LONG-TERM STUDY OF CHANGES IN MICROBIOTA IN A CYSTIC FIBROSIS PATIENT

by

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ABSTRACT

JOSHUA ROBERT STOKELL. Long-term study of changes in microbiota in a Cystic Fibrosis patient. (Under the direction of DR. TODD R. STECK)

Application of culture-independent techniques have revealed the presence of more types of bacteria than were previously thought [1-8], which led to the current description of Cystic Fibrosis (CF) being a polymicrobial disease [9]. We know this polymicrobial community changes over time [10-13] and during exacerbation events [14], and that interactions with non-pathogenic taxa can influence pathogen gene expression [15]. The polymicrobial nature of infection may explain why in vitro responses and susceptibility of bacteria such as *Pseudomonas aeruginosa* to antibiotics do not always correlate with *in vivo* outcomes [16, 17]. The ability of bacteria to adapt to the CF lung complicates long-term treatment strategies. Much is known about the involvement of *P. aeruginosa* in lung colonization and deterioration [18], and the genetic adaptations it undergoes over time [19-21]. Less is known about the adaptations that enable another CF pathogen, *Burkholderia multivorans,* to become resistant to antibiotics and persist in the lung environment. We identified a *B. multivorans* strain that acquired resistance *in vivo* to an antibiotic and became the dominant strain within a period of four days.

Expectorated sputum samples are the gold standard for identifying the pathogens present in the CF lungs. Sputum in CF is primarily composed of free DNA from host immune cells and bacterial cells which is markedly different from the normal mucus that lines the lung epithelia. This composition, along with the dehydrated nature of sputum, increases viscosity and heterogeneity of bacterial distribution. Culture-independent assays which examine bacterial diversity and abundance in sputum rely on bacterial DNA

extracted from aliquots which may not be representative of the whole sample. Sputum is typically homogenized through chemical means prior to DNA extraction but we have shown that adding a mechanical homogenization step significantly increases bacterial distribution within a sputum sample.

Acute bacterial infections are the major cause for pulmonary exacerbations (PE) in Cystic Fibrosis. PEs are connected to increased mortality and may result in a permanent impairment in lung function. Attempts at developing tools to predict an oncoming PE have been met with limited success due to the heterogeneity of patient characteristics. We analyzed bacterial DNA from 130 sputum samples collected weekly for three years to identify changes in bacterial diversity and abundance by combining frequent patient sampling, next generation sequencing, and quantitative PCR (qPCR). Approximately 81,000,000 sequences containing 150 taxa were identified. Changes in microbial diversity and abundance did not correlate to antibiotic treatment for a PE. A gradual increase in abundance of all bacteria, *Pseudomonas*, and *Burkholderia* was shown over the sampling period along with a gradual decline in lung function. Ours is the first to demonstrate a stable microbial diversity coupled with a gradual change in abundance of all bacteria, *Pseudomonas*, and *Burkholderia* over a multi-year period.

Regardless of the specific goal, it is clear that to understand CF infections requires knowledge of more than the dominant pathogen [22-24]. The data described in this dissertation demonstrate the importance of repeated, longitudinal sampling for studying microbial communities in human subjects [25] where some variation in microbial community composition can occur, even between sequential samples from a single clinically stable patient [26].

INTRODUCTION

The chapters in this dissertation reflect how my project changed over time as well as demonstrate how unexpected observations lead to interesting and publishable tangential projects. Except for chapter 1, each chapter in this dissertation is included as either a format of a published manuscript (chapters 2 & 4) or in preparation for submission (chapters 3, 5, & 6).

This dissertation was written based on a culmination around a central project in which I studied the changes in diversity and abundance of microbiota in the lungs of a single Cystic Fibrosis (CF) patient as described in chapter 1. Chapter 2 is a co-authored, published review on the viable but nonculturable (VBNC) state of bacteria which I co-wrote with my advisor, Dr. Todd Steck [1]. Chapters 3 & 4 in this dissertation were written based on data from two projects (examining the effect of mechanical homogenization on sputum and *in vivo* acquisition of an antibiotic resistant *Burkholderia multivorans* strain) that developed from observations made during the course of our primary study (Chapters 5 & 6).

Chapter 3 examines whether adding a mechanical homogenization step during sputum processing decreases the difference in means of bacterial counts obtained between sputum aliquots that are removed from the same sample. My goal was to determine if the difference in abundance I measured between adjacently collected sputum samples (the data from which formed the basis for results presented in chapter 5) was likely due to factors not associated with sputum processing such as unpredictability of where in the lung the sputum is derived.

During this long-term study, the CF patient I was following acquired a ceftazidime-resistant strain of *B. multivorans* as determined by clinical culture results. Since I had been collecting sputum twice weekly, I recognized I would be able to examine previously collected samples to ascertain the time of acquisition in effort to determine what environmental factor, if any, may have caused the resistance to occur. Chapter 4 is a published manuscript which describes the methods I used to narrow the time frame for acquisition of resistance and the potential impact this result had on my overall study [2].

The last two chapters, 5 & 6, describe the results of my three year study of a CF patient. To the best of my knowledge, no other study has examined the lung microbiota of a single patient with as many sputum samples and over such a long period of time. I was able to show the benefits of using both quantitative PCR and Illumina sequencing to show the changes in abundance and diversity of bacteria over time and in response to antibiotics in the CF lung.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
Bcc	*Burkholderia cepacia* complex
BCSA	*Burkholderia cepacia* selective agar
BCSB	*Burkholderia cepacia* selective broth
BLAST	basic local alignment search tool
bp	base pair
CDI	contact-dependent inhibition
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator
CFU	colony forming unit
CH	chemical homogenization
CT	cycle threshold
dsDNA	double-stranded DNA
DTT	dithiotreitol
EMA	ethidium monoazide
FCM	flow cytometry
FEV1	forced expiratory volume in one second
GMO	genetically modified organism
HGT	horizontal gene transfer

HMP	Human Microbiome Project
HTS	high-throughput sequencing
LB	Luria-Bertani
MH	mechanical homogenization
MIC	minimum inhibitory concentration
MLST	multi-locus sequence type
MPN	most probable number
mRNA	messenger RNA
OTU	operational taxonomic unit
PCoA	Principal Coordinate Analysis
PCR	polymerase chain reaction
PE	pulmonary exacerbation
PMA	propidium monoazide
qPCR	quantitative PCR
RDP	Ribosomal Database Project
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-qPCR	reverse transcription qPCR
s.e.m.	standard error of the mean
SMG	*Streptococcus milleri* group
TA	toxin-antitoxin
TMP-SMZ	Trimethoprim/sulfamethoxazole
T-RFLP	terminal restriction fragment length polymorphism

VBNC	viable but nonculturable
δ	effect size

CHAPTER 1: CYSTIC FIBROSIS: A POLYMICROBIAL DISEASE

Bacterial infections are the leading cause of death in the majority of those with Cystic Fibrosis (CF), a disease which affects an estimated 30,000 people in the US and 70,000 people worldwide [1]. It has been shown in CF patients that chronic infection with CF-related bacteria including Pseudomonas aeruginosa and Burkholderia cepacia complex (Bcc) is linked with an increase in mortality [2, 3]. Acute bacterial infections, which disturb the state of a stable, chronic infection, are the major cause for pulmonary exacerbations (PE)s which may develop 2-3 times per year in some CF patients [4, 5]. The frequency of PEs has been connected to increased mortality and results in a permanent impairment in lung function as measured by forced expiratory volume per one second (FEV1) [5]. The exact cause of a PE remains unclear but is often attributed to factors associated with established bacteria, viral infection and environmental insult such as ozone [6]. Diagnosis of a PE relies heavily on the observations of a clinician through x-rays, spirometric parameters and physical assessments as well as patients' subjective description of symptoms [7]. However, while several scoring systems and patient self-assessment forms, such as Fuchs [4], are now being used, no method encompasses the vast range of characteristics, including change in sputum, increased dyspnea and increased cough, that aid in determining if a patient is developing a PE [8]. Lack of a consensus for the cause of a PE continues to be one of the main reasons a consistent, objective measurement does not exist.

The microorganisms colonizing the lungs result in chronic infections that persist throughout the life of a patient [9]. Within the past decade, it was assumed the CF lungs were colonized with few different bacteria including P. aeruginosa, Haemophilus influenza, Staphylococcus aureus, and members of the Bcc which are all considered CF-related bacteria [10]. In 2004, a study by Rogers et al. used terminal restriction fragment length polymorphism (TRFLP) analysis to target the bacterial 16S rRNA gene to analyze DNA extracted from sputum of CF patients. This method of analysis revealed a complexity in the CF lung that included 15 species not previously identified in the lungs. The study by Rogers et al. laid the foundation for redefining CF as a polymicrobial disease. Since that time, additional studies and the use of pyrosequencing have described community profiles unique to each individual. Interestingly, some bacteria included in these profiles, such as SMG, Prevotella, Veillonella, and Rothia, which are considered avirulent in the human host, may contribute to polymicrobial infections [11, 12]. A 2008 study by Sibley et al. described three classes of these organisms including a bacterium which acts synergistically with P. aeruginosa but is avirulent on its own [13]. Veillonella and Rothia were isolated in a 2008 study, by Tunney et al., from patients which had exacerbations during the sampling period, leading the authors to believe each genus was potentially pathogenic in CF [12]. A 2010 study by Ulrich et al. observed clinically significant cell numbers of Prevotella in CF which are assumed to grow anaerobically in the lungs due to mucus plugs cause by colonization of P. aeruginosa [14]. The results of an additional 2010 study by Field et al. in which Prevotella was biochemically characterized, suggested that "the diversity and dynamics of this genus in CF may contribute to airway disease" [15]. It is still not clear what role these bacteria play in the

CF lung and but many suspect they contribute to the pathogenicity of existing pathogens such P. aeruginosa. To understand the dynamics of the microbial community and the role the less common bacteria play in the disease, long-term longitudinal patient sampling studies must be performed [10]. Candidate bacteria for use as biologically active markers for exacerbation onset can then be identified by correlating the changes in their abundance with onset or relief of a PE.

Methods of measuring an active PE are still disputed but are traditionally based on parameters defined for specific clinical trials [6]. The Cystic Fibrosis Foundation Clinical Practice Guidelines have widespread use in clinics and define exacerbations by patient symptoms including >10% reduction in predicted FEV1 value, excessive weight loss, increased frequency of cough and increased sputum production [7]. However, since the exact cause of PEs remains unclear, clinicians must rely on individualized observations which leave inconsistencies among the CF community with treatment and standardized care [4]. Due to the lack of a definition, treatment strategies vary widely among clinicians as well strategies for determining the severity of a PE [4]. Clinicians may opt to aggressively treat what might turn out to be a mild exacerbation with intravenous antibiotics and leave a severe PE insufficiently treated with oral antibiotics [4]. A center-based study measuring treatment outcomes showed that outcomes were improved with increased frequency of treatment which was postulated to be due to the increased frequency of clinic visits [16]. Frequently sampling from patients and assessing symptoms in this study was suggested to improve patient health. This study demonstrates that temporal changes occur in the CF lung which may not immediately present themselves through an increase in patient symptoms but if left untreated, may

result in the occurrence of a PE. Lack of continuous samples for examining changes in the microbial community in CF can be a limiting factor in community profiling studies since transient, minimally symptomatic or asymptomatic infections may cause unexplained results and a false perception of a stable bacterial profile [17]. While a single sputum sample can provide a snapshot of the community profile at any given time, continuous samples from multiple patients are necessary to draw conclusions on the role of the microbial community in disease progression [18, 19]. As a result, frequent sampling becomes a key point for correlating changes in factors such as bacterial abundance with PEs which would provide information to clinicians that may allow an exacerbation to be predicted.

Some researchers believe the occurrence of a PE is due to an expansion of existing bacterial populations, microbe-microbe interactions, viral infections or environmental insult [4]. Derived from development of culture-independent methods to study the microbial environment, researchers discovered a highly diverse microbial community in the lungs leading to the disease being redefined as polymicrobial [20]. Further examination using DNA-based techniques have allowed researchers to identify pathogens previously not associated with CF and provided unique information of the dynamics of the polymicrobial community [21]. Studies by Sibley et al. led authors to postulate that SMG was the cause of recurrent exacerbations when the abundance of the organism was found to increase in comparison to P. aeruginosa during an acute infection period [22]. Bacteria in CF have been associated with interspecies communication when a 2003 study by Duan et al. showed that an increased production of virulence factors by P. aeruginosa was linked to the presence of the microorganism, SMG [23]. A study by

Potvin et al. showed that increased production of virulence factors, such as pyocyanin by P. aeruginosa, is associated with acute infection periods [24]. The implications of observing measurable changes in bacterial populations, interspecies communication and virulence factors in the polymicrobial environment in CF provides researchers, as suggested by Rogers et al. in a 2011 review, with an opportunity to identify biomarkers to predict the onset of an acute infection [25].

