female with shoulder pain. Ann Emerg Med. 2010;55(1):e1-2.

48. Harding G, Sutter V, Finegold S, Bricknell K. Characterization of *Bacteroides melaninogenicus*. J Clin Microbiol. 1976;4(4):354-9.

49. De A, Varaiya A, Mathur M. Anaerobes in pleuropulmonary infections. Indian J Med Microbiol. 2002 Jul-Sep;20(3):150-2.

50. Brook I. *Prevotella* and *Porphyromonas* infections in children. J Med Microbiol. 1995;42(5):340-7.

51. Larsen JM, Steen-Jensen DB, Laursen JM, Søndergaard JN, Musavian HS, Butt TM, et al. Divergent pro-inflammatory profile of human dendritic cells in response to commensal and pathogenic bacteria associated with the airway microbiota. PloS one. 2012;7(2):e31976.

52. Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, et al. Human toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1-and 4′-phosphatase activities. Cell Microbiol. 2009;11(11):1587-99.

53. Hajishengallis G, Martin M, Schifferle RE, Genco RJ. Counteracting interactions between lipopolysaccharide molecules with differential activation of toll-like receptors. Infect Immun. 2002;70(12):6658-64.

54. Westphal O, Jann K. Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. Methods Carbohydr Chem. 1965 1965;5(5):83-91.

55. Fischer W, Koch HU, Haas R. Improved preparation of lipoteichoic acids. Eur J Biochem. 1983 Jul 1;133(3):523-30.

56. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: Repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol. 2000 Jul 15;165(2):618-22.

57. Caroff M, Tacken A, Szabo L. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the "isolated lipid A" fragment of the *Bordetella pertussis* endotoxin. Carbohydr Res. 1988 Apr 15;175(2):273-82.

58. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. Microextraction of bacterial lipid A: Easy and rapid method for mass spectrometric characterization. J Lipid Res. 2005;46(8):1773-8.

59. Chavez-Sanchez L, Madrid-Miller A, Chávez-Rueda K, Legorreta-Haquet M, Tesoro-Cruz E, Blanco-Favela F. Activation of TLR2 and TLR4 by minimally modified low-density lipoprotein in human macrophages and monocytes triggers the inflammatory response. Hum Immunol. 2010;71(8):737-44.

60. Arce R, Caron K, Barros S, Offenbacher S. Toll-like receptor 4 mediates intrauterine growth restriction after systemic campylobacter rectus infection in mice. Molecular Oral Microbiology. 2012.

61. Erridge C, Pridmore A, Eley A, Stewart J, Poxton IR. Lipopolysaccharides of *Bacteroides fragilis, Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. J Med Microbiol. 2004;53(8):735-40.

62. Berezow AB, Ernst RK, Coats SR, Braham PH, Karimi-Naser LM, Darveau RP. The structurally similar, penta-acylated lipopolysaccharides of *Porphyromonas gingivalis* and *Bacteroides* elicit strikingly different innate immune responses. Microb Pathog. 2009;47(2):68-77.

63. Carpenter S, O'Neill LA. Recent insights into the structure of toll-like receptors and post-translational modifications of their associated signalling proteins. Biochem J. 2009 Jul 29;422(1):1-10.

64. Hashimoto M, Asai Y, Tamai R, Jinno T, Umatani K, Ogawa T. Chemical structure and immunobiological activity of lipid A from *Prevotella intermedia* ATCC 25611 lipopolysaccharide. FEBS Lett. 2003;543(1-3):98-102.

65. Ogawa T, Uchida H, Amino K. Immunobiological activities of chemically defined lipid A from lipopolysaccharides of *Porphyromonas gingivalis*. Microbiology. 1994;140(5):1209-16.

66. Park SY, Park DJ, Kim YH, Kim YH, Choi YW, Lee SJ. Schisandra chinensis α-iso-cubebenol induces heme oxygenase-1 expression through PI3K/akt and Nrf2 signaling and has anti-inflammatory activity in *Porphyromonas gingivalis* lipopolysaccharide-stimulated macrophages. Int Immunopharmacol. 2011;11(11):1907-15.

67. Zhang D, Chen L, Li S, Gu Z, Yan J. Lipopolysaccharide (LPS) of *Porphyromonas gingivalis* induces IL-1β, TNF-α and IL-6 production by THP-1 cells in a way different from that of *Escherichia coli* LPS. Innate immunity. 2008;14(2):99-107.

68. Rossano F, Rizzo A, Sanges MR, Cipollaro de L'Ero G, Tufano MA. Human monocytes and gingival fibroblasts release tumor necrosis factor-α, interleukin-1α and interleukin-6 in response to particulate and soluble fractions of *Prevotella melaninogenica* and *Fusobacterium nucleatum*. Int J Clin Lab Res. 1993;23(1):165-8.

69. Roberts FA, Darveau RP. Beneficial bacteria of the periodontium. Periodontol 2000. 2002;30(1):40-5.

70. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology. 2004;4(6):478-85.

71. Coats SR, Reife RA, Bainbridge BW, Pham TTT, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide antagonizes *Escherichia coli* lipopolysaccharide at toll-like receptor 4 in human endothelial cells. Infect Immun. 2003;71(12):6799-807.

72. Ahmed N, Hayashi T, Hasegawa A, Furukawa H, Okamura N, Chida T, et al. Suppression of human immunodeficiency virus type 1 replication in macrophages by commensal bacteria preferentially stimulating toll-like receptor 4. J Gen Virol. 2010;91(11):2804-13.

Chapter 4.

Prevotella melaninogenica iron requirements and hemoglobin receptor characterization.

Abstract

Prevotella melaninogenica, an anaerobic Gram-negative bacterium, has been cultured from polymicrobial disease sites including the lungs of patients suffering with the genetic disease cystic fibrosis (CF). In the CF lung, like other disease sites, bacteria must acquire essential nutrients such as iron. To better understand how *P. melaninogenica* survives in the CF lung, we characterized the ability of the bacterium to utilize relevant host sources of iron. *In vitro* growth experiments showed that *P. melaninogenica* growth depends on the presence of heme or hemoglobin and that other host iron containing proteins such as lactoferrin and transferrin or various inorganic iron molecules cannot serve as a sole iron source. To identify potential hemoglobin acquisition systems of *P. melaninogenica,* we sequenced the genome of the typed reference strain 25845 and searched for homologues of known hemoglobin receptors. We found several putative hemoglobin receptors through amino acid homology and predicted membrane topology. We also used hemoglobin agarose beads to identify outer membrane proteins that bind hemoglobin and searched for surface proteins that were heme-iron regulated. Using a combination of approaches, we have identified a comprehensive list of putative *P. melaninogenica* hemoglobin receptors. Future studies, with the aid of a *P. melaninogenica* genetic system, will further the characterization of heme acquisition in *P. melaninogenica*.

Introduction:

P. melaninogenica, formerly *Bacteroides melaninogenicus*, is a black pigmented anaerobic, Gram-negative bacterium with several potential virulence characteristics including fimbrial hemagglutination and the production of hemolysin, β-lactamases, fibrinolysin and a variety of proteases (193-196). *P. melaninogenica* was historically considered an oral commensal; however, it has recently been described as a potential pathogen and is closely related to other species considered highly pathogenic (197-199). *P. melaninogenica* has been cultured as the sole infectious agent from abscesses associated with vertebral osteomyelitis, pyomyositis and vaginal mesh infections (200-202). In addition, *P. melaninogenica* has been documented in polymicrobial diseases including brain abscesses, pleuropulmonary infections, endocarditis, intra-abdominal infections, wound infections, necrotizing fasciitis, decubitus and diabetic ulcers (194, 203-205).

P. melaninogenica was the most common anaerobe reported in sputa from adult cystic fibrosis (CF) patients (197, 206). Both culture based methods and molecular techniques show *P. melaninogenica* in the CF lung (197, 207-214). CF is the most common lethal genetic disorder affecting Caucasians. In the lungs of CF patients, dehydrated mucus forms hypoxic and/or anaerobic plaques that are suitable environments for anaerobic bacterial colonization (215, 216). In CF, cycles of infection, followed by chronic airway colonization cause excessive and unproductive pulmonary inflammation eventually leads to lung dysfunction, respiratory failure and death. The ability of *P. melaninogenica* to survive in this CF airway environment and colonize mucus plaques is dependent on its capacity to acquire essential nutrients, such as iron.

Iron is a nutritional requirement of extreme importance. For aerobic bacteria, iron is a required cofactor for metabolism including the electron transport chain cytochromes (217). For anaerobic bacteria, fumarase, iron-sulfur compounds and other iron containing molecules protect against superoxide and are involved in ATP production through pyruvate aided fermentation (218). Within the human body, free iron is scarce due to the presence of iron 'withholding' proteins, such as hemoglobin, lactoferrin and transferrin, which protect from the host from hydroxyl radical production (219). These iron withholding proteins also deny colonizing bacteria access to iron as an essential growth factor (nutritional immunity) and thus represent an important host defense strategy against infection (218-220). The lung environment in chronically infected CF patients has more available iron than that of healthy individuals (217, 219, 221, 222). Significant amounts of iron is found in sputum and bronchoalveolar lavage (BALF) samples from CF patients in the form of lactoferrin, transferrin, heme and hemoglobin (217, 219, 222), suggesting that this environment would be prone to bacterial colonization.

Because hemoglobin stores more than two-thirds of the body's iron, bacteria have developed a highly complex mechanism to acquire iron from hemoglobin. (223). Iron is stored in the heme component of hemoglobin. Heme consists of iron complexed inside a protoporphyrin IX (PPIX) ring, which aids in oxygen transportation. The process of heme iron acquisition begins when hemoglobin is bound to a beta barrel transmembrane surface receptor; it is believe that hemoglobin is then degraded to release heme molecules, although the specific mechanism is unknown (224-227). Heme is then transported across the outer membrane via a TonB dependent

mechanism (228) that derives energy from the inner membrane proton motive force (42). Specialized heme binding motifs such as FRAP and NPNL domains aid in receptor binding and heme transport (226). Hemoglobin receptor expression is typically regulated by iron availability via the iron-regulated transcription factor Fur (229, 230). When iron is replete, Fur complexes with iron and binds to *fur* box promoter sequences and inhibits transcription (231, 232), Under iron depleted conditions, apo-Fur cannot bind to target promoters, thus allowing transcription of iron regulated genes, such as those encoding a bacterial hemoglobin receptor. Once in the periplasm, heme is transported to an ATP-binding cassette (ABC) transporter by a heme permease into the cytoplasm, where heme can be used as a bacterial iron source (broken down into PPIX, iron biliverdin and CO_2) or can be complexed with other molecules and used for oxygen resistance (catalase) and electron transport (cytochromes) among many other functions (233, 234).

In addition to iron, *Bacteroides* species like, *P. melaninogenica*, *Prevotella intermedia* and *P. gingivalis* require heme itself for growth. Numerous bacterial species, including *P. gingivalis* and *P. intermedia* must rely on exogenous heme for growth because they lack the full complement of genes encoding the enzymes necessary for de novo heme biosynthesis (235, 236). Outer surface black pigmentation, characteristic of *Prevotella* and *Porphyromonas* genera, is formed from a derivative of heme and can to be used for oxygen protection and nutrient storage (237, 238). The ability of *P. melaninogenica* to acquire heme iron from host proteins is not known. Defining the mechanism of heme iron acquisition through hemoglobin in *P. melaninogenica* would provide a greater understanding into how *P. melaninogenica* grows in the CF lung.

Because of the high occurrence of *P. melaninogenica* in CF patients and its pathogenic potential, this study aims to further understand *P. melaninogenica* colonization of the CF lung by elucidating essential host iron sources and the mechanism of heme acquisition. We found that *P. melaninogenica* is able to utilize CF-relevant iron sources such as hemoglobin and heme when grown *in vitro* but no other host iron carrying proteins such as transferrin or lactoferrin or inorganic sources of iron. We also demonstrated that *P. melaninogenica* can use heme as a sole iron source *in vitro*. To facilitate the investigation of iron procurement, specifically the mechanism of hemoglobin acquisition, we sequenced the complete genome of 25845 and identified candidate genes involved in hemoglobin (heme) binding and transport. We used an *E. coli* system, *E. coli* K12 *hemA* that lacks an endogenous hemoglobin receptor to test candidate receptor function. Finally, we investigated outer membrane hemoglobin binding and heme iron regulated proteins by hemoglobin affinity pull down experiments and outer membrane protein expression in heme deplete and replete conditions.

Materials and Methods:

Bacterial Strains: *P. melaninogenica* ATCC 25845 was obtained from ATCC and maintained on Columbian Blood Agar (Thermo Scientific). EB53 (K12::*hemAaroB*) and IR754 (K12::*hemAaroBtonB*) strains were maintained on Luria-Bertani media (LB) plates with carbenicillin 30 μg/ml with 300μM 5- aminolevulinic acid (ALA; Sigma)(239). All strains, plasmids and primers are listed in Table 1.

Bacterial Growth Conditions: *P. melaninogenica* ATCC 25845 was cultured from frozen glycerol stock anaerobically on Columbian Blood agar plates for two to four days until colonies displayed a characteristic red/brown phenotype. Colonies were then inoculated

into prereduced Tryptic Soy Broth (Becton Dickinson) supplemented with 5 μg/ml menadione (Sigma) and 150 μM of 2 2'-bipyridyl (BPD, Sigma). (240). All anaerobic Tryptic Soy Broth (ATSB) has menadione supplementation (241). All anaerobic growth was done in a Coy Anaerobic chamber at 37°C. Once growth had reached an optical density (OD) at 600nm OD 1.0, approximately $1x10^3$ CFU/ml was inoculated into ATSB with various iron and heme conditions and in some cases 100 μM BPD. Iron sources were normalized to 7.6 μM iron and bacteria were grown shaking anaerobically for the time stated. Aliquots of media were used to measure OD_{600} at various time points. Heme stock was dissolved in 1 M NaOH. Hemoglobin stock was dissolved in water. Lactoferrin, transferrin, ferric citrate, ferric chloride and ferrous chloride tetrahydrate stocks were dissolved in DMSO.

Genome Sequencing: Genomic DNA from *P. melaninogenica* ATCC 25845 was harvested from a one liter flask grown in Anaerobic Broth (BD Difco) and prepared using Fast DNA Spin Kit (MP Biomedicals). The gDNA was sequenced and assembled using 454 and Ilumina Solexa sequencing systems similar to (242).

Amino acid homology and topology mapping for hemoglobin receptors: To search for homologous proteins*, P. melaninogenica* sequences were investigated via BLAST-p and were compared using ClustaW (243, 244). Protein sequences were then imported into PREZ-TMD for topology mapping (245).

E. coli hemA system: The K12 Δ*hemAaroB* strain (EB53) is dependent on exogenous heme due to the inactivation of heme biosynthesis (*hemA*) and iron siderophore (*aroB)* pathways (239). The K12 Δ*hemAaroBtonB* strain has an additional mutation inactivating the TonB protein which restricts the ability to actively transport molecules to

the periplasm. K12 Δ*hemA* strains can be chemically complemented with 300 μM 5-aminolevulinic acid (ALA, Sigma) to restore a functional heme biosynthesis pathway and growth. *P. melaninogenica* candidate outer membrane receptors were expressed by an isopropyl b-D-thiogalactopyranoside (IPTG, Sigma) inducible promoter from plasmid pMMB which was adapted for Gateway cloning (Life Technologies) (246). *P. melaninogenica* TonB complex (*tonB, exbD (1), exbD (2), exbB*), was expressed by tetracycline cassette promoter on pACYC184. Strains were maintained on 300 μM ALA (Table 1). For hemoglobin candidate evaluation, strains were plated on 100 μM IPTG and 10 μM hemoglobin or 100 μM IPTG and 10 μM heme plates.

Hemoglobin agarose: Zwittergent solubilized bacteria grown under heme depleted conditions (no heme) were incubated with Affi-Gel 10 beads covalently attached to solubilized human hemoglobin. Hemoglobin bound proteins were eluted with elution buffer (1% octylglucoside/1.5% glycine pH 2.3) then run on 7.5% gradient SDS page gel as described (247). Putative *P. melaninogenica* hemoglobin binding proteins were viewed by Sypro Ruby staining (Bio-Rad). Proteins were excised and digested in gel with trypsin and the resulting peptides were analyzed by ABI 4800 MALDI TOF/TOF MS by the UNC Proteomics Core.

Outer membrane preparation (OMP): Bacteria were grown in one liter of ATSB under either heme deplete (0 μM heme) or heme replete (7.6 μM heme) conditions as described in (248, 249) until stationary phase. Bacteria were harvested by centrifugation at 10,000 x g for 25 minutes at 4°C. Supernatant were removed and the pellets were suspended in lysis buffer (50 mM NaH_2PO_4, pH 7, 300 mM NaCl) with 1 mg/ml lysozyme and incubated on ice for 30 minutes. The material was lysed by two passages

through a French pressure cell. Intact cells and cell debris were removed by centrifugation at 10,000 x g for 20 minutes at 4°C. Supernatant were centrifuged at 100,000 x g for 1 hour at 4°C. The pellet were solubilized in 1% Sarkosyl and resuspended with a syringe 27G (Becton Dickinson) 4 times and incubated for 30 minutes at room temperature with gentle shaking. The Sarkosyl soluble faction was subjected to centrifugation at 100,000 x g for 1 hour at 4°C. The supernatant fraction was removed and the pellet was resuspended in 100 µl PBS to yield the outer membrane preparation. 4 µgs of OMPs were suspended in SDS-PAGE sample buffer and heated to 100°C for 5 minutes. Samples were run on a 7.5% Mini-protean TGX (Bio-Rad) gel and were stained with GelCode Blue Stain Reagent (Thermo Scientific). iTRAQ analysis: 70 µgs of OMPs from either heme depleted or heme replete *P. melaninogenica* were collected and stored in 8 M urea. iTRAQ analysis was done by UNC Proteomics core.

Results:

Characterization of *P. melaninogenica* iron requirements.

Fastidious black pigmented *Bacteroides* species, including *P. melaninogenica*, are routinely grown in complex blood media and have a characteristic black heme derivative stored on the outer surface of the bacterium. In order to deplete heme iron stores *Bacteroides* species must be grown in serial passages on media lacking a heme source. To establish the iron growth requirements of *P. melaninogenica*, we first determined the effect of iron containing sources on the growth of *P. melaninogenica* after passages in heme depleted ATSB. In the absence of supplementation, *P. melaninogenica* is unable to grow in ATSB (Figure 1A). Supplementation of ATSB with

heme or human hemoglobin is sufficient to restore growth (Figure 1A and 1B). In contrast, other human iron containing proteins, readily available in infected airways (lactoferrin and transferrin) were not sufficient to support growth (Figure 1A). Inorganic forms of iron such as ferric citrate (Fe^{3+}) ferric chloride (Fe^{3+}) ferrous chloride tetrahydrate (Fe^{2+}) did not support *P. melaninogenica* growth in ATSB (Figure 1B). These inorganic forms were used to test if the oxygen state of the iron molecule and the iron solubility at physiological pH would promote growth in an anaerobic atmosphere (250). We also demonstrated that *P. melaninogenica* can use heme as a sole iron source in iron restricted chelated ATSB media (Figure 1C). However, growth in chelated conditions was not as robust on heme alone; suggesting that trace inorganic iron present in ATSB can be utilized. Additionally in the presence of inorganic iron, PPIX, a protoporphorin lacking iron, can be used to restore growth to similar rates as heme in ATSB (Supplementary Figure 1).

The *P. melaninogenica* genome sequence reveals iron acquisition system candidates.

To investigate the mechanism of iron/heme acquisition, we sequenced the *P. melaninogenica* genome to facilitate the identification of genes involved in hemoglobin (heme) binding and transport. In collaboration with Dr. Corbin Jones (UNC-CH Department of Biology) and Dr. Anthony Fodor (UNC-C Department of Bioinformatics and Genomics) we used 454 pyrosequencing in combination with the Ilumina-Solexa platform, to sequence the complete genome of *P. melaninogenica* ATCC 25845. The sequence is currently partially assembled. The genome is approximately 3.2 million

base pairs, contains over 2700 putative open reading frames (ORFs) and is predicted to be organized into two chromosomes. The combination of both platforms enabled confident structuring and assembly (242). However, prior to the completion of our genome sequencing efforts, the genome sequence for *P. melaninogenica* ATCC 25845 was submitted to NCBI by the J. Craig Venter Institute, as a part of the Human Microbiome Project (HMP) Reference Genomes (251).

The submitted *P. melaninogenica* genome sequence (ACSI00000000) was used to identify candidate genes involved in iron acquisition (Figure 2A and 2B). Using BLASTn, we found genes encoding secreted proteins such as hemagglutinin and hemolysin (196) , which are proposed to aggregate and lyse red blood cells. In terms of candidate heme and/or hemoglobin receptors, we identified 19 putative TonB dependent outer membrane proteins in addition to many TonB dependent plug domains (not shown). Even though there are no siderophore like proteins encoded by *P. melaninogenica* there is a TonB-dependent siderophore receptor that could be used to scavenge siderophores in multispecies environments.

The energy required for heme-iron transport across the outer membrane is likely supplied by a unique *P. melaninogenica* TonB complex. Four genes, encoding TonB, ExbB, ExbD1and ExbD2 proteins were found. Many bacteria have a single *exbD;* we hypothesized the additional gene could play an auxiliary role in iron acquisition. Once heme is transported to the periplasmic space, a putative heme permease transports heme to an inner membrane bound ABC transporter. *P. melaninogenica* also encodes *feoB* for inorganic iron transportation into the cytoplasm revealing the importance of inorganic iron to *P. melaninogenica* growth (Figure 1C). Further, *P. melaninogenica*

encoded several cytoplasmic proteins related to iron metabolism including two *fur* genes homologs, ferritin and bacterioferritin, but was missing key heme biosynthesis pathway genes such as ferrochelatase. RT-PCR studies with the two *fur* homologs show a different regulation pattern from each other suggesting different roles for each protein (Supplementary Figure 2). Additionally the lack of an encoded ferrochelatase, an enzyme that inserts iron into the PPIX ring, in the *P. melaninogenica* genome does not explain the apparent usage of PPIX in place of heme (Supplementary Figure 1).

Identification of *P. melaninogenica* candidate hemoglobin receptors through amino acid homology searches.

More extensive searches incorporating characteristic hemoglobin receptor motifs and membrane topology predictions revealed a small group of proteins resembling the well characterized hemoglobin receptor ShuA from *S. dysenteriae* (226) and HmuR *P. gingivalis* (230, 236) (Figure 3). Specifically, amino acid homology using conserved residues from other characterized hemoglobin receptors such as the FRAP and NPNL domains, TonB box (TVTATG) and outer membrane transmembrane beta barrel structure was used (226, 252). Two proteins, HMPREF0659_A5369 (designated HmuR) and HMPREF0659_A6271 (designated TonBr) showed similarity to both ShuA from *S. dysenteriae* and HmuR of *P. gingivalis* using these methods.

Characterization of candidate *P. melaninogenica* hemoglobin receptors using *E. coli hemA*.

To functionally characterize the genes identified in our analysis of the sequenced genome, we utilized an *E .coli* K12 based screening strain (239). *E. coli* K12

strains naturally lack an endogenous hemoglobin receptor but are capable of *de novo* synthesis of heme. To eliminate heme biosynthesis the screening strain carries a mutation in *hemA* (encoding glutamyl-tRNA reductase). The HemA gene product is required for the production of 5-aminolevulinic acid (ALA), a precursor in the heme biosynthesis pathway (253). To reduce growth under iron limiting conditions, the screening strain also carries a mutation in *aroB,* which is required for siderophore production. For growth under iron limiting conditions, the *E. coli* K12 *hemA, aroB double* mutant *(*K12 *hemAaroB)* can be chemically complemented with ALA or genetically complemented by expression of a functional heme/hemoglobin receptor (Figure 4A). We chose the following hemoglobin receptor candidates based on homology scores. 1) *P. melaninogenica* A5369 'HmuR', a TonB-dependent receptor, sharing 29% amino acid sequence similarity with the heme utilization receptor (HmuR) of *P. gingivalis*, 2) *P. melaninogenica* A7242 'OmpHb', a putative TonB-dependent receptor plug domain protein (44% similarity to PhuR of *Flavobacterium psychrophilum)* and 3) *P. melaninogenica* A6271 'TonBr', a putative TonB-dependent domain protein (45% similarity to an outer membrane heme/hemoglobin receptor of *S. dysenteriae*). The K12*hemAaroB* strain was used to test the candidate *P. melaninogenica* genes and known hemoglobin receptors from other species for their ability to restore growth in the absence of ALA. The panel of *P. melaninogenica* receptors showed no function in the heterologous *E. coli* background when grown on hemoglobin or heme, in contrast to a putative *E. coli* hemoglobin receptor (C1129, Figure 4B). To rule out the possibility that the candidate heme/hemoglobin receptors are incompatible with *E. coli* TonB, as has been reported in other systems (239), we cloned the putative *P. melaninogenica* TonB

complex *(tonB-exbD-exbD-exbB)* and expressed the candidate *P. melaninogenica* receptors and TonB complex genes in K12*hemAaroBtonB* an isogenic mutant of K12*hemAaroB* (Figure 4B). However, the *P. melaninogenica* TonB complex did not restore growth to K12*hemAaroBtonB* whereas expression of *E. coli* TonB protein restored function of the *E. coli* hemoglobin receptor (data not shown).