Attempts at developing tools to predict an oncoming PE have met with limited success due to the heterogeneity of patient characteristics and inadequate consensus among researchers on what a PE constitutes [26]. Additionally, patient symptoms are generally present only after an acute infection period has been established, inhibiting the chance of early intervention [25]. Biomarkers as measures for onset of a PE in CF would need to be sensitive to biological activity as well as the effect of treatment [27]. In a 2007 study by Mayer-Hamblett et al., the authors described a negative correlation between FEV1 values and sputum inflammatory markers such as free elastase, IL-8, neutrophil counts and percent neutrophils [27]. This study and other similar studies suggest that host-derived biomarkers from the inflammatory immune response are sensitive to the occurrence of an exacerbation [27-29]. However, the key issue with using inflammatory immune response is that the establishment of an acute infection must be in place in order to achieve a measureable change in the host-derived marker present in the lungs. Microbiological methods examining bacteria-derived biomarkers would have a distinct advantage in predicting onset of a PE because if bacteria are involved in eliciting a PE, bacterial population changes should occur prior to an elevation of patient signs and symptoms [25]. A direct reflection of the changes in the microbial community

dynamics could be measured from sputum samples and be used to identify factors involved in onset of an acute infection. Data generated in our lab from 454-FLX pyrosequencing of bacteria in sputum show that taxa richness decreases with treatment (Figure. 1) and suggest some bacteria are eliminated or reduced in number beyond the limit of detection. A 2005 study by van Ewijk et al. described reports of new bacterial colonization occurring during the viral season which correlated with an increased frequency of exacerbations [30]. Evidence from the 2005 study along with our data suggests that colonization of new bacteria or expansion of existing bacterial populations above the limit of detection correlates with occurrence of an exacerbation. Biomarkers which provide the most information include those which are most likely to trigger a PE such as an increase in richness caused by either acquisition of new bacterial species or expansion of existing bacterial populations, increases in virulence gene expression and changes in community structure initiated by interspecies communication. Measuring a change in any of these factors that coincide with an PE could indicate a valuable measure for clinicians to use in diagnosis. A 2011 review by Rogers et al. on bacterial biomarkers suggests that the future of disease management in CF will rely on predicting exacerbations and that the biological benefit of early detection will reduce both the duration and severity of a PE and increase the longevity of the patient [25].

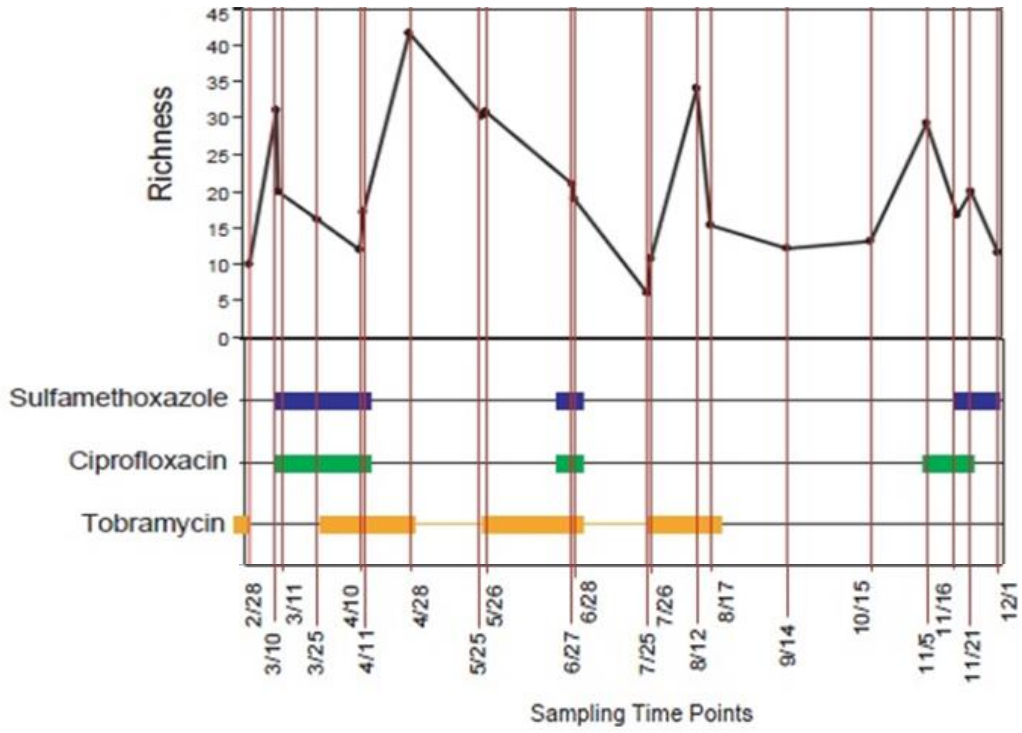


Figure 1.1: Change in taxa richness over time and in response to antibiotics. Taxa richness decreases in response to acute antibiotic therapy used to treat a PE. PEs were defined by use of TMP-SMZ and ciprofloxacin. The PCR products for 454-tagged sequencing, from DNA extracted from sputum (see Chapter 4: Methods), were prepared with primers and thermalcycling parameters described in Fierer et al.[31] The 454 Life Sciences primer B with a "TC" linker and bacterial 27F primer (5'-GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3') and 454 Life Sciences primer A with a "CA" linker, 12 mer barcode and bacterial primer 338R (5'-GCCTCCCTCGCGCCATCAGNNNNNNNNNNNNCATGCTGCCTCCCGTAGGAGT-3'), where the N's represent barcodes used to identify each sample, [32] were used to target the V1-V2 variable regions of the 16S rRNA gene [33]. PCRs were set up with Platinum Taq DNA polymerase (Invitrogen) according to the included protocol with 100ng of bacterial genomic DNA as a template. Each reaction was quantified by PicoGreen on a NanoDrop ND-3300 fluorospectrometer. Samples were pooled in equimolar amounts and concentrated in a vacuum centrifuge before being submitted the Environmental Genomics Core Facility at the University of South Carolina for 454-FLX sequencing.

CHAPTER 2: VIABLE BUT NON-CULTURABLE BACTERIA

1. Introduction

Bacteria have two types of population-level responses when exposed to harsh environments: undergo massive die-offs with surviving cells serving as the source for regrowth upon improved environmental conditions, or adjust cellular physiology to adapt to the harsh condition. Such adaptive mechanisms require the organism to carry additional genes. The existence of recognized bacterial responses, such as the stress and starvation responses, indicates that the increased energy cost to maintain these genes is sometimes offset by an increase in evolutionary fitness. One well-studied long-term survival mechanism is formation of endospores, a mechanism generally limited to gram-positive bacteria. This led to the question of whether there is a mechanism by which non-spore forming bacteria can withstand harsh environments for long periods of time. One possible adaptive mechanism was first described in 1981 when *Escherichia coli* and *Vibrio cholerae* were found to enter a dormant-like state in response to starvation [1]. Two years later, the term "viable but nonculturable" (VBNC or VNC) was used to describe this condition.

The VBNC state is empirically defined as one in which cells are viable yet they do not undergo sufficient division to give rise to visible growth on nonselective growth medium (i.e. medium lacking any selective or stress agents and containing nutrients that normally support growth of the strain). Since that time, over 70, mostly gram-negative,

bacterial species have been documented to have the ability to become VBNC [2]. The VBNC response differs from other bacterial responses such as cell stress, cell starvation, cell-wall deficient-bacteria, and persister cell formation in that only VBNC cells do not grow in or on nonselective growth medium, and cells can remain VBNC for long periods of time, up to multiple years; hence VBNC is not a transient response. These characteristics explain why the viable but nonculturable state is thought to be a long-term survival mechanism initiated in response to one or a combination of environmental stresses [3]. Starvation is the most common stress reported to induce bacteria to enter the VBNC state, either independently or in combination with other stresses; additional VBNC-inducing conditions reported include: UV light, intense visible light, high or low temperatures, changes in osmolarity or aeration, toxic forms of oxygen or biocidal agents such as copper, as well as unknown biological factors [4]. The percentages of cells in a culture that become VBNC vary in response to the specific environmental condition. That there is a wide range of seemingly unrelated environmental conditions that induce the VBNC state suggests a parallel with the better-characterized stress response.

Studying living cells that do not readily grow leads to controversy. Criticisms of early VBNC studies, especially of reports on the ability of VBNC cells to regain the ability to grow, have improved the quality of subsequent research [5-7]. Attempts to address these criticisms have led to a general, but not universal, acceptance of the VBNC condition even though the molecular mechanism behind VBNC remains uncharacterized [8, 9]. However, improved techniques that rapidly detect VBNC cells have led to a better understanding of their role in the environment [10, 11].

2. Identifying Cells as Being VBNC

The concentration of VBNC cells in a pure culture is determined by subtracting the concentration of viable cells from the concentration of culturable cells. Getting an accurate colony forming unit (CFU) count is dependent upon knowing which growth medium to use. This is straightforward when examining a pure culture of a characterized bacterial species, but is more difficult when examining an environmental sample containing multiple bacterial species. Analyzing samples on multiple types of growth media would allow detection of a wider range of species but would also lead to an overestimation of the CFU concentration if a given species could grow on more than one growth medium. The presence of uncharacterized bacterial species that do not grow on any of the media used to enumerate CFUs would lead to an underestimation of CFU concentration. As a result, there is no accurate estimate of the percentage of environmental microbes that are VBNC.

There are multiple methods to enumerate viable cell concentration, by necessity all are growth independent assays, and while microscopic based assays are still used, polymerase chain reaction (PCR)-based assays and flow cytometry analyses are recently developed accurate screens for viable cells [10, 11]. The original assay used to document the VBNC condition, the Kogure assay [12], incubates cells for approximately eight hours in medium containing low levels of yeast extract and a cell division inhibitor. After staining, cells are examined microscopically; all elongated cells are scored as being viable. That cells are able to elongate in this medium was taken as evidence for the cells retaining the potential to undergo cell division. Presumably, if the inhibitor was absent,

the cells would divide. This interpretation has led to speculation that VBNC cells may actually be able to undergo limited growth. Other viability assays that are indirect indicators of metabolic activity examine cell membrane potential, plasmolytic response to osmotic stress, or cell membrane integrity. The most commonly used commercial bacterial viability kit (LIVE/DEAD BacLight Bacterial Viability Kit; Molecule Probes Inc., Eugene OR) equates cell membrane integrity with viability. Two fluorescent nucleic acid-binding dyes are used to examine membrane integrity. A green fluorescing dye, SYTO 9, readily moves across the cell membrane; a red fluorescing dye, propidium iodide, does not. Because propidium iodide has a higher affinity for nucleic acids than does SYTO 9, green fluorescing cells are those with an intact membrane (i.e. viable), whereas red-fluorescing cells are those whose membrane integrity is compromised (i.e. dead). Figure 1 shows the result from such an assay. Multiple studies have compared viability assays; some find the examined assays yield similar results, others find significant differences between the various viability values [13]. There is no consistent explanation for these differences, when observed, as there is no consensus on which viability assay is the most accurate. Some researchers perform more than one type of viability assay to increase the accuracy of results.

Common to growth-independent viability assays is that a recently dead cell could yield a false positive result. This is possible because cellular processes may not cease and the integrity of cell membranes may not be lost immediately upon death. How one defines when a bacterial cell is dead is a topic beyond the scope of this dissertation [see 14]. What is relevant, however, is that the means to distinguish between a VBNC cell and a recently dead cell is based on one defining characteristic of a VBNC cell – the

ability to remain VBNC for extended periods of time. Confidence in identifying a cell as being VBNC increases by performing a viability assay on a sample after a period of time that exceeds the expected longevity of the target molecule in a dead cell. One report using the LIVE/DEAD kit established the intervening assay time by killing cells with UV irradiation, then determining the time until cell membranes became compromised to the point of allowing uptake of propidium iodide [15].

A recent variation is to combine the LIVE/DEAD assay with flow cytometry [10]. VBNC cells which may be harmful and undetected in food or water sources can be rapidly identified, even on large samples, using this FCM-based approach. Methods using reverse-transcription quantitative PCR (RT-qPCR) to target mRNA transcripts of housekeeping genes have also been developed [16]. Assessing viability using PCR based assays is difficult due to the possible amplification of DNA from dead cells, which can be avoided by addition of ethidium monoazide (EMA) or propidium monoazide (PMA) prior to DNA extraction [17].

Determining VBNC forms of a specific bacterial species in a sample containing multiple bacterial species requires having a means of identifying the target species. One such method uses taxa-specific RT-qPCR primers. Liu et al. [16] targeted the *rpoS* gene, which retains expression in VBNC *Escherichia coli* O157:H7 cells, to identify clinical and bovine isolates of as few as 23 VBNC cells of only *E. coli* O157:H7 in a river water sample containing other food-borne pathogens including *Yersinia enterolitica*, *Salmonella typhi*, and *Listeria monocytogenes*.

No growth-independent viability assay is definitive; all are indirect indicators of viability and subject to alternative interpretation. Only when a cell regains the ability to

grow, termed resuscitation, can it definitively be concluded that the cell was previously VBNC.