Our results suggest that the *P. melaninogenica* candidate hemoglobin receptors do not have the proposed function and/or the *P. melaninogenica* TonB complex and hemoglobin receptor systems are not functional in E. coli K12. Because we were unable to distinguish these possibilities, we chose several alternative methods to further pursue the identification of *P. melaninogenica* hemoglobin receptors.

Identification of *P. melaninogenica* hemoglobin receptors by hemoglobin affinity purification.

To purify *P. melaninogenica* hemoglobin binding proteins, we mixed heme starved *P. melaninogenica* lysate with Affi-gel 10 beads covalently attached to human hemoglobin (254). In parallel experiments hemoglobin agarose was used to isolate HgbA from heme starved *H. ducreyi* (239, 254). Using the hemoglobin pull down technique, several putative *P. melaninogenica* hemoglobin binding proteins were identified by MALDI TOF/TOF MS (Figure 5). The top candidates were HMPREF0659_A5491 putative outer membrane protein involved in nutrient binding, HMPREF0659_A7287 TonB-dependent receptor plug, HMPREF0659_A5179 putative RagA protein, HMPREF0659_A6276 TonB-dependent receptor and HMPREF0659_A6366 putative outer membrane protein. Despite the clear enrichment

of these proteins by hemoglobin affinity-based purification, none possessed all the predicted sequencing characteristics of known heme/hemoglobin receptors as described above.

Identification of *P. melaninogenica* hemoglobin receptors by enrichment under heme starved conditions.

Because iron acquisition is such an important growth requirement, bacteria regulate iron acquisition by controlling the amount of surface expressed iron receptor. Based on this widespread regulatory feedback mechanism, we hypothesized that *P. melaninogenica* hemoglobin receptors may be enriched under heme starved conditions. To enrich for iron regulated proteins, an outer membrane preparation was made under heme and iron limiting conditions sited in previous papers for other closely related bacteria (235, 238, 240, 248, 249). In addition, specific *P. melaninogenica* growth conditions were tested using *in vitro* growth curves that showed slow stunted growth characteristic of limited nutrient availability.

The *P. melaninogenica* proteins from heme replete and deplete conditions were analyzed by SDS-PAGE and isobaric tags for relative and absolute quantitation (iTRAQ), a quantitative method to identify peptides in a complex mixture (255). Both of the heme regulation experiment used (SDS-PAGE and iTRAQ) used the identical OMP preparation. SDS-PAGE analysis of the outer membrane preparations revealed multiple potential heme regulated proteins (Figure 6A and Figure 6B). The iTRAQ method showed four proteins that were regulated by heme, one of which fit the outer

membrane location and heme binding motif criteria characteristic of hemoglobin receptors (Figure 6C).

Using the collective information from the various analyses described above, we identified a total of 24 putative hemoglobin receptors. Of these, seven candidates possess known hemoglobin receptors predicted topology, heme binding motif signatures, hemoglobin binding and heme regulation.

Discussion

In order to colonize a host, bacteria must have the ability to acquire limited but essential nutrients. In the human host, iron, an essential nutrient, is sequestered by carrier molecules and proteins such as heme, hemoglobin, lactoferrin and transferrin. These high affinity iron binding molecules control iron toxicity and facilitate important metabolic processes (217-220). In CF chronic lung disease, a considerable quantity of free heme and hemoglobin is available inside the lumen of the airways (217, 219, 221) making a suitable niche for bacterial colonization.

Our results using *in vitro* growth conditions show that *P. melaninogenica* can use both heme and hemoglobin to support grow. Earlier studies, using a clinical isolate, established that *P. melaninogenica* can secrete a hemolysis capable of degrading hemoglobin (196). In addition, we demonstrated that *P. melaninogenica* can use heme as an iron source by growing *P. melaninogenica* in iron restricted media. With hemoglobin and heme as known growth sources, further experiments elucidated the mechanism for heme acquisition through hemoglobin receptors. Moreover, the *P. melaninogenica* genome does not encode for a typical ferrochelatase or ferrochelatase enzyme homolog that inserts or removes iron from the PPIX ring, but *P. melaninogenica*

can use heme as an iron source (Figure 1C) and can grow on PPIX in an iron rich environment (Supplementary Figure 1). Future studies are needed to identify the enzyme used for removal and insertion of iron into the heme molecule.

The *P. melaninogenica* genome provides clues to the mechanism(s) of heme acquisition. Based on the genome sequence, we identified nineteen putative TonB dependent outer membrane receptor/transporters and a complete ABC transport system and permease with homology to known periplasmic and inner membrane iron transport systems (Figure 2B). The TonB system of *P. melaninogenica*, encodes for two *exbD* genes like *Flavobacterium psychrophilum* (256) and *Xanthomonas campestris* (257). In *X. campestris,* a Gram-negative plant pathogen, only one of the *exbD* genes is involved in iron uptake. The function of *exbD1* and *exbD2* for *P. melaninogenica* are unknown. In addition, *P. melaninogenica* encodes two Fur proteins proposed to regulate fur dependent genes. Other bacteria like *Campylobacter jejuni* (258) and *Bacillus subtilis* (259) contain multiple *fur* homologues. In preliminary testing, only one candidate *fur* gene showed differential regulation in response to iron availability (Supplemental Figure 2). Interestingly, *P. melaninogenica* does not encode identifiable iron scavenging proteins like siderophores or gingipains, suggesting that the CF lung environment provides an ample supply of usable iron.

The *P. melaninogenica* genome search for hemoglobin receptors revealed multiple proteins that had hemoglobin receptor characteristics. One interpretation of these results is that *P. melaninogenica* does not just rely on one but several receptor proteins, as seen in *P. gingivalis* (260). *P. melaninogenica*, a black pigmented anaerobe like *P. gingivalis*, is covered in black heme-derived pigment that could be

attached by surface receptors that do have the ability to transport but when heme is not needed, hold the heme on the surface for storage and oxygen protection purposes (238). Theoretically there could be several hemoglobin receptors that each transport heme (or different heme forms) when needed.

Because *P. melaninogenica*, like other a black pigmented *Bacteroides* species, can store a heme iron derivative on its surface, heme starvation conditions are difficult to achieve without serial passage in iron depleted conditions. Optimal starvation conditions were needed to detect altered expression of iron regulated proteins in lysate preparations for hemoglobin agarose pull-downs and outer membrane preparations by gel electrophoresis and iTRAQ. The difficulty in achieving consistent depletion conditions may account for the variation in the results of each of these experiments. Additionally, because of the inability to genetically manipulate *P. melaninogenica*, an *E. coli* system was used as a method of localizing one protein and testing function instead of doing function assays with a *P. melaninogenica* mutant. The *E. coli hemA* system had been used in the past to demonstrate hemoglobin receptor function (253, 261). However, top candidates for *P. melaninogenica* hemoglobin receptor did not restore the *E. coli hemA* system. This led us to rethink the candidates for selection and the compatibility of *P. melaninogenica* proteins with *E. coli* protein machinery.

Taken together our data suggests that *P. melaninogenica* hemoglobin receptors are expressed and function to acquire hemoglobin as demonstrated in *in vitro* growth assays. Further studies in hemoglobin receptor identification will be greatly aided by the development of genetic systems in *P. melaninogenica*. Work done to further characterize hemoglobin receptors, to understand the mechanism of cytoplasmic heme-

iron removal, to characterize two putative Fur proteins and to investigate *fur*-dependent regulation will shed light on the mechanism of *P. melaninogenica* survival in the CF lung.

Figures:

A.

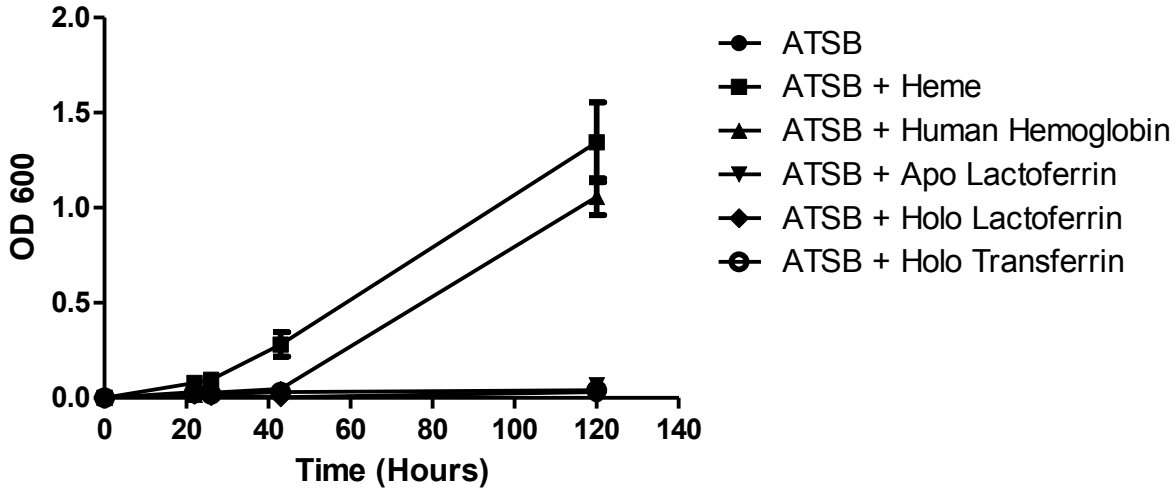
		Relevant genotype and/or phenotype	Source
Strains	*Prevotella melaninogenica* ATCC 25845		ATCC
	E. coli EB53	*hemA aroB rpoB, Carb*	Elkins *et. al* 1998
	E. coli IR754	EB53, but *tonB::Kan*	Elkins *et. al* 1998
Plasmids	pMMBV1GW	Gateway-adapted version of pMMB67EH; Carb	Lab stock
	pDONR203	Gateway cloning vector; Kan	Lab stock
	pACYC184	p15 replicon; Cm Tet	Lab stock

B.

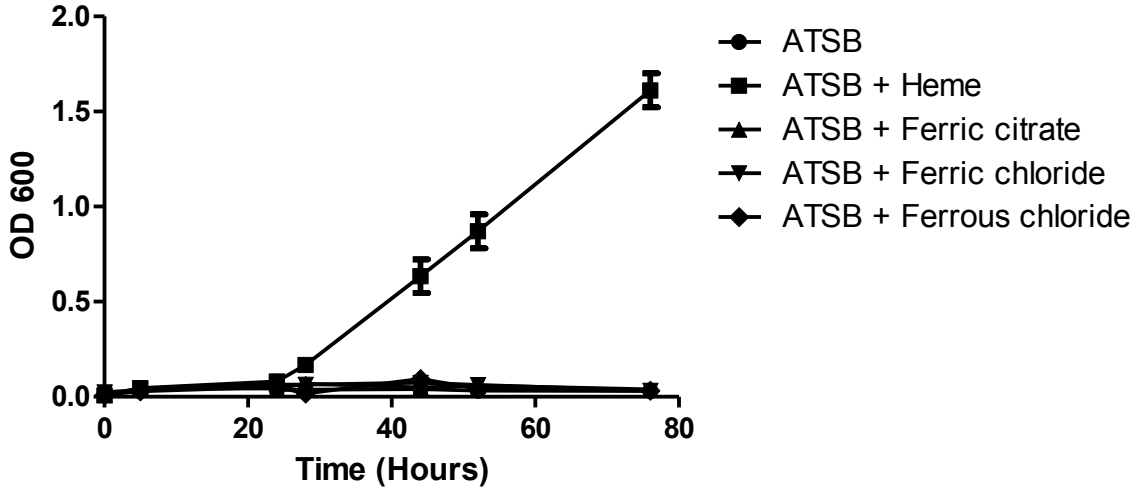
Primers		Sequence (5' to 3')
E. coli hemA primers (gateway)	C1129Ec-F	TACAAAAAAGCAGGCTCATATGTATATGAATGTAATCAGAACTG
	C1129Ec-R	TACAAGAAAGCTGGGTGGATCCTCACCACTGATAACGGGTATAAAGAC
	hmuRcompF	TACAAAAAAGCAGGCTCATATGTGGGCTGATGAGTTGCCTGATTCT
	hmuRcompR	TACAAGAAAGCTGGGTGGATCCAAATAGTTTATCAACATCAACTGACAT
	tonBrcompF	TACAAAAAAGCAGGCTCATATGCTCTTCGGTGATGTGAAGGCA
	tonBrcompR	TACAAGAAAGCTGGGTGGATCCTAGTTTAACGCTGAGACCGACATACCA
	ExbBExbDTonBgw1F	TCGAGGAGGATATTCATGGCAACTACACAACAAAAACCAG
	ExbBExbDTonBgw1R	CAAGAAAGCTGGGTTTCACTGCAGTTTGAAGGTAATA GG

Table 4.1 Bacterial strains, plasmids and primers used in this study.

A.


2.0
1.5
1.0
0.5
0.0
OD 600
0
20
40
60
80
100
120
140
Time (Hours)
ATSB
ATSB + Heme
ATSB + Human Hemoglobin
ATSB + Apo Lactoferrin
ATSB + Holo Lactoferrin
ATSB + Holo Transferrin

B.


2.0
1.5
1.0
0.5
0.0
OD 600
0
20
40
60
80
Time (Hours)
ATSB
ATSB + Heme
ATSB + Ferric citrate
ATSB + Ferric chloride
ATSB + Ferrous chloride

C.

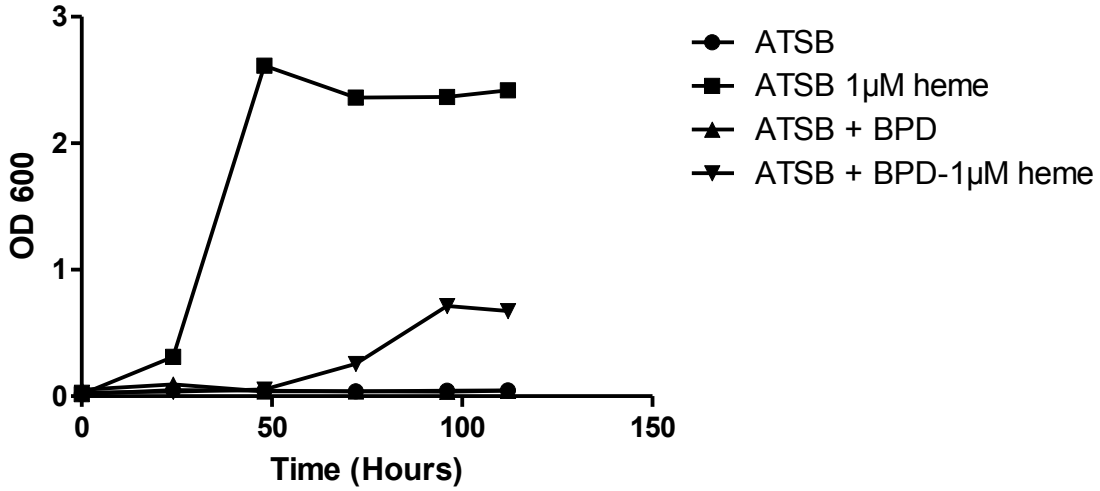
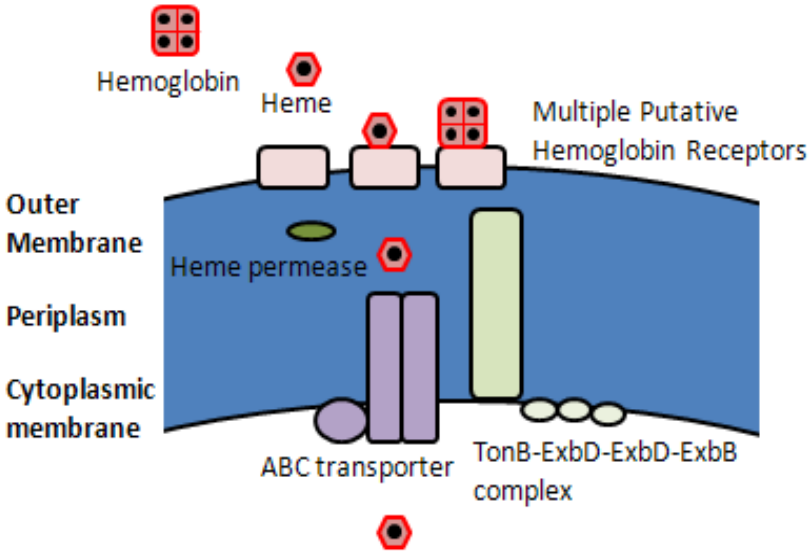


Figure 4.1 *P. melaninogenica* requires heme or hemoglobin to grow in ATSB. A) Growth of *P. melaninogenica* in ATSB with host iron sources normalized to 7.6 μM iron. Molar equivalences as follows: One mole hemoglobin contains four moles of heme. One molar heme molecule contains one molar iron. One mole lactoferrin/transferrin contains two mole of iron. B) Growth of *P. melaninogenica* in ATSB with inorganic sources of iron such as ferric citrate, ferric chloride and ferrous chloride tetrahydrate normalized to 7.6 μM iron. The average of three experiments was plotted and standard deviation of each point is depicted. C) Growth of *P. melaninogenica* in 100 μM BPD chelated ATSB. The experiment was completed twice with similar results.

A.


Hemoglobin
Heme
Multiple Putative
Hemoglobin Receptors
Outer
Membrane
Heme permease
Periplasm
Cytoplasmic
membrane
ABC transporter
TonB-ExbD-ExbD-ExbB
complex

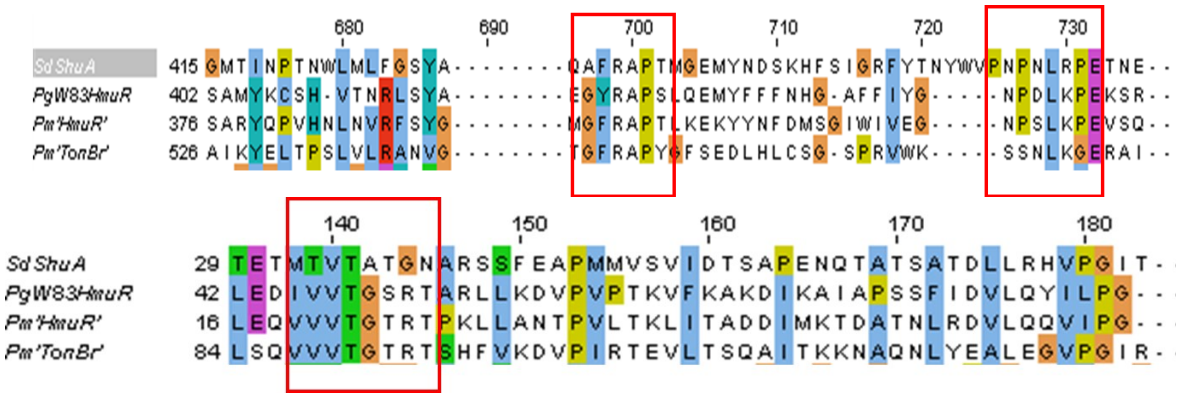
B.

Iron and Heme-Iron Acquisition

	Gene Description	Gene Name	Accession Number	Max Identity**	Name	Identity	Reference
Secreted proteins	Hemoloysin	*phyA*	HMPREF0659_A5220	60%	Hemolysin	YP_001929918.1	Allison *et al.* 1997
	Hemagglutinin		HMPREF0659_A6586	34%	Serine protease	YP_001928677.1	
Outer surface	TonB-dependent receptor plug domain protein	*'tonBr'*	HMPREF0659_A6271	29%	TonB-dependent receptor	YP_001928857.1	
	TonB-dependent receptor	*'hmuR'*	HMPREF0659_A5389	29%	TonB-dependent receptor HmuR	YP_001928673.1	
	TonB-dependent receptor		HMPREF0659_A6063	25%	Receptor antigen A	YP_001928409.1	
	TonB-dependent receptor		HMPREF0659_A6078	28%	TonB-dependent outer membrane receptor	YP_001930069.1	
	TonB-dependent receptor		HMPREF0659_A5280	26%	Receptor antigen A	YP_004509191.1	
	TonB-dependent receptor		HMPREF0659_A5310	29%	Receptor antigen A	YP_001928409.1	
	TonB-dependent receptor		HMPREF0659_A6564	33%	Receptor antigen A	YP_001928409.1	
	TonB-dependent receptor		HMPREF0659_A7354	43%	Hypothetical protein PGN_1437	YP_001929553.1	
	TonB-dependent receptor		HMPREF0659_A6481	26%	TonB-linked outer membrane receptor	YP_001928820.1	
	TonB-dependent receptor		HMPREF0659_A5458	28%	TonB-dependent receptor HmuR	YP_001928673.1	
	TonB-dependent receptor		HMPREF0659_A7167	32%	Hypothetical protein PGN_1275	YP_001928219.1	
	TonB-dependent receptor		HMPREF0659_A7294	29%	Receptor antigen A	YP_001928409.1	
	TonB-dependent receptor		HMPREF0659_A5445	30%	Hypothetical protein PGN_0400	YP_001928516.1	
	TonB-dependent receptor		HMPREF0659_A6413	27%	Receptor antigen A	YP_001928409.1	
	TonB-dependent receptor		HMPREF0659_A6587	24%	TonB-linked receptor Tlr	YP_001928799.1	
	TonB-dependent receptor		HMPREF0659_A5544	26%	TonB-linked outer membrane receptor	YP_001928820.1	
	TonB-dependent receptor		HMPREF0659_A5027	33%	TonB-dependent receptor exported protein	YP_001929463.1	
	TonB-dependent receptor		HMPREF0659_A5303	43%	TonB protein	YP_001928925.1	
	TonB-dependent receptor		HMPREF0659_A6954	29%	TonB-dependent receptor exported protein	YP_001929463.1	
	TonB-dependent siderophore receptor		HMPREF0659_A5535	31%	TonB-linked receptor Tlr	YP_001928799.1	
	Iron chelate uptake ABC transporter, permease protein	*fhuB*	HMPREF0659_A6581	49%	Iron ABC transporter permease	YP_001928823.1	
Periplasm	Periplasmic protein TonB	*tonB*	HMPREF0659_A5407	50%	TonB protein	YP_001928925.1	
	Ferrous iron transport protein B	*feoB*	HMPREF0659_A7165	50%	Ferrous iron transport protein B	YP_004510887.1	
Inner membrane	Iron complex transport system substrate-binding protein	*fhuD*	HMPREF0659_A6582	25%	Iron ABC transporter substrate-binding protein	YP_001928803.1	
	Ferrichrome transport ATP-binding protein	*fhuC*	HMPREF0659_A6312	47%	Iron ABC transporter ATP-binding protein	YP_001928824.1	
	Transporter, MotA/TolQ/ExbB	*exbB*	HMPREF0659_A5404	28% $	MotA/TolQ/ExbB proton channel family protein	NP_904916.1	
	Hypothetical protein	*exbD1*	HMPREF0659_A5405	42% $	Biopolymer transport protein ExbD	NP_904917.1	
	Hypothetical protein	*exbD2*	HMPREF0659_A5406	26% $	Biopolymer transport protein ExbD	NP_904917.1	
	Ferric uptake regulation protein	*fur*	HMPREF0659_A5773	29%	Ferric uptake transcriptional regulator	YP_001929619.1	
cytoplasmic	Ferric uptake regulation protein	*fur*	HMPREF0659_A6606	27%	Ferric uptake transcriptional regulator	YP_001929619.1	
	Ferritan	*ftn*	HMPREF0659_A5828	45% $	Ferritin	NP_905466.1	
	Bacterioferritin		HMPREF0659_A5226	62%	Bacterioferritin comigratory protein	YP_001929174.1	
	Uropophyrinogen III synthase	*hemD*	HMPREF0659_A6329	54% $	Uroporphyrinogen-III synthase	NP_904537.1	
	Coproporphyrinogen dehydrogenase	*hemN*	HMPREF0659_A5198	26%	Oxygen-independent coproporphyrinogen III oxidase	YP_001929610.1	
	Protophorhyrinogen oxidase	*hemG*	HMPREF0659_A5199	48%	Protoporphyrinogen oxidase	YP_001928520.1	

Figure 4.2 *P. melaninogenica* iron and heme-iron acquisition system. A) Pictorial representation of putative *P. melaninogenica* heme/hemoglobin acquisition machinery. Light pink boxes are genes encoding for putative hemoglobin receptors. The dark green oval represents heme permease and white boxes represent the *P. melaninogenica* TonB system. Purple boxes are representing ABC transporter. All locations were determined by author's discretion. B) *P. melaninogenica* genes homologous to characterized heme iron acquisition systems. ** All Amino Acid Identity comparisons are homologous to proteins in *Porphyromonas gingivalis* ATCC 33277 except for those noted with $. $ Amino acid identity comparisons are from *Porphyromonas gingivalis* W83.