3. Resuscitation

If the VBNC state is a long-term survival strategy, then it must be reversible. Initially, resuscitation was commonly reported by subjecting a VBNC culture that contained no detectable culturable cells to an environmental change, such as a temperature upshift, and subsequently observing CFUs. Even from these early studies it was clear that simply reversing the VBNC inducing condition would often not lead to resuscitation. More recent resuscitation studies have benefited from the criticism leveled at these early studies [18]. Details on current controversies within the VBNC field of study are discussed in a following section; those concerning resuscitation are discussed here.

The main problem with reports of resuscitation is that it cannot be stated with certainty that there were no culturable cell forms present in the culture prior to resuscitation (knowing there are no culturable cells present would require attempting to culture the entire microcosm, thus ending the experiment). That is, the physiological status of every bacterium in a population under study cannot be determined through sampling methods. It is possible that a microcosm could contain a few culturable cells, which, by chance, were not included in a sample to be examined for CFUs. Regrowth of a few undetected culturable cells has been ruled out based on the kinetics of regaining culturable cells. However, it was reported that dispersal of clumps of culturable cells could occur in response to VBNC inducing conditions and give the appearance of there

being at most only a few culturable cells in a microcosm [19]. Dispersal of these clumps could give the appearance of resuscitation.

The most definitive method to document resuscitation has been to perform a dilution study (e.g., MPN assay) in which a sample is diluted into multiple tubes to a degree that statistically eliminates the possibility of any undetected culturable cells (even if clumped) being present. Observing growth can then be attributed to resuscitation.

3.1 Current Model to Explain Lack of Growth

Although no universal resuscitation conditions have been reported, resuscitation is more likely to occur when cells are placed in a medium containing low nutrients, perhaps combined with an environment stress (the nature of which may be based upon the particular VBNC inducing stress), prior to exposure to a rich medium [20]. This observation led to the current strategy for resuscitation and the hypothesis to explain why VBNC cells would not grow when placed on standard rich medium normally used to propagate bacteria [2].

VBNC cells can be assumed to have low metabolism. To maintain viability and be able to reinitiate growth if environmental conditions change, some cellular process (e.g., DNA repair and protein replacement) need to occur. However, due to decreased metabolism, the level of some protective proteins will drop, which will limit the ability of VBNC bacteria to adapt to a new environment. Placing VBNC cells in a nutrient-rich medium at optimal growth temperatures can result in be a burst in production of superoxide and free radicals. If not removed, these byproducts of metabolism could kill, or prevent growth in the cells. The requirement for an initial incubation period in a poor nutrient medium, supplementation of the medium with peroxide inhibitors, or in a living

host reflects a need to for optimum conditions to undergo a slow growth transition phase as a part of resuscitation.

3.2 Comparison to Other Growth Regulation Systems

Bacteria have several growth regulation systems such as contact-dependent inhibition (CDI), toxin-antitoxin modules, and persister cell formation which are induced as a result of various types of environmental stress and which allow cells to survive deleterious environments [21]. One function of CDI appears to be as a protective system to inhibit growth of neighboring bacterial cells, especially in biofilms containing high numbers of bacteria [22]. CDI relies on outer membrane proteins contacting receptor proteins on other cells to reduce metabolic activity by reducing proton gradient, ATP formation, and cell/growth/division of target cells. Persister cells are slow-growing or dormant cells which, due to the lack of active targets required for killing, makes them highly multi-drug tolerant. This resistance is not genetically programmed as removal of antibiotics and growth of the persister cells will give rise to a microbial population exhibiting the same sensitivity profile as the original population [23]. In Cystic Fibrosis (CF), persister *Pseudomonas aeruginosa* cells are believed to be a survival mechanism against exposure to a multitude of antibiotics given as treatment to CF patients [24]. While no mechanism is known for persister cell formation, several genes are identified as inducing the phenotype including toxin-antitoxin gene pairs, such as *hipBA* and *tisAB*. *hipA*7 mutants show a 1000-fold increase in persister cell formation [24].

Identification of genes or changes in gene expression patterns in a phenotype can be used to propose mechanisms explaining a physiological response as well as to determine how similar related bacterial physiological responses are. Currently, no

specific mechanism has been identified which allows a bacterium to enter the VBNC state. There is little data on genes or changes in gene expression in cells entering or exiting the VBNC state, which helps explain why acceptance of the VBNC state is not universal.

4. Source of Controversy

The VBNC condition continues to be controversial. The areas of controversy include: terminology, definitions of bacterial physiological states and viability, validity of viability assays, distinguishing resuscitation from growth of undetected culturable cells, and distinguishing VBNC from other bacterial responses. It is clear that the term viable but nonculturable does not accurately describe living bacteria that, while they may not be growing, have the ability to grow (albeit under different and perhaps not yet discovered conditions).

Documenting the VBNC condition is based upon the validity of viability assays and the interpretation of data from resuscitation experiments [8]. Although the use of dilution studies has diminished criticism of some resuscitation reports, true resuscitation has been recorded in only a subset of VBNC reports. Hence, viability assays remain the main tool to document the presence of VBNC cells. Through observing individual cells (e.g., viability assays) or populations of cells (e.g., enumerating CFUs), insight as to the physiological status of individual cells is inferred. One cannot determine through simple observation whether a specific bacterial cell is alive or dead; one can only determine if a cell was alive or dead based on whether that cell undergoes subsequent growth. The problem is that with a bacterial condition such as VBNC a lack of growth is a necessary prerequisite to describe the condition.

A related criticism focuses on the validity of culturability assays. It is possible for a cell to undergo a change that inhibits growth in media that normally support growth (part of the definition of VBNC), yet would not be considered to be VBNC. For example, in one study, the concentration of culturable cells gradually decreased to undetectable levels in *Vibrio vulnificus* over multiple weeks when placed in sterile sea water. Warming the VBNC culture to room temperature in the absence of nutrients resulted in the appearance of CFUs, suggesting resuscitation had occurred. When this study was reproduced by another research group, addition of catalase or sodium pyruvate during the initial gradual CFU decline period resulted in up to 1000-fold higher CFU counts [25]. These authors concluded that there was a subpopulation of cells which were not VBNC, but were in a hydrogen peroxide-sensitive state, and speculated that the previously reported resuscitation was instead growth of hydrogen peroxide-sensitive cells. A study by Kong et al. [26] demonstrated that "low temperature inhibits *oxyR*-mediated catalase activity" in VBNC cells, indicating that the non-culturability is not due to a distinct hydrogen-peroxide-sensitive state. These studies highlight the difficulty in studying a bacterial phenotype (lack of growth) that can arise from various physiological responses, only some of which are characterized. At a broader level, these studies also highlight the difficulty in inferring molecular mechanisms using data obtained from experiments designed to observe phenomena for which there are always alternative interpretations.

Molecular studies of the VBNC condition have documented differences between VBNC and other bacterial responses. Changes in peptidoglycan composition have been observed in VBNC *E. coli*, [27] and *Enterococcus faecalis* [28]. Both species have an

increase in total cross-linking and a change in penicillin binding proteins. Changes to the cell wall in VBNC cells may explain increased resistance to antibiotics; Lleo et al. [28] found that the peptidoglycan in *E. faecalis* lack the pentapeptide target for vancomycin which was theorized to be due to peptidoglycan turnover rather than *de novo* synthesis. These chemical modifications to the peptide are different from those observed with UV-killed, starved, or exponentially growing cells. Proteomic analysis has also been applied to the study of VBNC *E. faecalis*. VBNC cells were found to generate a protein profile that was different from starved and exponentially growing bacteria [29]. Another protein involved in the VBNC state is the cytoskeletal protein MreB which is fragmented as cells are exposed to stress condition. Reports of VBNC *V. parahemolyticus* becoming smaller in size and more spherical in shape as a response to stress conditions further suggest MreB as a structural component [30]. These studies confirm that the VBNC state is distinct, and that changes to the cell wall and protein profile may be indicative of the VBNC state.

4.1 VBNC Associated Genes

Definitive proof of the VBNC condition will likely come only when the corresponding genes and gene products are identified. Although no gene has yet been identified that is specific for the VBNC state, multiple genes involved in the VBNC condition have been identified [18]. *rpoS* is reported to be involved in the maintenance of *E. coli* in the VBNC state [31]. That *rpoS* continues to be expressed in VBNC cells has led to it being targeted in cell viability assays as a way to identify which species in a mixed population are VBNC. Down-regulation of *katG* gene expression, encoding a periplasmic catalase in *V. vulnificus*, while in the VBNC state [32] may explain why

VBNC cells are sensitive to hydrogen peroxide. The one gene product proposed to be involved in resuscitation is Rpf (resuscitation promoting factor) [33]. This muralytic enzyme is thought to be necessary for cell wall expansion when cells resume growth from nutrient-poor conditions or resuscitation of VBNC cells.

Microarray analysis has been applied to identify genes that are expressed while in the VBNC state in *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* [34]. Advances in high-throughput sequencing analysis such as RNA-sequencing should allow for more accurate comparisons of transcriptomic profiles between VBNC and other cell responses. Experimental analysis using RNA-sequencing or RT-qPCR would be useful in not only identifying candidate genes for VBNC but also for determining the role and effect of VBNC cells in the environment [35].

5. Role of VBNC Cells in the Environment

The lack of information on the prevalence of VBNC bacteria in the environment, the biological activities VBNC cells can engage in, and the ability to resuscitate makes it difficult to estimate the impact of the VBNC condition on environmental microbial processes even though VBNC cells of targeted species have been identified in environmental samples. That only a fraction (0.1-10%) of bacteria observed microscopically to be present in an environmental sample are able to be cultured suggests most of these uncultured cells are not dead. Therefore, 90+% of environmental microbes represent either uncharacterized bacterial species that do not grow on media commonly used in environmental microbiology, or characterized species that are VBNC. Care must be taken to not confuse VBNC cells with dormant or injured bacteria (cells that are not growing, but would if provided nutrients) as the VBNC state is considered to be a

separate, genetically controlled adaptive physiological response, and specific stimuli, separate from nutrients, may be required before growth is reinitiated. This distinction removes from consideration in this review literature concerning the role of dormant cells in the environmental processes, such as in bioremediation. Most studies on the importance of VBNC cells have focused on their role in disease.

VBNC bacteria have been reported to retain a number of pathogenic features. *E. coli* continued to produce enterotoxin after entry into the VBNC state when incubated in rabbit intestinal loops [36], and VBNC uropathogenic *E. coli* cells were found in the bladder of mice after antibiotic resolution of a urinary tract infection [37]. VBNC *E. coli* O157:H7 was shown to retain stable expression of *stx1* and *stx2* genes, necessary for production of Shiga-toxin, and remain toxic to VERO cells [38]. The food safety risk of VBNC cells was demonstrated when Dinu and Bach recorded a constant level of Shiga-toxin, produced by VBNC *E. coli* O157:H7, for up to 3 days on the surface of lettuce [39]. *Shigella dysenteriae* retained several virulence factors when associated with human cells [36]. VBNC *Campylobacter jejuni* were able to infect rats [36, 40]. *Aeromonas hydrophila* retained virulence in goldfish, although at a decreased level when compared to cultured bacteria [41]. VBNC *V. vulnificus* were reported to be virulent in mice [42], and VBNC *V. cholerae* resulted in clinical cholera symptoms in human volunteers [43]. On a more global level, the appearance of cholera outbreaks has been suggested to correspond to resuscitation of marine reservoirs of VBNC *V. cholera* [44]. The ability of VBNC forms of *Salmonella enterica* to retain pathogenicity was suggested to explain an infectious outbreak in Japan in 1999; a hypothesis that was subsequently supported by in vivo mouse studies [45]. A recent study was the first to describe stress-induced entry of

an enterohemorrhagic (EHEC)/enteroaggregative *E. coli* (EAEC) hybrid strain into a VBNC state which was linked to an outbreak of hemolytic uremic syndrome and bloody diarrhea in northwestern Germany [46]. Resuscitation of nonculturable enteric bacteria was recently demonstrated by Senoh et al. [47] when co-cultured with select eukaryotic cells including HT-29, Caco-2, T-84, HeLa, Intestine 407 and CHO cells. In addition to retaining virulence, VBNC cells may also have increase resistance to antibiotics. VBNC *Enterococci* cells have been shown to retain increased vancomycin resistance, and antibiotics were not able to eliminate VBNC *Helicobacter pylori* cells from infected cats. If VBNC cells are shown to be involved in disease etiology, then strategies to both identify and treat VBNC cells will need to be developed.

Although lesser studied, multiple plant pathogenic bacteria have been shown to have the ability to become VBNC, including *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas campestris*. For *R. solanacearum*, evidence has been presented supporting the following steps in how VBNC is involved in the disease/life cycle [48]. In the cycle, infecting bacteria enter the VBNC stage as the plant undergoes necrosis, the VBNC form falls to the soil where they remain until resuscitated in response to encountering a host plant root system. Consistent with this model is a recent study by Santander et al. [49] which demonstrated that VBNC *Erwinia amylovora* were able to regrow only *in planta*. The ability of pathogens to become VBNC can explain the persistent nature of both animal and plant diseases, and why disease outbreaks can reoccur on fields, or in patients, treated with biocidal agents.