A.



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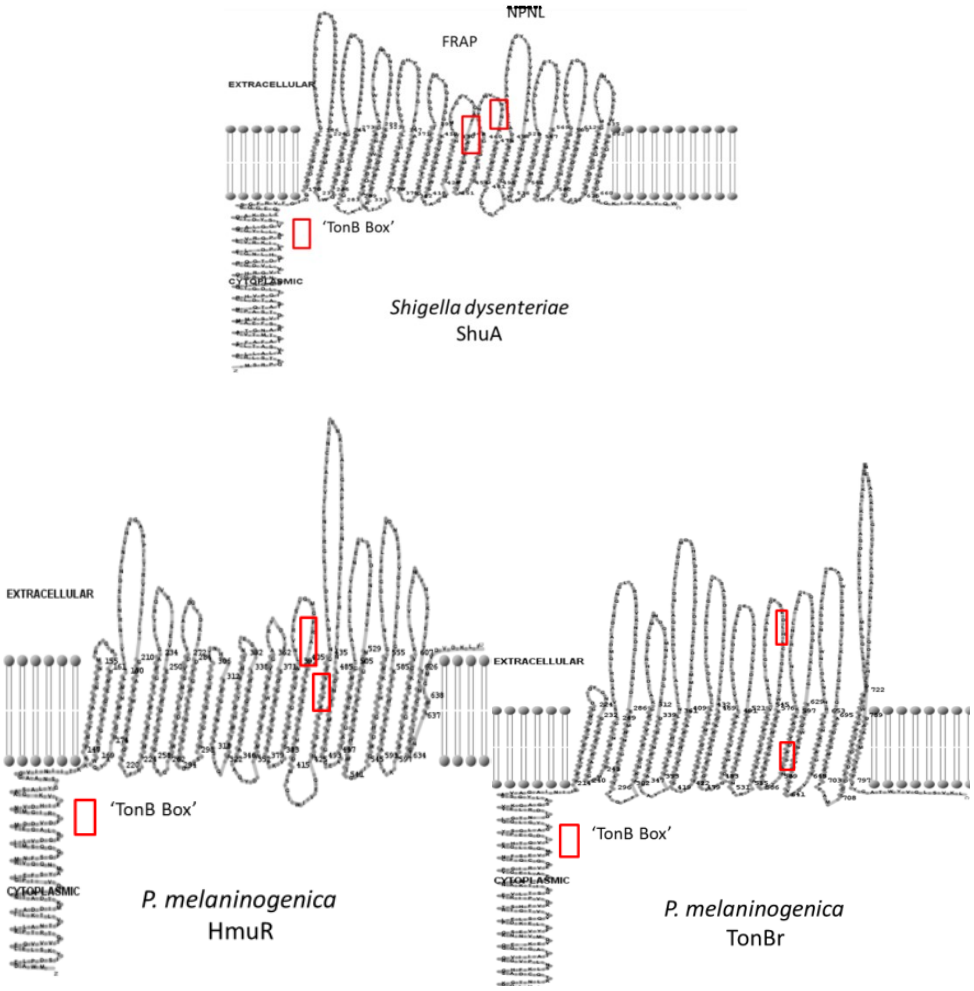
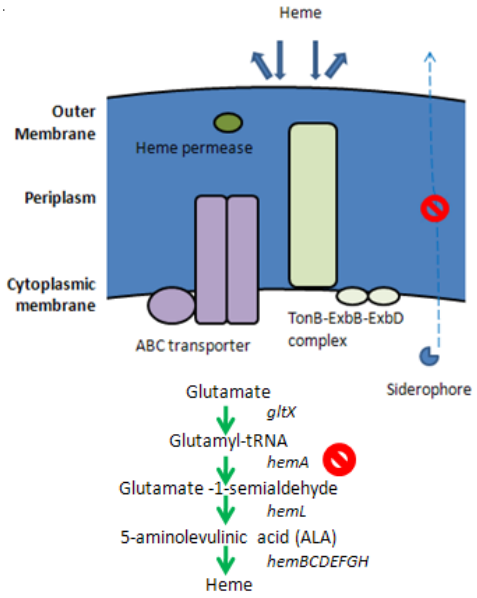


Figure 4.3 *P. melaninogenica* proteins homologous to characterized hemoglobin receptors. A) ClustalW map of BLAST-p search of *P. melaninogenica* proteins. ShuA *Shigella*. *P. gingivalis* W83 HmuR. *P. melaninogenica* TonBr and *P. melaninogenica* HmuR. Colors are dependent on amino acid residue algorithms. B) PREZ-TMD depiction of ShuA and candidate *P. melaninogenica* hemoglobin receptors. Red boxes depict TonB Box, FRAP and NPNL motifs.

A.



B.

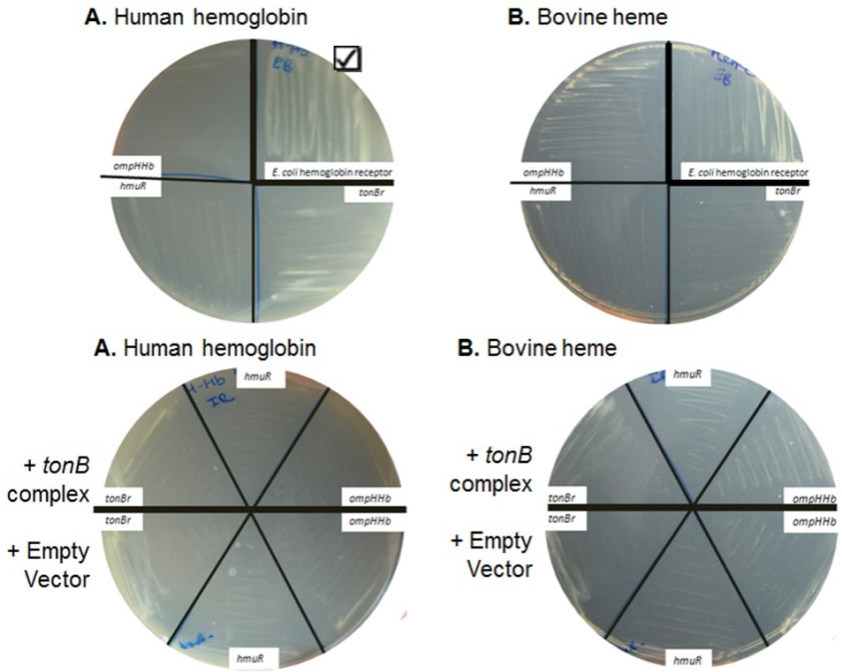
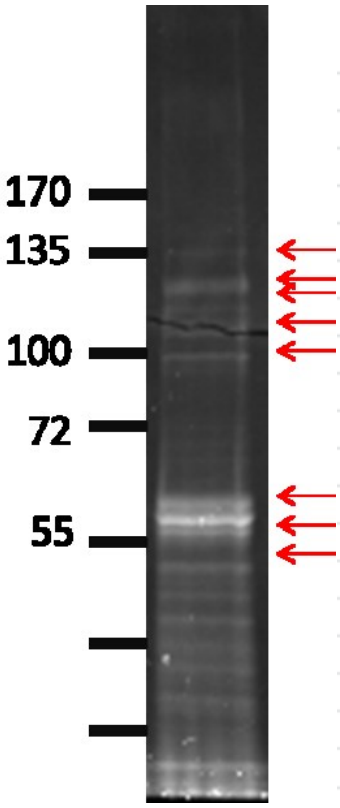


Figure 4.4: Outline of experiments with E. coli K12 hemA system. A) Schematic representation of the properties of the *E. coli hemA* system. B) Growth of *E. coli* hemoglobin receptor cloned into *hemA* system on hemoglobin and heme plates. Lack of growth of candidate *P. melaninogenica* hemoglobin receptors cloned in *E. coli* K12 *hemA* system on hemoglobin and heme plates with and without the *P. melaninogenica* TonB system shown. *P. melaninogenica* A7242 'OmpHHb'. *P. melaninogenica* A5369 'HmuR'. *P. melaninogenica* 'A6271 TonBr'.

A.

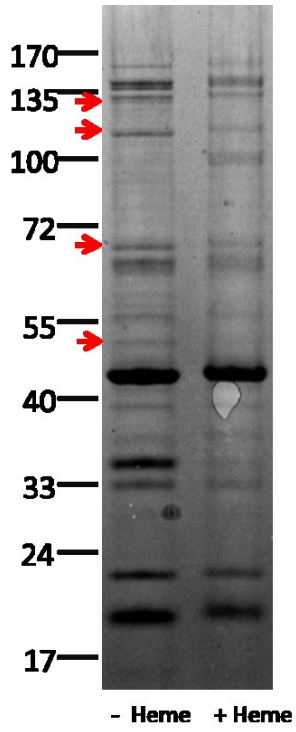


B.

Accession Number	Protein Name
HMPREF0659_A5491	putative outer membrane protein, nutrient binding
HMPREF0659_A7287	TonB-dependent receptor, plug
HMPREF0659_A5439	putative outer membrane protein, nutrient binding
HMPREF0659_A7373	TonB-dependent receptor, plug
HMPREF0659_A5179	putative RagA protein
HMPREF0659_A7294	TonB-dependent receptor, plug
HMPREF0659_A6276	TonB-dependent receptor
HMPREF0659_A5313	translation elongation factor G
HMPREF0659_A7121	conserved hypothetical protein
HMPREF0659_A6587	TonB-dependent receptor domain protein
HMPREF0659_A7002	putative liporotein
HMPREF0659_A7001	conserved hypothetical protein
HMPREF0659_A6371	phosphoenolpyruvate carboxykinase
HMPREF0659_A7001	conserved hypothetical protein
HMPREF0659_A7002	putative liporotein
HMPREF0659_A6689	conserved hypothetical protein
HMPREF0659_A6004	peptidase, M20/M25/M40 family
HMPREF0659_A5274	conserved hypothetical protein
HMPREF0659_A6366	putative outer membrane protein

Figure 4.5: Functional assessment of *P. melaninogenica* proteins using hemoglobin agarose. A) Hemoglobin agarose pull down of *P. melaninogenica* proteins stained with Sypro ruby on gradient gel. Red arrows indicate gel excised proteins B) Mass-spec data assignment of gel excised hemoglobin binding proteins.

A.



B.

Gene Name	NCBI
HMPREF0659_A7294	TonB-dependent receptor, plug
HMPREF0659_A7131	conserved hypothetical protein
HMPREF0659_A5274	conserved hypothetical protein
HMPREF0659_A6275	putative outer membrane protein, nutrient binding
HMPREF0659_A6689	conserved hypothetical protein
HMPREF0659_A7292	glycoside hydrolase family 18

C.

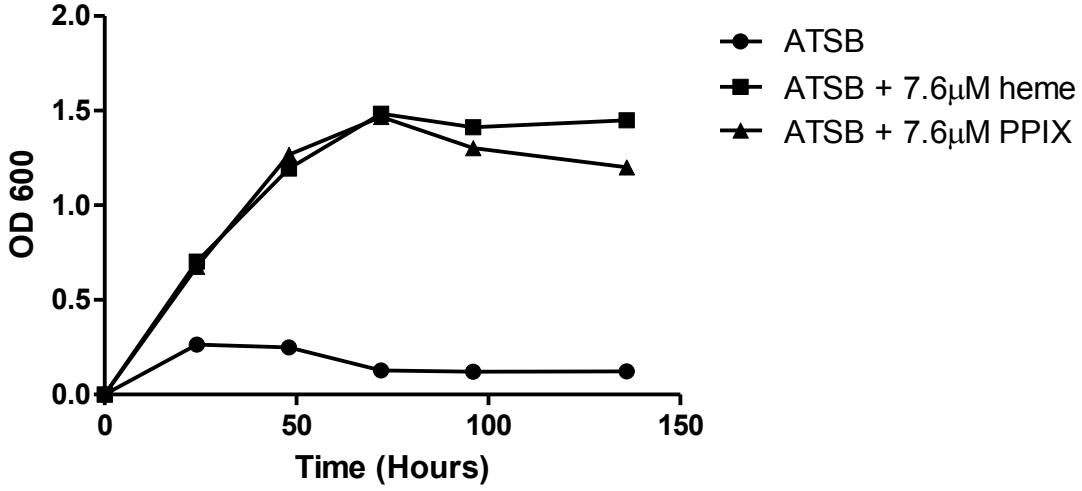
Induced under heme deplete conditions (0μM)	
HMPREF0659_A6587	TonB-dependent receptor
HMPREF0659_A5339	30S ribosomal protein S13
Induced under normal heme conditions (7.6μM)	
HMPREF0659_A5477	efflux transporter, outer membrane factor lipoprotein, NodT family
HMPREF0659_A6032	ribosomal protein L25, Ctc-form

Figure 4.6 Characterization of heme regulated *P. melaninogenica* OMPs. A) Gradient gel electrophoresis of *P. melaninogenica* grown in heme depleted and heme repeat conditions. B) Results from excised gel bands noted with red arrow. C) iTRAQ results from *P. melaninogenica* grown in heme depleted and heme repeat conditions.