VBNC cells appear to be ubiquitous in the environment; they have been reported to be found in surface water bodies, bulk soil, the rhizosphere, the phyllosphere, and even as a normal constituent in the human and mice urinary tract [15]. In addition to having a possible role in disease etiology, the VBNC condition may also impact other environmental phenomenon. A recent study on the "rhizobial competition problem" suggested that indigenous populations of bacteria co-inhabiting legume root nodules may be able to induce the VBNC state in rhizobia through triggering host production of salicylic acid which can create reactive oxygen species [50]. Two VBNC-related questions related to release of a genetically modified organism (GMO) are: 1) will GMOs become VBNC and potentially escape ready detection and monitoring strategies, and 2) do VBNC forms of indigenous microbes in the field explain why it is difficult to form stable populations of released GMOs in the environment.

6. Summary

Until recently, microbiologists have studied bacteria under controlled conditions, and usually when grown in an excess of nutrients. This contrasts with the natural environment for bacteria in which there are limited nutrients, changing environmental conditions, and exposure to deleterious agents. Bacteria studied under more natural conditions have revealed the existence of multiple physiological responses. One of these responses is called "viable but nonculturable."

The VBNC condition is difficult to study because it is defined as viable bacteria that do not readily grow. Empirically defining a non-growing cell as being alive based on growth-independent viability assays is problematic. So while there are numerous reports of multiple bacterial species entering into the VBNC state in response to changes in a

variety of environmental conditions, not all of these species have been reported to resuscitate. The history of the VBNC field may correspond to that of discovery of endospores – documentation of endospores as being alive was met with skepticism until the conditions that allowed them to grow, and the genes involved in this response, were discovered. While VBNC resuscitation conditions have been reported, few genes have been proposed to be involved in the response.

That there is a long-term dormant-like survival strategy available to non-sporulating bacteria is not surprising. TA systems and persister cell formation have also been reported as a strategy pathogens and non-pathogens in response to environmental stress conditions. While the exact role of these growth-regulations systems is not fully understood, microarray and RT-qPCR have been useful in detection of these pathogens, which may also retain virulence in a VBNC state, in food and water sources. What remains unresolved is the genetics and biochemical mechanism of this physiological response. Future research should benefit from techniques advancements in transcriptomics/RNA sequencing and proteomics. However, only when genes involved in the VBNC response are discovered will the VBNC condition be universally accepted.

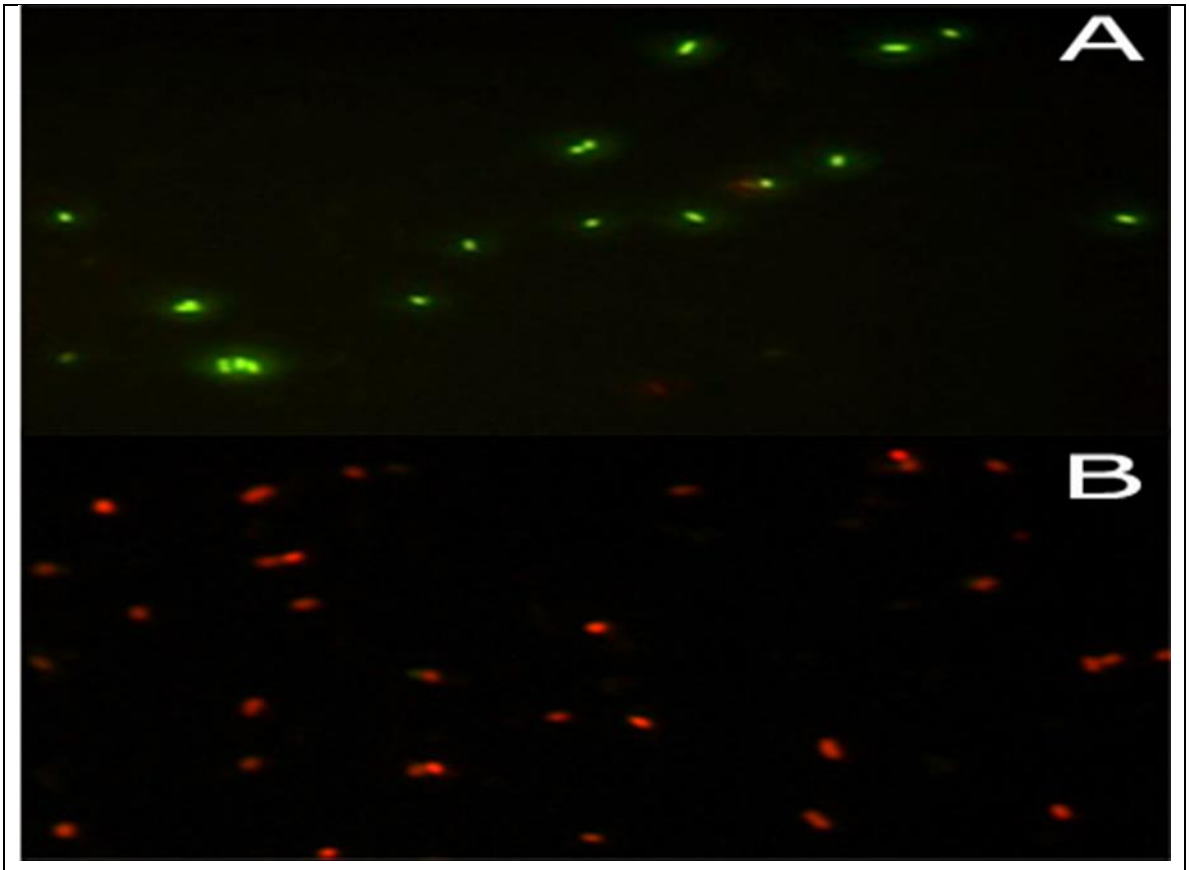


Figure 2.1. *Escherichia coli* cells viewed via epifluorescent microscopy after staining with the LIVE/DEAD Bacterial Viability Kit. Panel A – exponentially growing culture; Panel B – cells placed in boiling water bath for 1 min. Green fluorescing cells have an intact cell membrane and are tentatively identified as being viable; red fluorescing cells lack an intact cell membrane and are scored as dead. Each cell is approximately 2 microns in length.

CHAPTER 3: MECHANICAL HOMOGENIZATION DECREASES VARIABILITY BETWEEN SPUTUM SAMPLES

1. Introduction

Sputum samples are regularly used in studies to represent the microbial environment found in the Cystic Fibrosis (CF) lung. Expectorated sputum is often provided by the patient and then stored away in a lab freezer at -80ºC. Since sputum is known to be thick and difficult to process, the viscosity is reduced through chemical means prior to use in an assay. The primary chemical method includes mixing a ratio of sputum to dithiothreitol (DTT) commonly known as Sputasol, Sputolysin®, or Cleland's reagent [1]. Although it has been shown that this method is useful for reducing sputum viscosity, it is not clear if this method effectively homogenizes the sputum allowing for an evenly distributed bacterial composition throughout the entire sample.

Aliquots of 130 sputum samples, obtained in our lab over a three-year period, were used to assay the change in total bacterial load over time and with antibiotic therapy through qPCR (see Chapter 5). For sputum processing, each sample was mixed with a DTT solution for liquefaction. To further reduce sputum viscosity and ensure complete homogenization prior to obtaining an aliquot, we subjected each sample to a high performance disperser for one minute at 12,000 rpm. From the samples collected, we observed over a 2300-fold variation between the highest and lowest bacterial load in sputum. An average fold change of 7.2 was observed between sequential samples with the maximum difference being a 172-fold change for samples collected four days apart.

We suspected that a factor contributing to the variation in total bacterial load between samples may be the heterogeneity of sputum resulting in an uneven distribution of bacteria [2]. Due to the highly viscous nature of sputum, an aliquot may not be representative of the sample. Examination of whether aliquots serve adequately as the whole sputum sample has not been performed in detail.

Analyzing and processing sputum through chemical homogenization (CH) using a DTT solution is the standard practice for molecular detection assays [3]. While adding DTT prior to aliquoting is preferred, sputum is often homogenized after an aliquot has been removed [4, 5]. Mechanical homogenization (MH) may also be performed in addition to using DTT but the efficacy of this method as an additional processing step has not been evaluated.

In this experiment, we compared aliquots of sputum to determine if non-homogenized sputum results in aliquots that are not representative and if the process of homogenization evenly distributes the bacterial composition. All sputum samples in this study were treated with a DTT solution. Total bacterial abundance was compared between DTT-treated samples and those additionally subjected to MH using a high-performance dispersing instrument. The abundance of *Burkholderia multivorans*, which was present in the sputum samples, was also measured. Since *B. multivorans* was shown to be of lower in abundance in the sputum samples obtained for the three-year study, we suspected the distribution of this bacterium would be less uniform in sputum than the distribution of all bacteria. If so, the effect of MH on decreasing the variability of *B. multivorans* abundance between aliquots would likely be greater than for all bacteria.

We also used a descriptive statistic to determine if the mechanical disruption of sputum using a high-performance disperser would have an effect on bacterial abundance.

2. Methods

2.1 Samples

Nine expectorated sputum samples were obtained from a CF patient (IRB Protocol Approval # 11-12-36). The patient was clinically stable as judged by a treating physician at the time of collection. Samples were collected each morning by the patient expectorating sputum into a 15 mL Falcon tube, placed on ice during transport to the lab and immediately processed. Sputum color was noted to determine if a relationship might exist between the color and heterogeneity of the sample.

2.2 Chemical Homogenization (CH)

Each sputum sample was mixed with a 1:3 ratio of sputum to a 0.1% dithiothreitol solution, vigorously vortexed, and then incubated at 37°C for one hour. Samples were divided into two equal portions. The first portion (A) was divided into six 400µL aliquots, with four aliquots used for MH and two without further homogenization. The second portion (B) was divided into six 400µL aliquots with each being subjected to MH.

2.3 Mechanical Homogenization

Two aliquots from portion A of each sputum sample were subjected to MH for two minutes using a high-performance dispersing instrument (IKA ULTRA-TURRAX® T-25 digital, Staufen /Germany) set to 12,000 rpm. The metal shaft of the disperser was disinfected between each aliquot using a combination of steps which included placing the shaft in five percent of a bleach solution and 70% ethanol for at least 30 seconds. The

shaft was then rinsed thoroughly with deionized, distilled water. All aliquots were weighed and then stored on ice until DNA extraction.

2.4 DNA Extraction

DNA was extracted from each sputum aliquot using the IT 1-2-3 VIBE Sample Purification Kit (Biofire Diagnostics, Inc, Salt Lake City, Utah) and its concentration determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). All extracted DNA was immediately stored at -20ºC until its use for quantitative polymerase chain reaction (qPCR).

2.5 Quantitative PCR

The qPCR mixture contained 10 µL Perfecta SYBR Green FastMix Reagent Low ROX (Quanta Biosciences, Gaithersburg, MD), .5 µL of 100 pmole/µL of each primer, 5 µL of DNA, and 4 µL of nuclease-free water to a final volume of 20 µL. Universal primers [11] were used to target a 16S rDNA fragment of 466bp and measure the abundance of all bacteria in the sample. *Burkholderia*-specific primers [12] were used to target *B. multivorans* and generate a fragment of 333bp. qPCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with an initial step of 10 min at 95°C, then 40 cycles of 15 sec at 95°C and 1 min at 60°C. Melting curves were determined following the qPCR by 1 cycle of 15 sec at 95°C, 1 min at 60°C, 30 sec at 95°C and 15 sec at 60°C. Standard curves were created for each primer pair using 10-fold dilutions of amplicons generated using an *Escherichia coli* strain as the DNA template for the 16S rDNA primers and *B. multivorans* for the *Burkholderia*-specific primers. DNA copy number per gram of sputum was calculated for each sample based on a standard curve with a $1x10^5$ fold linear range in CT values.

2.6 Statistical Analyses

To determine if the aliquots from each portion (A, B) were representative of the total, a two-way between subject analysis of variance (ANOVA) for each group was performed. Individual sputum samples were divided into two equal portions based on their weight (in grams), "A" and "B". Portion A was used to determine the effect of MH on the difference of the means of abundance by comparing homogenized and non-homogenized aliquots. Portion B was used to determine if the effect of MH reduces the difference in abundance between aliquots from the same portion. We paired the samples based on each portion and the aliquot designation (Table 1). Aliquots were designated by the sample number and homogenization status (CH or CH+MH) as two independent variables of the $\log_{10}$ total bacterial abundance or $\log_{10}$ *Burkholderia* abundance. Using a two-way between subject ANOVA in R programming language with the following model to compare the abundance of all bacteria and of *B. multivorans* as a function of the nine samples using the CH and CH+MH aliquots as the interaction term:

$$Y_i = B_0 + B_1 X_i + e_i$$

If we hypothesize that B_0 = no difference in the means, the full equation becomes:

$$Y_i = B_1(\text{sample}) + B_2(\text{aliquot}) + B_3(\text{sample} * \text{aliquot}) + e_i$$

A meta-analysis was used to summarize the effect of MH on the total and *Burkholderia* abundance of each sample. The Hedges' *d* effect size (δ) measure was used by treating each sputum sample independent of one another [6]. Each portion was treated as a separate group within each sputum sample and the number of aliquots within each group as the sample size.