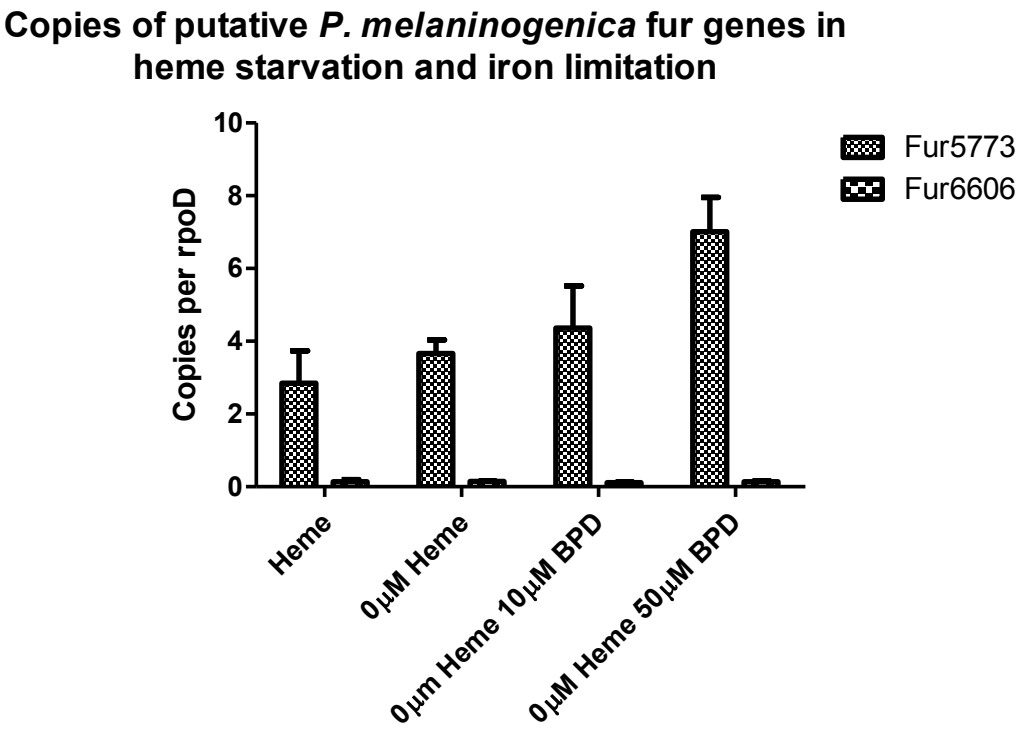
Gene Name	Method (Date when ID)	Gene description
HMPREF0659_A7294	OMP heme -+ (7/17/11) Hb agarose (4/18/11)	TonB-dependent receptor, plug
HMPREF0659_A7001	Hb agarose (4/18/11) w/ Western OMP	conserved hypothetical protein
HMPREF0659_A6689	OMP heme -+ (7/17/11) Hb agarose (4/18/11)	conserved hypothetical protein- OM no prediction
HMPREF0659_A6587	Hb agarose (4/18/11) w/ Western OMP iTRAQ (7/11/11)	TonB-dependent receptor domain protein
HMPREF0659_A6271	Homology to hemoglobin receptor	TonBr' TonB-dependent receptor plug domain protein
HMPREF0659_A5369	Homology to Pg HmuR	HmuR' TonB-dependent receptor
HMPREF0659_A5274	OMP heme -+ (7/17/11) Hb agarose (4/18/11)	conserved hypothetical protein Opacity family porins OmpA Neisseria
Pg W83 HmuR	Olczak papers	
Ec UTI C1129	Complementation of *E. coli K12 hemA* strain/ homology with *Shigella* ShuA	

Table 4. 2 Summary of *P. melaninogenica* candidate hemoglobin receptors. Four green highlighted genes where represented in two or more methods.

Supplementary Data:



Supplementary Figure 4.1S: *P. melaninogenica* can use heme and PPIX for growth in ATSB. The experiment was completed twice with similar results.



Supplementary Figure 4.2S: Two fur genes differentially regulated by iron. A) RNA was collected from four *P. melaninogenica* growth conditions and RTPCR was done showing that Fur5773 is regulated by iron availability. Experiment done once with three replicates. Standard deviation graphed.

During the mining of *P. melaninogenica* for heme/hemoglobin acquisition proteins, two putative fur proteins were found. HMPREF0659_A6606 is a transcriptional regulator in the Fur family located on chromosome 2, with 27% homology with *P. gingivalis* W83 Fur protein and 22% homology to *E. coli* O157:H7 strain. HMPREF0659_A5773, a transcriptional regulator in the Fur family, is on chromosome 1, with 28% *P. gingivalis* W83 Fur protein and 29% homology to *E. coli* O157:H7 strain. Comparing the two proteins Identities = 38/130 (29%), 8e-12. RT primers were then made and tested with various heme and chelated conditions. Growth conditions were done in a similar manor as growth curves with the exception of the addition of BPD. The heme condition (7.6μM) represents a standard baseline condition for optimal growth of *P. melaninogenica*. Noheme+10BPD is represented as a low level stress (50BPD=high). Only A5773 showed regulation by heme limitation and iron chelation.

References

1. Haraldsson G, Meurman JH, Könönen E, Holbrook WP. Properties of hemagglutination by *Prevotella melaninogenica*. Anaerobe. 2005 10;11(5):285-9.

2. Talan DA, Abrahamian FM, Moran GJ, Citron DM, Tan JO, Goldstein EJ, et al. Clinical presentation and bacteriologic analysis of infected human bites in patients presenting to emergency departments. Clin Infect Dis. 2003 Dec 1;37(11):1481-9.

3. Botta GA, Arzese A, Minisini R, Trani G. Role of structural and extracellular virulence factors in gram-negative anaerobic bacteria. Clin Infect Dis. 1994 May;18 Suppl 4:S260-4.

4. Allison HE, Hillman JD. Cloning and characterization of a *Prevotella melaninogenica* hemolysin. Infect Immun. 1997;65(7):2765-71.

5. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2004 Nov;42(11):5176-83.

6. Robert R, Grollier G, Frat JP, Godet C, Adoun M, Fauchere JL, et al. Colonization of lower respiratory tract with anaerobic bacteria in mechanically ventilated patients. Intensive Care Med. 2003 Jul;29(7):1062-8.

7. Falagas ME, Siakavellas E. *Bacteroides, Prevotella,* and *Porphyromonas* species: A review of antibiotic resistance and therapeutic options. Int J Antimicrob Agents. 2000 Jun;15(1):1-9.

8. Mukhopadhyay S, Rose F, Frechette V. Vertebral osteomyelitis caused by *Prevotella (Bacteroides) melaninogenicus.* South Med J. 2005 Feb;98(2):226-8.

9. Odeh M, Oliven A, Potasman I, Solomon H, Srugo I. Pyomyositis of the thigh due to *Prevotella melaninogenica*. Infection. 2000 Jan-Feb;28(1):49-50.

10. Athanasiou S, Matthaiou DK, Falagas ME. Vaginal mesh infection due to *Bacteroides melaninogenicus*: A case report of another emerging foreign body related infection. Scand J Infect Dis. 2006;38(11-12):1108-10.

11. Harding GK, Sutter VL, Finegold SM, Bricknell KS. Characterization of *Bacteroides melaninogenicus.* J Clin Microbiol. 1976 Oct;4(4):354-9.

12. De A, Varaiya A, Mathur M. Anaerobes in pleuropulmonary infections. Indian J Med Microbiol. 2002 Jul-Sep;20(3):150-2.

13. Brook I. *Prevotella* and *Porphyromonas* infections in children. J Med Microbiol. 1995 May;42(5):340-7.

14. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med. 2008 May 1;177(9):995-1001.

15. van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME journal. 2010;5(5):780-91.

16. Sibley CD, Rabin H, Surette MG. Cystic fibrosis: A polymicrobial infectious disease. Future microbiology. 2006;1(1):53-61.

17. Rogers G, Hart C, Mason J, Hughes M, Walshaw M, Bruce K. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003;41(8):3548-5.

18. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proc Natl Acad Sci U S A. 2007 Dec 18;104(51):20529-33.

19. Kolak M, Karpati F, Monstein HJ, Jonasson J. Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. Int J Med Microbiol. 2003 Aug;293(4):309-17.

20. Field TR, Sibley CD, Parkins MD, Rabin HR, Surette MG. The genus *Prevotella* in cystic fibrosis airways. Anaerobe. 2010;16(4):337-44.

21. Filkins L, Hampton T, Gifford A, Gross M, Hogan D, Sogin M, et al. The prevalence Of *Streptococci* and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol. 2012.

22. Madan J, Koestler D, Stanton B, Davidson L, Moulton L, Housman M, et al. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: Interaction between intestinal and respiratory tracts and impact of nutritional exposures. mBio. 2012;3(4).

23. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest. 2002;109(3):317-25.

24. Boucher R. New concepts of the pathogenesis of cystic fibrosis lung disease. European Respiratory Journal. 2004;23(1):146-58.

25. Lamont IL, Konings AF, Reid DW. Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. Biometals. 2009;22(1):53-60.

26. Nakano MM, Zuber PA. Strict and facultative anaerobes: Medical and environmental aspects. Wymondham : Horizon Bioscience, c2004.; 2004.

27. Reid DW, Anderson GJ, Lamont IL. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;297(5):L795-802.

28. Skaar EP. A precious metal heist. Cell Host & Microbe. 2009;5(5):422-4.

29. Cosgrove S, Chotirmall SH, Greene CM, McElvaney NG. Pulmonary proteases in the cystic fibrosis lung induce interleukin 8 expression from bronchial epithelial cells via a heme/meprin/epidermal growth factor receptor/toll-like receptor pathway. J Biol Chem. 2011;286(9):7692-704.

30. Ghio AJ, Roggli VL, Soukup JM, Richards JH, Randell SH, Muhlebach MS. Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients. Journal of Cystic Fibrosis. 2012.

31. Stojiljkovic I, Perkins-Balding D. Processing of heme and heme-containing proteins by bacteria. DNA Cell Biol. 2002;21(4):281-95.

32. Gaudin CFM, Grigg JC, Arrieta AL, Murphy MEP. Unique heme-iron coordination by the hemoglobin receptor IsdB of *Staphylococcus aureus*. Biochemistry (N Y). 2011.

33. Smalley JW, Byrne DP, Birss AJ, Wojtowicz H, Sroka A, Potempa J, et al. HmuY haemophore and gingipain proteases constitute a unique syntrophic system of haem acquisition by *Porphyromonas gingivalis*. PloS one. 2011;6(2):e17182.

34. Cobessi D, Meksem A, Brillet K. Structure of the heme/hemoglobin outer membrane receptor ShuA from *Shigella dysenteriae*: Heme binding by an induced fit mechanism. Proteins. 2010 Feb 1;78(2):286-94.

35. Byrne DP, Wawrzonek K, Jaworska A, Birss AJ, Potempa J, Smalley JW. Role of the cysteine protease interpain A of *Prevotella intermedia* in breakdown and release of haem from haemoglobin. Biochem J. 2009;425(1):257.

36. Krewulak KD, Vogel HJ. TonB or not TonB: Is that the question? Biochemistry and Cell Biology. 2011;89(2):87-9.

37. Mills M, Payne SM. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an analogous system in *Escherichia coli* O157: H7. J Bacteriol. 1995;177(11):3004-9.

38. Simpson W, Olczak T, Genco CA. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of *Porphyromonas gingivalis*. J Bacteriol. 2000;182(20):5737-48.

39. Bagg A, Neilands J. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry (N Y). 1987;26(17):5471-7.

40. Carpenter BM, Whitmire JM, Merrell DS. This is not your mother's repressor: The complex role of fur in pathogenesis. Infect Immun. 2009;77(7):2590-601.

41. Létoffé S, Heuck G, Delepelaire P, Lange N, Wandersman C. Bacteria capture iron from heme by keeping tetrapyrrol skeleton intact. Proceedings of the National Academy of Sciences. 2009;106(28):11719-24.