3. Results

To determine if an aliquot of sputum is representative of the whole sample, we compared CH and CH+MH aliquots from nine sputum samples collected from a CF patient. Each sputum sample was divided in half (portion A and B) and each half into six equal aliquots. Four of the six aliquots from portion A were subjected to MH. Each of the aliquots from portion B were subjected to MH, with the first two aliquots paired with the CH aliquots from portion A and the other remaining four aliquots paired with the CH+MH aliquots from portion A.

Our two-way ANOVA model showed a significant difference in the means of total bacterial abundance ($p = .04$) and abundance of *B. multivorans* ($p = .05$) between the CH and CH+MH aliquots in portion A (Figure 1A and 1C). Using the same model for the aliquots in portion B, we found no significant difference in the means of total bacterial abundance ($p = 0.76$) and abundance of *B. multivorans* ($p = 0.99$) between the two CH+MH aliquots paired with the two CH aliquots from portion A and the four CH+MH aliquots paired with the four CH+MH aliquots in portion A (Figure 1B and 1D).

We examined the effect of MH on bacterial abundance by using the Hedges' *d* effect size analysis of total bacterial and *B. multivorans* abundance as shown by Rogers et al. [6]. The mean $\log_{10}$ copy number for all bacteria and *B. multivorans* in the CH aliquots was 9.74 and 6.88 with a range of 8.75 to 10.34 and 5.85 to 8.71, respectively. Similarly, the mean $\log_{10}$ copy number for all bacteria and *B. multivorans* in the CH+MH aliquots was 9.77 and 6.96 with a range of 8.81 to 10.23 and 5.90 to 8.31, respectively. Jacob Cohen provides a guideline, if necessary, for interpreting the effect size by stating that an δ of 0.20 is a small effect, an δ of 0.50 is a medium effect, and an δ of ≥ 0.80 is a

large effect [7]. Table 1 shows the effect size on total bacterial abundance and *B. multivorans* for each of the nine sputum samples. Since the effect size was quite variable from one sample to the next, we calculated the overall effect size for total bacterial and *B. multivorans* abundance using a method similar to Rogers et al., in which the overall effect size (E) is weighted by the variance of each sample (V). We then calculated the standard error of the mean for the combined samples (s.e.m.$_E$.) [6]:

$$E = \frac{\sum_{i=1}^{9} \frac{1}{V_i} \times \delta_i}{\sum_{i=1}^{9} \frac{1}{V_i}}$$

$$s.e.m._E = \frac{\sqrt{\frac{1}{\sum_{i=1}^{9} \frac{1}{V_i}}}}{\sqrt{9}}$$

The overall effect size for total bacterial and *B. multivorans* abundance was -0.25 and -0.37 standard deviations from the mean, respectively.

4. Discussion

The method of processing sputum from CF patients varies from one study to the next [4, 8, 9]. DTT has been recommended for use as an agent to liquefy sputum since 1955 [10]. Additional liquefaction steps, such as MH, may be included but these are not routinely performed. Most labs will only use a small aliquot of the sputum and store the remainder at -80°C until further use.

A recent survey was performed in our lab that measured total bacterial abundance in multiple aliquots of sputum samples collected from a single CF patient in order to determine if standard sputum sampling methods contribute to variations in bacterial abundance. Many factors may contribute to sample-to-sample variation in bacterial

abundance such as sample viscosity, sample heterogeneity, and location in the lungs from which the sputum was expectorated. While the location in the lungs is indeterminable using expectorated sputum, ensuring consistency in aliquots from processed sputum is important since there is a possibility that individual aliquots used for later analyses may provide different results from DNA-based assays.

4.1 Aliquot Size and Storage Temperature May Affect Intrasample Variability

In this study, we immediately processed the sputum samples collected from the CF patient to ensure no other factors such as storage time and temperature would affect its characteristics or composition. For example, intrasample variability may be masked by the growth of the dominant bacterium in samples that remain too long at room temperature (see Nelson et al.) [3]. The ratio of aliquot volume to overall sputum+DTT volume may also affect the variability of the bacterial concentration between aliquots by diluting the sputum and potentially separating further the small areas of sputum which may consist of higher bacterial density. The aliquot size may vary according the amount of sputum obtained, amount needed for the DNA-based analysis, and the volume of DTT added. The results obtained here are based on our use of 400uL aliquots according to the manufacturer's recommendation for the DNA extraction kit.

4.2 Intrasample Variability is Reduced with Mechanical Homogenization

DTT is used in most sputum processing protocols, therefore; we removed it as a variable factor in our analysis by adding it to all samples. Although DTT is effective for liquefying sputum, some sputum samples with high viscosity remain difficult to pipette even after DTT treatment. With the high-speed disperser, the viscosity of the sputum is reduced which makes the sputum easier to aliquot through standard pipetting methods.

The volumes of majority of the sputum samples collected in this study were approximately one milliliter. Protocols for DNA extraction, such as the VIBE 1-2-3 Sample Purification Kit used in this study, usually require a portion of sputum smaller than that which may be expectorated from an adult with CF. As a result, only a small fraction of the sputum is used for analysis with the remainder placed in cold storage. Without processing the whole sputum sample with homogenization prior to obtaining an aliquot, there is a chance that any fraction of the sputum may not represent the whole sample due to the heterogeneity of bacterial distribution.

By treating the aliquots as a group within the independent sputum samples, we found that MH significantly reduces variability in total bacterial abundance and *B. multivorans* abundance. The two-way ANOVA, which compared the variability between the MH and non-MH aliquots, revealed a dramatic decrease in means of bacterial abundance between the CH and CH+MH aliquots (all bacteria, $p = 0.04$; *B. multivorans*, $p = 0.05$) in portion A and the difference in means between the CH+MH and CH+MH aliquots (all bacteria, $p = 0.76$; *B. multivorans*, $p = 0.99$) in portion B (Figure 1). These results indicate that using the high performance disperser increased the distribution of bacteria in the sputum.

4.3 The Effect of MH on Bacterial Abundance

Hedges' *d* effect size provides a measure for determining the number of standard deviations between the means of two groups. For our purposes we used effect size to determine the impact MH had on the total bacterial and *B. multivorans* abundance between groups of MH and non-MH aliquots. Through this measure, we can confirm that

mechanical homogenization does or does not have an impact on bacterial abundance which would generate a variable in downstream analysis.

We observed effect size variability between the sputum samples which did not reveal any discernible pattern (Figure 2). Based on Cohen's guidelines (see Results), we would conclude that MH had a large effect (>0.80) on some sputum samples and a small effect (<0.20) on others. However, Cohen cautions the use of these guidelines as a universal tool since the context of effect size can vary based on the experiment. Visually, we observed little difference between the MH and non-MH aliquots which indicates MH likely has little effect on abundance. Our observations were confirmed when we calculated the overall effect size of MH on total bacterial abundance and *B. multivorans* abundance (Figure 3). The standard error of the mean for each measure was greater than the overall size indicating that MH has no effect on abundance.

5. Conclusion

Mechanical disruption is not a new method for processing sputum [11]. However, the impact of mechanical means on decreasing the innate heterogeneity of highly viscous sputum has not, to the best of our knowledge, been reported. We recognize that the physical properties of our sputum samples are not reported here which is due to the lack of a consistent measure for the those properties, such as color, which might differentiate between sputum with high or low viscosity. And although we did not measure viscosity of our sputum samples, we did note that none of the samples could be pipetted until after the addition of DTT.

Mechanical disruption of the sputum had little effect on bacterial abundance as measured by qPCR but had a dramatic effect on decreasing the difference in mean

abundance between aliquots taken from the same sputum sample. Other methods of MH were not measured here, such as mixing through pipetting, but we show that the use of a high performance disperser is an effective method for homogenizing sputum without having an impact on bacterial abundance. Adding MH as an additional sputum processing step prior to obtaining an aliquot will ensure consistency in downstream analysis of any further aliquots obtained from the remainder of sputum that is typically stored in cold storage until further use.

Table 3.1. Effect of mechanical homogenization on the abundance of all bacteria and *B. multivorans* in nine sputum samples

	Total bacterial abundance			*B. multivorans* abundance		
Sample Number	δ	V	s.e.m.δ	δ	V	s.e.m.δ
A	-0.24	0.61	0.32	-0.22	0.61	0.32
B	0.25	0.61	0.32	-0.16	0.61	0.32
C	-1.32	0.60	0.32	-0.92	0.60	0.32
D	0.52	0.61	0.32	0.80	0.62	0.32
E	-2.04	0.60	0.32	-1.27	0.60	0.32
F	-1.00	0.62	0.32	-0.83	0.62	0.32
G	0.33	0.62	0.34	-1.50	0.63	0.34
H	1.44	0.60	0.32	1.80	0.61	0.32
I	-0.20	0.61	0.32	-1.03	0.61	0.32

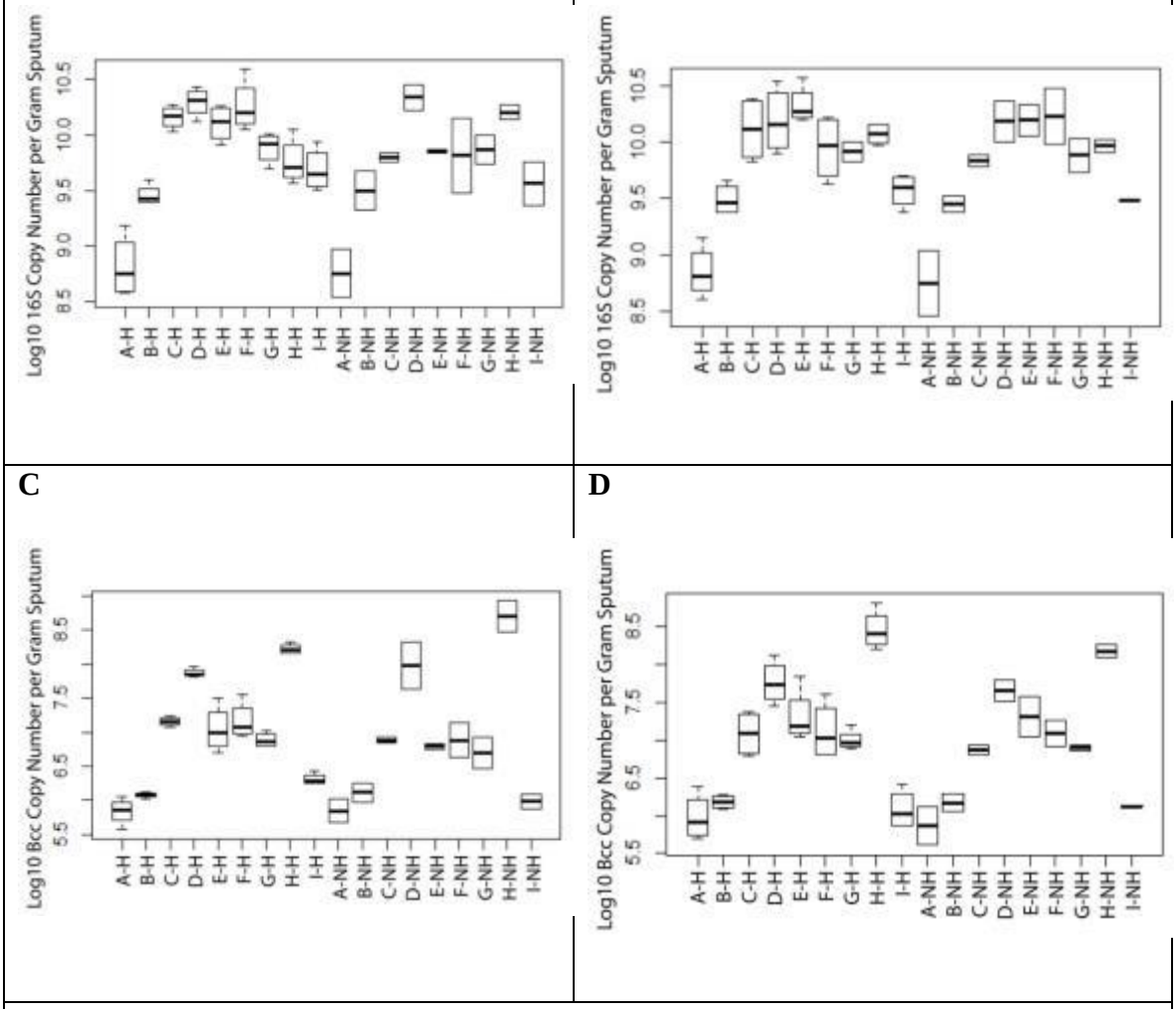
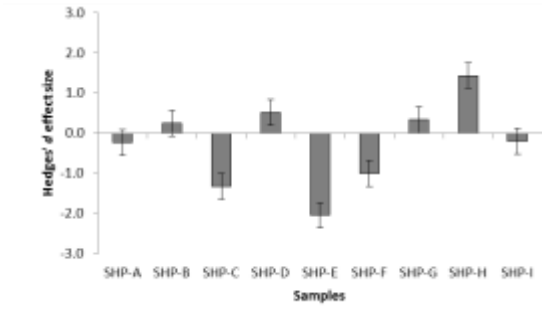


Figure 3.1. Effect of MH on variation of total bacterial and *B. multivorans* abundance between aliquots of nine sputum samples. Comparison of non-MH aliquots to MH aliquots showed significant variation (all bacteria, p = 0.04 (A); *B. multivorans*, p = 0.05 (C)). Comparison of MH aliquots to MH aliquots within the same sputum sample showed no significant difference in variation (all bacteria, p = 0.76 (B); *B. multivorans*, p = 0.99 (D))



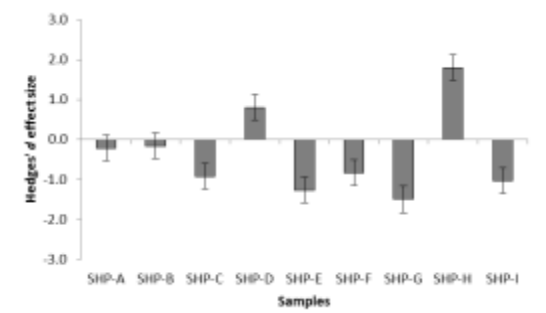


Figure 3.2. The effect of mechanical homogenization on the total bacterial abundance (A) and *B. multivorans* (B). The abundance is measured by qPCR for paired samples that have been homogenized (MH) or non-homogenized (no MH). The columns represent the effect size of mechanical homogenization on abundance. The error bars are determined by the s.e.m. of the effect size (δ). Any error bars which cross zero indicate no effect.

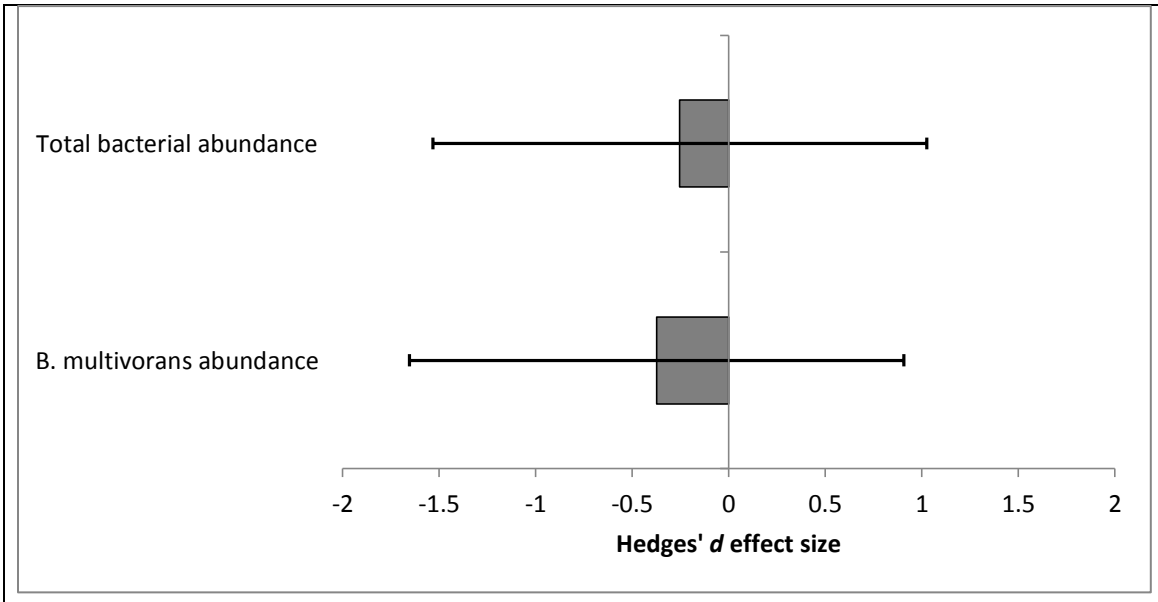


Figure 3.3. The overall effect of mechanical homogenization on the abundance of bacteria and *B. multivorans* in sputum. The abundance is measured by qPCR for paired samples, MH and non-MH. The columns represent the effect size of mechanical homogenization on abundance. The error bars are determined by the s.e.m. of the overall effect size (δ). Any error bars which cross zero indicate no effect.

CHAPTER 4: RAPID EMERGENCE OF A CEFTAZIDIME-RESISTANT *Burkholderia multivorans* IN A CYSTIC FIBROSIS PATIENT

1. Introduction

Burkholderia cepacia complex (Bcc) bacteria pose a serious health threat to Cystic Fibrosis (CF) patients due to the innate resistance to multiple antibiotics and ability to adapt to adverse environmental conditions through multiple mechanisms [1, 2]. Bcc bacteria infect approximately 2.5% of CF patients in the US and *B. cenocepacia*, *B. cepacia* and *B. multivorans* have been associated with a form of septicemia known as "cepacia syndrome" [3, 4]. While studies have described the adaptive responses of *Pseudomonas aeruginosa*, equivalent studies that examine the adaption of Bcc species in the CF lung are lacking [5]. Here we document *in vivo* the emergence of a resistant strain of *B. multivorans* during in-hospital antibiotic treatment for a pulmonary exacerbation (PE) and the decreased response to subsequent antibiotic treatment.

2. Methods

2.1 Patient and Sample Collection

A 30-year-old male diagnosed with CF at two weeks of age was hospitalized on January 4, 2010 for a course of intravenous antibiotics due to an acute worsening of signs and symptoms consistent with a PE. Approximately four weeks later, the patient was readmitted to the hospital with symptoms of a PE. Sputum culture results from the second admission revealed the presence of ceftazidime-resistant *B. multivorans*.

Expectorated sputum samples were obtained from the CF patient twice weekly for a period of almost three years (IRB Protocol Approval # 11-12-36). Samples were collected each morning by the patient, placed on ice during transport to the lab, and then stored at -80°C. Single colonies of *B. multivorans* were obtained from sputum enriched in *Burkholderia cepacia* selective broth (BCSB) at 37°C for 48 hours by spreading diluents onto *Burkholderia cepacia* selective agar (BCSA) plates [6]. A single colony from the BCSA plate was used to inoculate 2mL of LB broth and incubated 48 hours at 37°C to be used for antibiotic susceptibility testing.

2.2 *B. multivorans* Ceftazidime-Resistance Determination

Ceftazidime-resistant colonies were identified by transferring single colonies from BCSA onto LB agar with and without 15 µg/mL ceftazidime (MIC determined using a standard macrodilution tube method [7]) and scoring for growth after 48 hours at 37°C. Ceftazidime-resistance was measured in isolates from sputum samples spanning a 26-month period beginning March 2009, two months following hospitalization for PE treatment in January 2010 and ending May 2011, 18 months after hospitalization. Percent resistance was measured by replica plating approximately 200-400 colonies isolated from each of 13 sputum samples onto LB +/- 15 µg/mL ceftazidime, and scoring for growth following incubation at 37°C for 48 hours.

2.3 Sample Processing

Each sputum sample was mixed with a 1:3 ratio of sputum to a 0.1% dithiothreitol solution and incubated at 37°C for 1 hour followed by mechanical homogenization for 1 minute using a micro blender. Propidium monoazide (Biotium, Hayward, CA) was then added to a final concentration of 50 µmol/mL, and DNA-cross linking was induced using

a 400-watt halogen light source [8]. DNA was extracted using the IT 1-2-3 VIBE Sample Purification Kit (Idaho Technologies, Salt Lake City, UT).

2.4 qPCR Methods

qPCR using Perfecta SYBR Green FastMix Reagent Low ROX (Quanta Biosciences, Gaithersburg, MD) was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with a detailed protocol described in [9]. Universal primers [10] targeting a 16S rDNA fragment of 466bp were used to measure total bacterial abundance. Bcc-specific primers [11] targeting members of the *Burkholderia cepacia* complex generated a fragment of 333bp. Standard curves were created using 10-fold dilutions of amplicons generated using *B. multivorans* DNA as a template.

2.5 MLST Analysis

Multilocus sequence typing (MLST) was performed *in silico* to determine the relatedness of isolates examined during the study period. Concatenated sequences of *B. multivorans* DNA collected as part of a whole-genome Illumina sequencing analysis, performed by Dr. Raad Gharaibeh in collaboration with Dr. Anthony Fodor and not included in this dissertation, were used to determine the sequence identity of the seven housekeeping genes used to differentiate strains according to Baldwin et al. [12]. ClustalW was used to align the sequence of each MLST locus and determine the similarity of alleles between isolates [13]. To obtain an allelic profile and clonal complex designation, PubMLST was used to align each locus to the *Burkholderia cepacia* complex database. These will be available at the *Burkholderia cepacia* complex database (http://pubmlst.org/bcc/). Both PubMLST and NCBI BLAST were used to align the *recA*

gene and a DNA sequence spanning the V1-V3 region of the 16S rRNA gene, respectively, to verify the species identity [14].

3. Results

In vivo acquisition of ceftazidime-resistant *B. multivorans* was detected by examining changes in the percentage of resistant colonies. In the four sputum samples collected before January 14, 2010, no ceftazidime-resistant *B. multivorans* colonies were detected. In the January 14, 2010 sample, 78% of colonies were resistant. This percentage ranged from 69% to 97% in six of the subsequent eight samples collected over a 26-month period (Figure 1). A decrease in the percentage of resistant colonies to 1% and 7% in two sputum samples collected five months after the resistant strain of *B. multivorans* was first detected did not correlate with administration of antibiotics or patient health. To determine if the percent resistance was connected to changes in total bacterial abundance, qPCR was used to measure abundance of all bacteria and *B. multivorans* from all sputum samples. The results (Figure 1) indicate there was a correlation between abundance of *B. multivorans* and percent resistance but not for total bacterial counts.

The change in abundance resulting from antibiotic treatment was compared for three exacerbations that occurred prior to the detection of ceftazidime resistance (32 samples) with four subsequent exacerbations (34 samples). There was a statistically significant (Student's t-test) decrease in abundance caused by non-ceftazidime antibiotics for the first three exacerbations but not for the four post-acquisition exacerbations (Figure 2).

Appearance of ceftazidime-resistant Bcc could occur due to acquisition of a new Bcc strain or selection of an existing Bcc strain. To determine if the ceftazidime-resistant and -sensitive strains are clonal, the sequences of five of the seven genes used for MLST analysis were compared and found to be 100% identical for 12 isolates (Table 1). The only exception was *gyrB* which was 96% identical for the AS130A, AS130B, and AS131 isolates due to a partial lack of sequence coverage in the first 41 nucleotides. The *trpB* locus for each isolate was not usable for alignment due to multiple N's within the sequence generated during assembly (data not shown). An exact allele match was found for six loci when compared to the PubMLST database while the *trpB* locus showed a near match to two different alleles (Table 1). All isolates shared 100% identity in six alleles with each allele corresponding to a *B. multivorans* isolate in the PubMLST database. BLAST showed a sequence similarity of 99% in the V1-V3 region of the 16S rRNA gene when compared to the *B. multivorans* ATCC 17616 reference strain and exact match of the *recA* gene to *B. multivorans* in the PubMLST database was found for all isolates, confirming their identity as *B. multivorans* (Table 1).

In vitro testing of four ceftazidime-sensitive and four ceftazidime-resistant isolates for resistance to two other antibiotics commonly used to treat *B. multivorans* infections, trimethoprim/sulfamethoxazole (TMP-SMZ) and minocycline, showed no significant difference and was consistent with the result of clinical susceptibility tests performed during the sampling period (data not shown). No other beta-lactam, such as meropenem, was tested since *B. multivorans* was shown to be consistently resistant to this carbapenem in clinical analyses of isolates from sputum samples collected prior to

and during the study period; indicating the acquisition of ceftazidime resistance occurred independent of treatment with any other antibiotic.

4. Discussion

Here we document a dramatic *in vivo* change in the resistance profile of *B. multivorans*, a major CF pathogen. Clinical analysis of *B. multivorans* showed a change in the susceptibility profile from sensitive to intermediate ceftazidime resistance in a period of one month (data not shown). We were able to narrow that window to a period of four days, suggesting that administration of the antibiotic caused the increase in *in vivo* resistance. The increase in percent resistance occurred without a change in the total *B. multivorans* abundance. Although phenotypic variability has been shown to exist within multiple isolates from the same patient in a given sputum sample [15], repeated testing of every isolate collected during the 26-month period for antibiotic susceptibility and MIC indicates the phenotype is stable. While the lower percentage of resistant isolates shown in Figure 1 cannot be explained at this time, the stability of antibiotic resistance profile for each isolate from every time point indicates this observation reflects a change in the population profile (ratio of ceftazidime-resistant/sensitive) and not a chance observation. A transient decrease in percentage of *B. multivorans* that were ceftazidime-resistant (Figure 1) may reflect normal changes that occur in the microbial community, though this model would differ from the current consensus on the stability of the lung microbiome [16]. Alternatively, this phenomenon may have occurred due to an unidentified environmental change. And although spatial heterogeneity of microbial communities has been observed in ex-plant and post mortem CF lungs, we attempted to resolve some of

this potential variability by using identical methods for obtaining each sputum sample [17].