42. Wandersman C, Delepelaire P. Bacterial iron sources: From siderophores to hemophores. Annu Rev Microbiol. 2004;58:611-47.

43. Guan SM, Nagata H, Shizukuishi S, Wu JZ. Degradation of human hemoglobin by *Prevotella intermedia.* Anaerobe. 2006;12(5-6):279-82.

44. Olczak T, Simpson W, Liu X, Genco CA. Iron and heme utilization in *Porphyromonas gingivalis*. FEMS Microbiol Rev. 2005;29(1):119-44.

45. Smalley JW, Silver J, Birss AJ, Withnall R, Titler PJ. The haem pigment of the oral anaerobes *Prevotella nigrescens* and *Prevotella intermedia* is composed of iron (III) protoporphyrin IX in the monomeric form. Microbiology. 2003;149(7):1711-8.

46. Dashper SG, Ang CS, Veith PD, Mitchell HL, Lo AWH, Seers CA, et al. Response of *Porphyromonas gingivalis* to heme limitation in continuous culture. J Bacteriol. 2009;191(3):1044-55.

47. Elkins C, Totten PA, Olsen B, Thomas CE. Role of the *Haemophilus ducreyi* ton system in internalization of heme from hemoglobin. Infect Immun. 1998;66(1):151-60.

48. Leung KP, Folk SP. Effects of porphyrins and inorganic iron on the growth of *Prevotella intermedia*. FEMS Microbiol Lett. 2002 Mar 19;209(1):15-21.

49. Gibbons RJ, Macdonald JB. Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J Bacteriol. 1960;80(2):164-70.

50. Reinhardt JA, Baltrus DA, Nishimura MT, Jeck WR, Jones CD, Dangl JL. De novo assembly using low-coverage short read sequence data from the rice pathogen *Pseudomonas syringae pv. oryzae.* Genome Res. 2009;19(2):294-305.

51. Altschul SF, Gertz EM, Agarwala R, Schaffer AA, Yu YK. PSI-BLAST pseudocounts and the minimum description length principle. Nucleic Acids Res. 2009 Feb;37(3):815-24.

52. Align sequences using ClustalW2 | EBI [Internet].; cited 1/7/2013]. Available from: http://www.ebi.ac.uk/Tools/msa/clustalw2/.

53. TMpred server [Internet].; cited 1/7/2013]. Available from: http://www.ch.embnet.org/software/TMPRED_form.html.

54. Jones AK, Fulcher NB, Balzer GJ, Urbanowski ML, Pritchett CL, Schurr MJ, et al. Activation of the *Pseudomonas aeruginosa* AlgU regulon through mucA mutation inhibits cyclic AMP/vfr signaling. J Bacteriol. 2010;192(21):5709-17.

55. Afonina G, Leduc I, Nepluev I, Jeter C, Routh P, Almond G, et al. Immunization with the *Haemophilus ducreyi* hemoglobin receptor HgbA protects against infection in the swine model of chancroid. Infect Immun. 2006;74(4):2224-32.

56. Guan SM, Nagata H, Maeda K, Kuboniwa M, Minamino N, Shizukuishi S. Purification and characterization of a hemoglobin-binding outer membrane protein of *Prevotella intermedia*. FEMS Microbiol Lett. 2004;235(2):333-9.

57. Bramanti TE, Holt SC. Roles of porphyrins and host iron transport proteins in regulation of growth of *Porphyromonas gingivalis* W50. J Bacteriol. 1991;173(22):7330-9.

58. Weber KA, Achenbach LA, Coates JD. Microorganisms pumping iron: Anaerobic microbial iron oxidation and reduction. Nature Reviews Microbiology. 2006;4(10):752-64.

59. Genome R. ***Prevotella melaninogenica* ATCC 25845**. A reference genome for the human microbiome project. 6-Aug-2010.

60. Burkhard KA, Wilks A. Characterization of the outer membrane receptor ShuA from the heme uptake system of *Shigella dysenteriae*. J Biol Chem. 2007;282(20):15126-3.

61. Wang L, Elliott M, Elliott T. Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in *Salmonella typhimurium*. J Bacteriol. 1999;181(4):1211-9.

62. Elkins C. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi.* Infect Immun. 1995;63(4):1241-5.

63. Zieske LR. A perspective on the use of iTRAQ™ reagent technology for protein complex and profiling studies. J Exp Bot. 2006;57(7):1501-8.

64. Álvarez B, Álvarez J, Menéndez A, Guijarro JA. A mutant in one of two exbD loci of a TonB system in *Flavobacterium psychrophilum* shows attenuated virulence and confers protection against cold water disease. Microbiology. 2008;154(4):1144-51.

65. Wiggerich HG, Klauke B, Köplin R, Priefer UB, Pühler A. Unusual structure of the tonB-exb DNA region of *Xanthomonas campestris pv. campestris*: tonB, exbB, and exbD1 are essential for ferric iron uptake, but exbD2 is not. J Bacteriol. 1997;179(22):7103-10.

66. van Vliet AHM, Baillon MLA, Penn CW, Ketley JM. *Campylobacter jejuni* contains two fur homologs: Characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. J Bacteriol. 1999;181(20):6371-6.

67. Bsat N, Herbig A, Casillas-Martinez L, Setlow P, Helmann JD. *Bacillus subtilis* contains multiple fur homologues: Identification of the iron uptake (fur) and peroxide regulon (PerR) repressors. Mol Microbiol. 1998;29(1):189-98.

68. Genco C. Regulation of hemin and iron transport in *Porphyromonas gingivalis*. Adv Dent Res. 1995;9(1):41-7.

69. Ghigo JM, Letoffe S, Wandersman C. A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in *Escherichia coli*. J Bacteriol. 1997;179(11):3572-9.

Chapter 5

Conclusion

Cystic fibrosis lung disease

Cystic fibrosis (CF) is the most common fatal genetic disease affecting the Caucasian population, with more than 30,000 individuals with CF in the United States (1). The prevailing phenotype in CF is a defect in mucociliary clearance of the airways and the accumulation of dehydrated viscous mucus, which creates an optimal niche for bacterial colonization. In CF, chronic bacterial pulmonary infection and recurring episodes of acute pulmonary exacerbation produce an irreversible decline in lung function that ultimately leads to respiratory failure and death. While life expectancy has improved, it is still only 37 years (2). Recently, our understanding of the microbial landscape of the CF airway has expanded to include a more diverse polymicrobial community of species. The contribution of these non-traditional pathogens to CF airway infection and disease progression is unknown.

CF Microbiome Studies

In the past 5 years, our understanding of CF pathogenesis has dramatically expanded with the application of high throughput sequencing, which has redefined CF airway microbiology. Historically, clinical bacterial culture in CF has focused on the frequency, abundance and antimicrobial susceptibility of aerobic pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa.*

During childhood, *S. aureus* and *H. influenzae* are isolated in high abundance and these are gradually replaced by *P. aeruginosa* and *Burkholderia cepacia* during adolescence and adulthood (3, 4). While these observations still hold true, more recent metagenomic studies estimate the presence of more than 60 different bacterial genera by deep sequencing and over 43 different phyla by 16S ribosomal RNA (rRNA) gene-based phylogenetic microarrays (5). The most abundant genera commonly sampled are *Pseudomonas*, *Streptococcus*, *Fusobacterium*, and *Prevotella.* As technology has advanced, lesser known facultative and strict anaerobic organisms associated with the oral cavity have been detected; however, their role in CF pathogenesis is unclear.

Key Questions about CF

Despite the apparent polymicrobial nature of CF, treatment decisions are still largely based on the identification of traditional CF pathogens (*P. aeruginosa /S. aureus)* and antibiotics with broad spectrum activity against Gram-negative and Gram-positive bacteria are used. Unfortunately, chronic antibiotic treatment management has become the norm (1). Chronic bacterial infection throughout life produces a new baseline of 'tolerable' and manageable colonization and forces clinicians into individualized management of symptoms. Antibiotic management of exacerbation status, a state when there is a sudden onset of worsening lung function, appears to minimally impact species diversity and microbial community structure, yet patients respond clinically and show improved lung function (5). This

disconnect reveals the complexity of CF microbiology and highlights the need for a deeper understanding of the CF airway microbiota.

Are oral anaerobes in CF samples contamination or are they colonizing microbes in the CF lung?

Most respiratory specimens are collected through the oropharynx except retrieval of bacteria from explanted lung samples. Recently, two studies have described the presence of oral anaerobes as less abundant in explanted lungs than others who have cultured oral anaerobes from sputum (6, 7). Both of these studies, though very thorough, sampled end stage and/or deceased patients when bacterial diversity has been shown to be significantly reduced. However, these studies call into question the reliability of microbiome studies that use samples that have passed through the oral cavity. Yet, aspiration into the respiratory tract from the oral cavity is expected and may contribute to colonization of the lower airways (5, 8). Furthermore, it is possible that the oral cavity could serve as a reservoir for pulmonary infection in CF (4, 9).

Most studies reporting a high abundance of anaerobes in the lungs of CF patients have been based on analysis of spontaneous expectorated sputum (5, 8-25). These studies represent the traditional method of evaluating microbial diversity in the lungs. Comparison of concomitant mouthwash samples to lung sputum samples show similar species but the abundance of anaerobes in lung samples points to colonization verses contamination (26). Additionally, anaerobic species have been cultured consecutively in longitudinal studies, demonstrating a constant

presence of anaerobes in the lungs (5, 22). While contamination is unavoidable in expectorated sputum samples, studies have shown a similar pattern of oral bacterial species in bronchoalveolar lavage fluid (BALF), obtained directly from the lower airways (17, 27).

With the demonstration of steep oxygen gradients in CF mucus plugs, the credibility of the presence of strict anaerobes has begun to increase (28). Anaerobes, specifically oral-related anaerobes, have been identified in numerous studies (5, 8-25, 27) with the most abundant member being *Prevotella melaninogenica* (8, 11, 15, 17, 18, 22, 23, 27). In chapter 2, we demonstrate, in a UNC cohort, that *P. melaninogenica* was present in 19 of 28 adult sputum samples and 19 out of 33 samples from pediatric patients, representing the most abundant and frequent strict anaerobe. Furthermore independent of culture results, we show an increase in antibody response to *P. melaninogenica* in CF patients, even in young CF children, compared to non-disease controls (Chapter 2). In this context, antibody response points to an increased exposure or presence of *P. melaninogenica* that is occurring outside the oral cavity.

What is the timing of microbial development in CF?

In CF, the loss of normal clearance mechanisms results in extensive mucus accumulation on the apical surface of the airways providing an ideal environment for bacterial colonization (19, 29). The presence of bacteria in the CF lung can occur as early as two months of age (10). Using oropharyngeal swab samples, a time-dependent development of CF airway microflora, beginning with *Streptococcus,*

Veillonella, and *Prevotella* has been reported for pediatric patients (10). It is unclear whether these bacteria truly colonize the lung or are just aspirated and cleared. In chapter 2, we demonstrated a similar spectrum of anaerobic species from patients (as young as 1 year old) with *P. melaninogenica* being the most prevalent and abundant strict anaerobe.

Early bacterial colonization is corroborated by porcine studies (CFTR−/− genotype) that show an impact of defective mucociliary clearance in a matter of hours after birth with impaired bacterial elimination occurring in pigs as young as 8 to 10 hours postpartum (30). An influx of neutrophils and increased inflammatory cytokines is seen in CF pediatric patients compared to normal controls and other chronic respiratory disease patients (31-33), consistent with early bacterial exposure.

Bacterial colonization could contribute to these already exaggerated CF dependent inflammatory responses. In chapter 3, we demonstrate that *P. aeruginosa*, the classic pathogen associated with CF, can produce a robust LPS dependent IL-8 response corroborating previous studies (34). In contrast, we demonstrated that *P. melaninogenica* LPS produces a low IL-8 and an undetectable IL-1β response in the same cell line. These results suggest that the contribution of *P. melaninogenica* to the overall inflammatory response seen in CF may be minimal.

As the patient ages, a diverse polymicrobial infection becomes dominated by *P. aeruginosa* and lung function decreases eventually leading to respiratory failure (8, 35, 36). During the transition between acute and chronic infection, *P. aeruginosa* isolates acquire mutations causing them to overproduce an alginate

exopolysaccharide, a process referred to as mucoid conversion (37, 38). There is direct evidence that *P. aeruginosa* are enmeshed in alginate and form biofilm aggregates within luminal mucus plugs (39). Inside mucus plugs, low oxygen and in some cases anaerobic conditions have been measured (28). The presence of anaerobes has been correlated with the presence of *P. aeruginosa* in several studies suggesting a potential benefit for each bacterial species in the development of the microbial niche (17, 19). In chapter 2, we also demonstrate a positive correlation of antibody response against *P. aeruginosa* and *P. melaninogenica* in children and adults.