Bcc are intrinsically resistant to multiple antibiotics including β-lactams, aminoglycosides, and fluoroquinolones [18]. Acquisition of resistance to one of the few antibiotics known to be effective against Bcc such as ceftazidime is suggested to arise from antibiotic stress, however, the exact mechanism has yet to be elucidated [2, 19]. Our observation of no recognizable pattern of resistance to TMP-SMZ or minocycline associated with the ceftazidime-resistant *B. multivorans* strain suggests resistance is not due to a typical multi-drug resistant mechanism as seen in previous Bcc studies [20]. A statistically significant decrease in abundance of *B. multivorans*, but not of total bacteria, caused by non-ceftazidime antibiotics for the first three exacerbations (Figure 2) suggests that acquisition of resistance affected the response of *B. multivorans* to treatment with more than ceftazidime and a measurable change in the phenotype.

Documentation of the acquisition of a change in phenotype in a given strain requires confirming the identity of the isolates and that those collected over time are clonal. We were able to identify each isolate to the species level by aligning the V1-V3 region of the 16S rRNA gene to a *B. multivorans* reference genome and the *recA* locus to the PubMLST database. Each MLST locus, included *recA*, matched an allele that only corresponded to a *B. multivorans* isolate in the PubMLST database. The comparison of MLST loci along with the 99% similarity of the 16S rRNA gene sequence of the isolates to the *B. multivorans* ATCC 17616 reference genome indicates all of the isolates are of the same species. We characterized the allelic profile of six genes used for MLST analysis in each of the sequential isolates collected during the 26-month period and found

a near match to three sequence types (ST), including 198, 417, and 659. A near match for clonal complex 198 was identified, based on the ST for each isolate, suggesting all isolates are clonal. Through MLST analysis, we were able to determine that ceftazidime resistance is likely due to *in vivo* acquisition of resistance.

We have shown that acquisition of antibiotic resistance can lead to decreased reduction in the abundance of *B. multivorans* during subsequent antibiotic treatment for exacerbations. While there is low expectation of eradicating pathogens in CF due to complex issues surrounding treatment of persistent bacterial infections, identifying the emergence of resistant strains impacts treatment options.

Table 4.1. Characterization of *B. multivorans* isolates and comparison to the PubMLST *Burkholderia cepacia* complex MLST database

Isolate No.	Date of Isolation	Ceftazidime Susceptibility[d]	MLST[e] Isolate:Isolate Sequence Identity[a]							BLAST result[f]	Allele Identity Based on MLST[e] Isolate:PubMLST database comparison[b]							Nearest match clonal complex from PubMLST database
			atpD	gltB	gyrB	recA	lepA	phaC	16S rRNA[c]	16S rRNA[c]	atpD	gltB	gyrB	recA	lepA	phaC	trpB	
AS137	02/01/10	S	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS139	02/01/10	S	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS142	03/07/09	S	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS144	03/07/09	S	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	357	198
AS149	05/26/11	S	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	357	198
AS150	05/26/11	S	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS130A	02/01/10	R	100%	100%	96%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS130B	02/01/10	R	100%	100%	96%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS131	02/01/10	R	100%	100%	96%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS132	02/01/10	R	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS154	05/26/11	R	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198
AS155	05/26/11	R	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	357	198
AS158	05/26/11	R	100%	100%	100%	100%	100%	100%	100%	99%	9	50	84	141	37	96	7	198

[a] Comparison of gene sequence between isolates

[b] Each PubMLST allele is an exact match for the *B. multivorans* strain except allele 357 which has a partial match at the trpB locus

[c] Based on primers targetting the V1-V3 region of the 16S rRNA gene

[d] Based on a minimum inhibitory concentration (MIC) of 15µg/mL (S - sensitive; R - resistant)

[e] MLST (multilocus sequence typing)

[f] Comparison of gene sequence to *Burkholderia multivorans* reference strain ATCC 17616

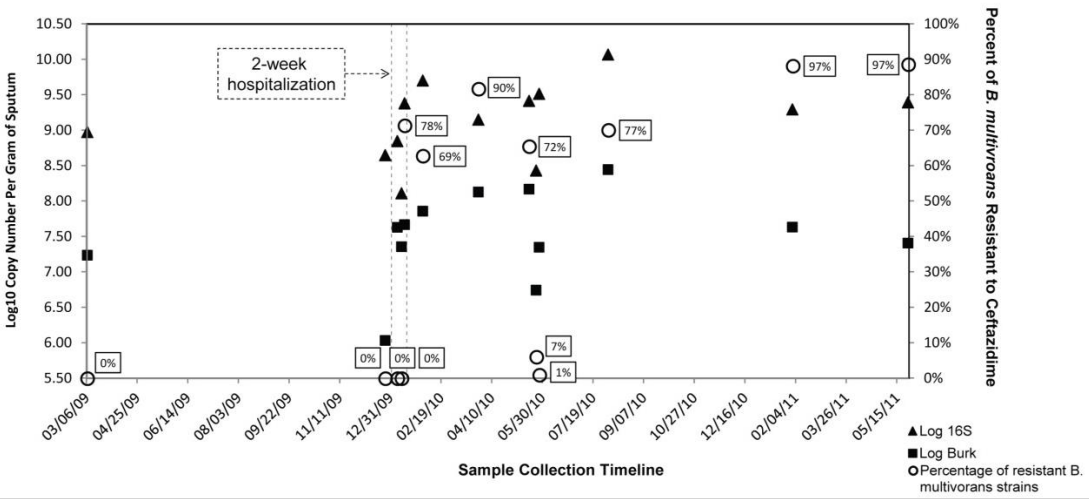


Figure 4.1. Total bacterial abundance, *Burkholderia*-specific abundance, and percent of ceftazidime-resistant *B. multivorans* colonies measured from sputum samples. DNA isolated from sputum samples as described in the methods, was subjected to qPCR using *Burkholderia cepacia* complex-specific 16S rDNA primers (9). The abundance values, expressed as copy number, represent the averages of the three replicates. The dates of the study period are given. During the 2-week hospitalization, the patient was treated with 300(mg) inhaled tobramycin twice per day, 750(mg) of oral ciprofloxacin twice per day, 2(g) intravenous injection of ceftazidime three times per day, 100(mg) oral minocycline once per day, and 500(mg) oral azithromycin for two days while in the hospital and then sent home to continue intravenous treatment for 10 additional days. After 18 days, ciprofloxacin and minocycline were discontinued following the relief of symptoms.

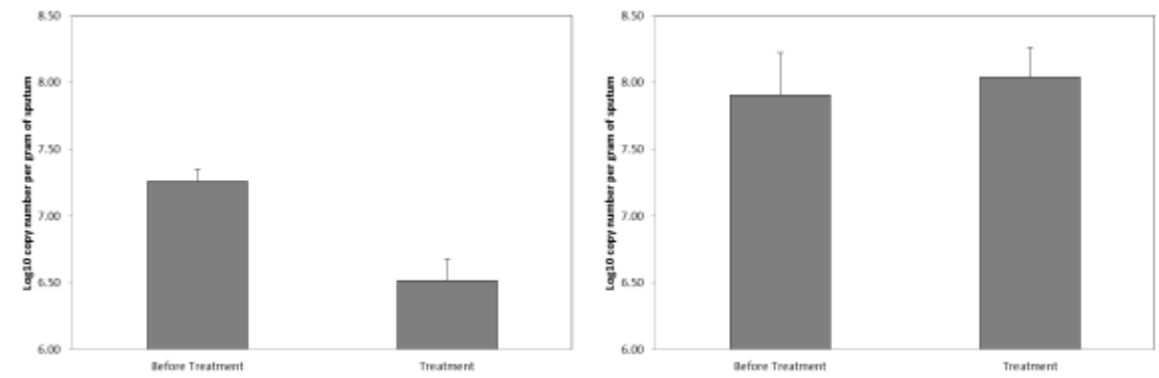


Figure 4.2. Effect of antibiotics on abundance of *B. multivorans* in sputum samples before and after treatment. Antibiotics given prior to the acquisition of the ceftazidime-resistant strain (A) caused a significant decrease (P = .01 by Student's t-test) in abundance during treatment for an exacerbation (three exacerbations spanning the following dates: 3/13/09-4/09/09; 6/23/09-6/26/09; 11/05/09-11/30/09). Antibiotics given to treat an exacerbation that occurred after the acquisition of the ceftazidime-resistant strain (B) showed no significant change (P = .63 by Student's t-test) in abundance during treatment for an exacerbation (four exacerbations spanning the following dates: 1/04/10-1/22/10; 4/12/10-4/18/10; 7/09/10-7/25/10; 3/03/11-3/16/11).

CHAPTER 5: ANALYSIS OF CHANGES IN DIVERSITY AND ABUNDANCE OF THE MICROBIAL COMMUNITY IN A CYSTIC FIBROSIS PATIENT OVER A MULTI-YEAR PERIOD

1. Introduction

Chronic airway infections are the leading cause of death in the majority of those with Cystic Fibrosis (CF) [1]. Acute bacterial infections are the major cause for pulmonary exacerbations (PE) [2, 3]. The frequency of PEs has been connected to mortality and may result in permanent lung function impairment [2]. Early intervention could reduce the length and severity of a PE; however, attempts at developing tools to predict a PE have been met with limited success [4, 5].

DNA-based techniques developed in the past 15 years have shown a diverse community of microbes exists in the CF airways leading to reclassification of infections in CF as polymicrobial [6]. These culture-independent studies have caused a paradigm shift away from treating CF lung infections as being caused by a single-agent towards a focus on multiple species. If bacteria are involved in eliciting a PE, then bacterial population changes could be used as an early indicator of a PE [5]. Identifying such changes to the microbial community that precede PE onset requires continuous samples from multiple patients since a single sputum sample can only provide a snapshot of the community profile at any given time [7, 8].

Combining T-RFLP and weekly sampling of 12 patients over a 12-month period, Daniels et al. were able to show that mean taxa richness decreases in response to

antibiotic treatment using sputum samples obtained up to four weeks bracketing the start of treatment for a PE [9]. Daniels et al. used quantitative PCR (qPCR) and the same set of patients and samples in another study and showed that bacterial density does not change in one to three weeks prior to the onset of a PE [8]. Data from the Cystic Fibrosis Foundation Patient Registry shows the changes in the percent of dominant pathogens that occur as an individual with CF becomes an adult [3]. While *Staphylococcus aureus* and *Haemophilus influenza* dominate in early childhood, their abundance slowly decreases as their prevalence is replaced by *Pseudomonas aeruginosa*. Little change in the abundance of the dominant pathogens appears to occur even with the use of antibiotics which indicates that once established the primary pathogens remain dominant in the lungs. Our long-term study was designed to determine if changes in bacterial diversity and abundance prior to the onset of antibiotic treatment or a PE could be observed within the preceding week and if next generation sequencing could reveal population changes undetected in previous studies.

We hypothesized that quantitative analysis of bacterial DNA in sputum samples would reveal changes in abundance of all bacteria, *Pseudomonas* spp. and members of the *Burkholderia cepacia* complex (Bcc), including an increase within 30 priors to a PE, a decrease during antibiotic treatment, and increase to baseline within 30 days after a PE. We also hypothesized that sequencing the bacterial DNA in frequently collected sputum samples would reveal taxa which contribute to onset of a PE by identifying changes in their relative abundance prior to, during treatment of, and after the PE.

We used Illumina sequencing and qPCR to examine changes in the microbial community diversity and abundance of all bacteria, *Pseudomonas*, and Bcc in sputum

samples collected from a 30-year old CF patient at least once a week over a three year period that included nine PEs. Our study is the first to demonstrate a stable microbial diversity coupled with a gradual change in abundance of all bacteria, *Pseudomonas*, and Bcc during a long-term sampling period. Monitoring these changes to individual taxa as well as total bacteria allowed us to determine how different pathogens change over time and respond to antibiotic therapy

2. Methods and Materials

2.1 Patient Characteristics

The 30-yr-old adult male subject, diagnosed with CF at two weeks of age, voluntarily participated in this study. His treatment regimen during the study included oral enzymes for CF-related malabsorption, along with various antibiotics (Table S1) for PEs. The subject has a heterozygous deltaF508/unknown CFTR genotype and no other CF-associated complications. The FEV1 (forced expiratory volume in one second) values measured during clinic appointments over the course of the study were consistently <30% which is indicative of advanced stage lung disease.