Outside of the mucus plugs, cellular debris and neutrophils contribute to the nutrient rich inflammatory environment. Elevated iron in the form of ferritin, lactoferrin, heme and hemoglobin has been observed in the lumen of CF patients compared to healthy individuals and disease controls (40, 41). Work in chapter 4 focuses on the ability of *P. melaninogenica* to use available host based iron sources for growth, and describes potential heme acquisition systems. This combination of anaerobiosis and usable nutrients provides the 'perfect storm' for colonization by a large range of species including fastidious anaerobic bacteria.

What is the role of anaerobes in CF pathogenesis?

Fastidious organisms, not commonly cultivated by standard laboratory procedures, are now being detected in the CF lung but their role in CF airway disease remains unclear and controversial.

The *Streptococcus milleri* group (SMG)(composed of *S. constellatus, S. intermedius,* and *S. anginosus)* and oral related *Streptococcus* species *(S. salivarius and S. parasanguis)* have been detected in several studies using culture independent techniques (9, 12, 20, 42). The original motivation for investigating *Streptococcus* species was their ability to change *P. aeruginosa* pathogenicity by modulating virulence factor expression in a polymicrobial infection model (43). These studies lead Sibley and colleagues to investigate the connection between CF exacerbation and the presence of the SMG (9). By culturing samples from a small cohort of patients longitudinally, they found that abundance of the SMG correlated with exacerbation state. Later, Filkins et al., sampled 35 patients and noted the abundance of the SMG and oral related *Streptococcus* species was not associated with exacerbation. In fact, they found that the presence of *Streptococcus* species in respiratory specimens correlated with stable CF disease. The authors noted that *Streptococcus* colonization was the strongest predictor of stable disease and concluded that it was advantageous in keeping higher diversity of the patient microbiota (12). Each of these groups, one while studying a single snapshot of the lung microbiota and the other studying longitudinal samples of a few patients, came to differing conclusions leading to uncertainty about the impact of *Streptococcus* species in infection. It is important to note here that in a complex polymicrobial community such as in CF it is extremely difficult if not impossible to assign cause and effect. Each bacteria or community of bacteria can have a clear association with health or disease state but that does not equate to cause (4, 44, 45).

The diversity of microbes present in the CF microbiome appears to be beneficial to lung health if total bacterial eradication is not an option (5, 12). Bacterial 'space holders' taking the place of a traditional pathogen could represent a more favorable environment than a chronic *P. aeruginosa* infection. Even though there is not a correlation of *P. melaninogenica* with health or disease stability (Chapter 2), we hypothesize that community composition rather than total bacterial burden may impact disease state (12). With the identification of lesser-known species in high abundance in CF airways, mechanistic studies are sorely needed to elucidating the contribution of these organisms to disease pathogenesis.

Anaerobic species have been detected in many studies of the CF microbiome (5, 8-25, 27) and have the potential to effect the pathogenesis of CF. *P. melaninogenica*, the subject of this dissertation, was the most frequently associated strict anaerobic species in CF in both adults and pediatric patients (Chapter 2). Oral species occupying the CF niche could lead to nutrient fluctuation and depravation in the environment causing a regulatory change in the primary pathogen (9, 43). Duan et al. shows that in the presence of an oral commensal, *P. aeruginosa* virulence can be greatly enhanced or reduced depending on the commensal present. In a mouse model of pneumonia, the addition of a *Prevotella* species with *Streptococcus constellatus* caused a 6 fold increase in mortality of the mouse (46). Additionally, the presence of anaerobes could preserve a diverse multispecies community in order to delay or prevent the dominance of *P. aeruginosa* in the CF lung.

It remains unclear as to whether anaerobes are detrimental or beneficial in the context of CF airway infection and disease progression. Investigating the role of

P. melaninogenica in the CF lung in chapter 2, we found no significant correlation with the presence (CFU/ml) or IgG response to *P. melaninogenica* with clinical measurements of lung function. The clinical relevance of *P. melaninogenica* or other oral anaerobic bacteria must be assessed by clinical trials that treat aerobic bacteria compared to treatment regimes that combine treatment towards anaerobic and aerobic bacteria (18).

Conclusions

We have shown that oral anaerobes are present in samples collected from pediatric and adult CF patients. Exposure to oral anaerobes, specifically *P. melaninogenica*, is corroborated by our studies investigating *P. melaninogenica* reactive antibody response. We show that CF patients, both pediatric and adult, demonstrate a higher antibody response than non-diseased and healthy control groups. Initial studies investigating *P. melaninogenica* LPS illustrate that *P. melaninogenica* could minimally contribute to the overall increased cytokine response seen in CF patients. Additionally, within the CF lung, we establish that *P. melaninogenica* has the ability to survive and growth with available host iron proteins demonstrated to be in high abundance in the CF lung. The presence of oral anaerobes in our studies is undeniable but their role in CF infection remains to be determined.

In summary, I believe that early aspiration of oral commensals into the lungs of pediatric CF patients leads to initial exposure to oral bacteria. As the anaerobic niche develops, transient exposure of anaerobes leads to stable colonization. These

anaerobic and highly nutrient rich mucus plug likely house anaerobic bacteria until colonization of *P. aeruginosa* becomes predominant. Chronic *P. aeruginosa* infection then dominates the CF landscape leading to a decrease in microbial diversity, overwhelming inflammation, lung dysfunction and respiratory failure. Treatments that simultaneously tip the balance from an inflamed and infected to hydrated and healthy airway surface are desperately needed to circumvent the progression of CF disease into life threatening chronic bacterial infection.

References

1. Ratjen F, Döring G. Cystic fibrosis. The Lancet. 2003 2/22;361(9358):681-9.

2. LiPuma JJ. The changing microbial epidemiology in cystic fibrosis. Clin Microbiol Rev. 2010;23(2):299-323.

3. Razvi S, Quittell L, Sewall A, Quinton H, Marshall B, Saiman L. Respiratory microbiology of patients with cystic fibrosis in the united states, 1995 to 2005. CHEST Journal. 2009;136(6):1554-60.

4. Nelson A, De Soyza A, Perry JD, Sutcliffe IC, Cummings SP. Polymicrobial challenges to Koch's postulates: Ecological lessons from the bacterial vaginosis and cystic fibrosis microbiomes. Innate Immunity. 2012;18(5):774-83.

5. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLOS ONE. 2012;7(9):e45001.

6. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proceedings of the National Academy of Sciences. 2012;109(34):13769-74.

7. Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, et al. Spatial distribution of microbial communities in the cystic fibrosis lung. The ISME Journal. 2011.

8. van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME journal. 2010;5(5):780-91.

9. Sibley CD, Duan K, Fischer C, Parkins MD, Storey DG, Rabin HR, et al. Discerning the complexity of community interactions using a drosophila model of polymicrobial infections. PLoS pathogens. 2008;4(10):e1000184.

10. Madan J, Koestler D, Stanton B, Davidson L, Moulton L, Housman M, et al. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: Interaction between intestinal and respiratory tracts and impact of nutritional exposures. mBio. 2012;3(4).

11. Stressmann FA, Rogers GB, Klem ER, Lilley AK, Donaldson SH, Daniels TW, et al. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. J Clin Microbiol. 2011;49(1):281-9.

12. Filkins L, Hampton T, Gifford A, Gross M, Hogan D, Sogin M, et al. The prevalence of *Streptococci* and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol. 2012.

13. Twomey KB, O'Connell OJ, McCarthy Y, Dow JM, O'Toole GA, Plant BJ, et al. Bacterial cis-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of *Pseudomonas aeruginosa.* The ISME Journal. 2011.

14. Rogers GB, Skelton S, Serisier DJ, Van Der Gast CJ, Bruce KD. Determining cystic fibrosis-affected lung microbiology: Comparison of spontaneous and serially induced sputum samples by use of terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2010;48(1):78-86.

15. Rogers G, Carroll M, Serisier D, Hockey P, Jones G, Bruce K. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2004;42(11):5176-83.

16. Rogers G, Hart C, Mason J, Hughes M, Walshaw M, Bruce K. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003;41(8):3548-5.

17. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. American journal of respiratory and critical care medicine. 2008;177(9):995-1001.

18. Tunney M, Klem E, Fodor A, Gilpin D, Moriarty T, McGrath S, et al. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. Thorax. 2011;66(7):579-84.

19. Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: A complex fungal and bacterial Community—Implications for therapeutic management. PloS one. 2012;7(4):e36313.

20. Sibley CD, Grinwis ME, Field TR, Eshaghurshan CS, Faria MM, Dowd SE, et al. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. PloS one. 2011;6(7):e22702.

21. Field TR, Sibley CD, Parkins MD, Rabin HR, Surette MG. The genus *Prevotella* in cystic fibrosis airways. Anaerobe. 2010;16(4):337-44.

22. Worlitzsch D, Rintelen C, Böhm K, Wollschläger B, Merkel N, Borneff-Lipp M, et al. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. Clinical Microbiology and Infection. 2009;15(5):454-60.

23. Bittar F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, et al. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One. 2008;3(8):e2908.

24. Ulrich M, Beer I, Braitmaier P, Dierkes M, Kummer F, Krismer B, et al. Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis. Thorax. 2010;65(11):978-84.

25. Thomassen M, Klinger J, Badger S, Van Heeckeren D, Stern R. Cultures of thoracotomy specimens confirm usefulness of sputum cultures in cystic fibrosis. J Pediatr. 1984;104(3):352-6.

26. Rogers G, Carroll M, Serisier D, Hockey P, Jones G, Kehagia V, et al. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. J Clin Microbiol. 2006;44(7):2601-4.

27. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proceedings of the National Academy of Sciences. 2007;104(51):20529.

28. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest. 2002;109(3):317-25.

29. Peters BM, Jabra-Rizk MA, Graeme A, Costerton JW, Shirtliff ME. Polymicrobial interactions: Impact on pathogenesis and human disease. Clin Microbiol Rev. 2012;25(1):193-21.

30. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, et al. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. Science translational medicine. 2010;2(29):29ra31.

31. Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW. Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. J Infect Dis. 1997;175(3):638-47.

32. van de Weert-van Leeuwen, PB, Slieker M, Hulzebos H, Kruitwagen C, van der Ent C, Arets H. Chronic infection and inflammation affect exercise capacity in cystic fibrosis. European Respiratory Journal. 2012;39(4):893-8.

33. Bonfield TL, Konstan MW, Berger M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. J Allergy Clin Immunol. 1999;104(1):72-8.

34. Ernst RK, Eugene CY, Guo L, Lim KB, Burns JL, Hackett M, et al. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. Science. 1999;286(5444):1561-5.

35. Brumback LC, Davis SD, Kerby GS, Kloster M, Johnson R, Castile R, et al. Lung function from infancy to preschool in a cohort of children with cystic fibrosis. Eur Respir J. 2013 Jan;41(1):60-6.

36. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. Proceedings of the National Academy of Sciences. 2012;109(15):5809-14.

37. Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS pathogens. 2009;5(3):e1000354.

38. Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, Molin S, et al. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. J Med Microbiol. 2004;53(7):679-90.

39. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. 2009;44(6):547-58.

40. Ghio AJ, Roggli VL, Soukup JM, Richards JH, Randell SH, Muhlebach MS. Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients. Journal of Cystic Fibrosis. 2012.

41. Lamont IL, Konings AF, Reid DW. Iron acquisition by pseudomonas aeruginosa in the lungs of patients with cystic fibrosis. Biometals. 2009;22(1):53-60.

42. Parkins MD, Sibley CD, Surette MG, Rabin HR. The *Streptococcus milleri* group—an unrecognized cause of disease in cystic fibrosis: A case series and literature review. Pediatr Pulmonol. 2008;43(5):490-7.

43. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol Microbiol. 2003;50(5):1477-91.

44. Hauser AR, Jain M, Bar-Meir M, McColley SA. Clinical significance of microbial infection and adaptation in cystic fibrosis. Clin Microbiol Rev. 2011;24(1):29-70.

45. Bjarnsholt T, Nielsen XC, Johansen U, Nørgaard L, Høiby N. Methods to classify bacterial pathogens in cystic fibrosis. Cystic fibrosis: diagnosis and protocols, vol.II.Methods and resources to understand cystic fibrosis. 2011:143-71.

46. Shinzato T, Saito A. A mechanism of pathogenicity of *Streptococcus milleri* group" in pulmonary infection: Synergy with an anaerobe. J Med Microbiol. 1994;40(2):118-23.