2.2 Samples

Expectorated sputum samples were obtained with from our CF patient twice weekly for a period of almost three years (IRB Protocol Approval # 11-12-36). Samples were collected each morning by the patient expectorating sputum into a 15 mL Falcon tube, placed on ice during transport to the lab and then stored at -80°C until use. Samples were chosen for analysis based on the day of collection nearest a PE.

2.3 Sputum Homogenization, Viable Cell Selection, and DNA Extraction

Each sputum sample was mixed with a 1:3 ratio of sputum to a 0.1% dithiothreitol solution and incubated at 37°C for one hour followed by mechanical homogenization for one minute at 7,000 rpm using a high-performance disperser (IKA ULTRA-TURRAX® T-25 digital, Staufen /Germany). Propidium monoazide (Biotium, Hayward, CA) was then added to a final concentration of 50 µmol/mL, and DNA-cross linking was induced using a 400-watt halogen light source [10]. DNA was extracted using the IT 1-2-3 VIBE Sample Purification Kit (Idaho Technologies, Salt Lake City, UT) and its concentration determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). All extracted DNA was immediately stored at -20ºC until its use for quantitative polymerase chain reaction (qPCR).

2.4 Abundance of All Bacteria, *Pseudomonas*, and *Burkholderia* in Sputum

The qPCR mixture contained 10 µL Perfecta SYBR Green FastMix Reagent Low ROX (Quanta Biosciences, Gaithersburg, MD), .5 µL of 100 pmole/µL of each primer, 5 µL of DNA, and 4 µL of nuclease-free water to a final volume of 20 µL. Universal primers [11] were used to target a 16S rDNA fragment of 466bp and measure the abundance of all bacteria in the sample. Bcc-specific primers [12] and *Pseudomonas*-specific primers [13] were used to target each genus and generate a fragment of 333bp and 93 bp, respectively. qPCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with an initial step of 10 min at 95°C, then 40 cycles of 15 sec at 95°C and 1 min at 60°C. Melting curves were determined following the qPCR by 1 cycle of 15 sec at 95°C, 1 min at 60°C, 30 sec at 95°C and 15 sec at 60°C. Standard curves were created for each primer pair using 10-fold dilutions of

amplicons generated using an E. coli strain as the DNA template for the 16S rDNA primers, *Pseudomonas aeruginosa* for the *Pseudomonas*-specific primers, and *Burkholderia multivorans* for the Bcc-specific primers. DNA copy number per gram of sputum was calculated for each sample based on a standard curve with a $1x10^5$ fold linear range in CT values.

2.5 Illumina Sequencing Library Preparation

Samples were prepared for 16S rRNA gene Illumina sequencing targeting the V6 hypervariable region with a two-stage PCR strategy. Samples were PCR amplified in the first stage using primers that included barcodes in both the forward and reverse oligonucleotides for sample identification in a multiplex fashion. A secondary stage of PCR utilized a set of primers that overlapped the 5' ends of the first set of primers, and added bases complementary to the Illumina flow cell adapters for sequencing (Table S2). Thermalcycling conditions were as follows: An initial denaturation step at 94°C for 3 minutes was followed by a touchdown protocol beginning at 94°C for 45 seconds; 61°C for 45 seconds with 1°C drop each cycle for a total of 5 cycles; an additional 15 cycles at 51°C for 45 seconds; 72°C for 45 seconds and a final elongation at 72°C for 2 minutes. Fifteen µl of the first PCR products were utilized in the second stage of PCR. The second PCR consisted of one denaturation step of 94°C for 3 min; 15 cycles at 94°C for 45 seconds; 65°C for 45 seconds; 72°C for 45 seconds and a final extension step at 72°C for 2 minutes.

PCR fragments were visualized on a gel, quantitated on a NanoDrop ND-3300 (Thermo Scientific, Wilmington, DE) using PicoGreen® to determine the concentration of dsDNA and pooled in equimolar amounts for sequencing.

2.6 Sequence Mapping/Assembly

Illumina HiSeq2000 technology was used to sequence the 112 samples for this study. Raw paired-end sequences were processed as described previously [14] except that we required a minimum of 70 continuous matching nucleotides across the length of the ungapped alignment to produce each merged sequence. A total of 85,048,458 sequences with an average length of ~ 75 bases met our merging and extending criteria and those were fed into the program AbundantOTU+ v.0.93b (http://omics.informatics.indiana.edu/AbundantOTU/otu+.php) with the "-abundantonly" option. AbundantOTU+ clustered those sequences into 182 Operational Taxonomic Units (OTUs), incorporating 84,721,799 (99.62%) of all the merged sequences. The sequences that were not incorporated into an OTU were excluded from further analyses. For the purpose of detecting chimeric OTUs, we used UCHIME (http://www.drive5.com/uchime/) in conjunction with the Gold reference database; UCHIME did not report any chimeras in the 182 OTUs.

Taxonomic classification was achieved by first aligning the OTU sequences to the Silva database (release 108, http://www.arb-silva.de/) using BLASTn v. 2.2.26+ with an expectation value of e^{-5}. Then, the standalone version of the RDP classifier [15] v. 2.5 was used to classify the full-length Silva sequences with the best BLASTn match to the OTU sequence requiring an RDP confidence score $\geq 80\%$. This was done to compensate for the short read length of the generated OTUs. Raw counts for each OTU were normalized and log transformed according to the following equation:

$$\log_{10}\left\langle\left(\frac{\text{OTU raw count}}{\text{Number of sequences in sample}} \times \text{Average number of sequences per sample}\right)+1\right\rangle$$

and Principle Co-ordinate Analysis (PCoA) was done through mothur v.1.25.0 using Bray-Curtis dissimilarity matrix generated from the log normalized counts.

3. Results

3.1 Bacterial Abundance Measures

Quantitative PCR was used to measure the abundance of all bacteria and two targeted bacteria in the sputum samples. The total bacterial abundance was measured in each sample using universal primers and SYBR green chemistry to target the sequence spanning the V3-V4 region of the 16S rRNA gene. From the 130 samples we examined over the three year period, we found no pattern of short-term changes prior to or after the occurrence of an exacerbation. Using a linear regression model, a positive relationship ($p < .001$) was found between time and the abundance all bacteria, *Pseudomonas* and Bcc (Figure 1) which coincides with our visual observation of the data that the abundance of bacteria increases over time.

We used statistical modeling to test our hypothesis that a change in abundance occurs during antibiotic treatment for PE. Using the R programming language, we factored each category of samples into four levels according to the treatment status at the time of collection, samples collected ≤ 30 days prior to antibiotic treatment for a PE were categorized as “Before Treatment”, samples collected during antibiotic treatment for a PE were categorized as “Treatment”, samples collected ≤ 30 days after antibiotic treatment

for a PE were categorized as “Recovery”, and all other samples were categorized as “Stable”.

We observed a reduction in the abundance of all bacteria, which was not significant, when samples collected during “Treatment” ($p = 0.0522$) were compared to those samples collected during the “Stable” category. An increase in the abundance of all bacteria was observed when the “Stable” samples were compared to the “Before Treatment” (0.1781) and “Recovery” ($p = 0.2125$) samples (Figure 1A). A significant decrease in the abundance of *Pseudomonas* was measured when samples collected during “Treatment” were compared to those collected during the “Stable” ($p = 0.00020$), “Before Treatment” ($p = 0.00163$), and “Recovery” ($p = 0.00279$) periods (Figure 1B). When compared to “Stable”, the abundance of Bcc showed a significant increase in samples collected “Before Treatment” ($p = 0.00598$) and during “Recovery” ($p = 0.00101$) (Figure 1C). Little difference was seen in the abundance of Bcc in samples collected during “Treatment” ($p = 0.30715$) when compared to samples collected during the “Stable” category (Figure 1C).

To compare the change in abundance of the primary pathogens over time, we compared the abundance of *Pseudomonas* and Bcc to the abundance of all bacteria in the sputum samples. We observed a greater increase in abundance of Bcc as the abundance of all bacteria increased when compared to the change in abundance of *Pseudomonas* (Figure 2).

3.2 Bacterial Diversity Measures

We analyzed the diversity and richness of the microbial community across 72 of the sputum samples that were collected. We classified the sequencing reads to the genus

level with at least 80% RDP classification confidence. Out of the more than 84 million sequences, *Pseudomonas*, a typical CF pathogen, was the dominant genus representing greater than 90% of all sequences. Two other typical CF pathogens, *Burkholderia* and *Streptococcus*, were similar in abundance and made up approximately 6% of all sequences. The remaining sequences were mostly non-typical CF-associated bacteria [16] and classified as either *Veillonella*, *Rothia*, *Fructobacillus*, each of which consisted of ≥2% of all sequences, or other less prevalent genera, consisting of <1% of all sequences.

The microbial profiles of the individual sputum samples showed little variability across the study period (Figure 3). No pattern of change in relative abundance of the top nine most abundant genera was observed surrounding the occurrence of a PE. *Pseudomonas* was the most abundant genus and showed little change in relative abundance in the majority of the sputum samples. An increase in relative abundance of *Burkholderia*, *Streptococcus*, or *Rothia* was observed only those samples in which the relative abundance of *Pseudomonas* showed a short-term decrease (Figure 3A). The lesser abundant genera also varied little over time (Figure 3B).

No pattern of bacterial diversity was observed over time or surrounding the occurrence of a PE (Figure 4B). Shannon diversity ranged from 0.068 to 2.44 with no significant difference measured in samples collected during any one of the treatment status categories. A decrease in bacterial richness was observed and found to be significantly correlated with time ($p < .01$). The decrease in richness was highly significant when samples collected during "Treatment" ($p = 0.0001$) were compared to those samples collected during the "Stable" category. A significant decrease in bacterial

richness was measured when the “Stable” samples were compared to the “Recovery” (0.036053) samples with only a moderate decrease in richness in the “Before Treatment” ($p = 0.136562$) samples (Figure 4A).

4. Discussion

In this study of a single CF patient, we collected sputum samples twice weekly over a three year period. Our patient experienced nine PEs which required antibiotic intervention. Each PE was diagnosed by a CF specialty pulmonologist at an adult CF clinic. The focus of our analysis was on sputum samples collected before, during, and after treatment for a PE. For comparison, we also included samples collected during periods of stability, the time during which no antibiotics were taken within 30 days before or after of the occurrence of a PE.

Our goal was to determine if examining the changes in abundance may allow for prediction of an oncoming PE. While other studies have examined the use of biomarkers to aid in prediction of a PE and the progression of lung disease in CF [5, 17], our study was based upon the assumption that a PE is caused by a detectable shift in the bacterial community within 30 days preceding the PE. Testing this assumption required a longitudinal study to reveal the relationship between disease progression, occurrence of a PE, and the change in diversity or abundance in the microbial community [18]. We hypothesized that if a bacteria-derived biomarker can be identified, antibiotic treatment can be initiated early in the course of a PE and reduce the symptoms which would otherwise cause permanent damage to the lungs and permanent loss of lung function. Cross-sectional studies have demonstrated the diversity of the microbiota in the lungs of CF patients, but the time of collection between samples, which can be more than three

months, does not reveal potential short-term changes which may cause a PE [19]. Frequent patient sampling, or collecting multiple samples in a single month, becomes necessary since the length of time between changes in the bacterial factors associated with PE onset is unknown [20]. For this reason, we analyzed 130 sputum samples from a single patient over the three year period.

4.1 Change in Bacterial Abundance Over Time

No short-term changes in the absolute abundance of total bacteria, *Pseudomonas*, or Bcc occurred which would suggest a change in any of these factors initiated a PE. This finding is consistent with that of Stressman et al. who found no evidence of changes in bacterial density in sputum samples obtained 21, 14, and 7 days prior to the occurrence of a PE [9]. However, due to our extended sampling period, we were able to examine multiple samples spanning nine PEs and sample collected fewer than 7 days prior to a PE. Using this sampling frequency, we were able to capture multiple samples per PE and group them into categories relative to the occurrence of a PE. Although no change could be used to predict onset of a PE, our strategy revealed moderate changes that occur in absolute abundance of all bacteria before, during, and after a PE. Using the samples collected during the "Stable" period as a baseline for absolute abundance, total bacterial numbers increased prior to a PE, decreased during treatment, and then quickly recovered within 30 days following a PE. While we observed no statistically significant change in total bacterial abundance for any of the treatment categories, we did observe significant changes relative to the occurrence of a PE for *Pseudomonas* and Bcc. For *Pseudomonas*, the abundance seen during the "Treatment" period was significantly lower than the abundance in any of the other treatment status categories. This decrease indicates

antibiotic treatment is having an effect on reducing the bacterial load of *Pseudomonas* but does not indicate the effect on bacteria other than *Pseudomonas*. However, this effect may be due to the increased efficacy of antibiotics given to target the most abundant pathogen, *Pseudomonas*, and potentially decreased efficacy against other bacteria present in the samples. For example, the other most abundant pathogen measured in our samples, Bcc, showed a significant increase in abundance before and after a PE when compared to "Stable" and little difference in abundance during "Treatment". Based on the lack of a significant decrease in abundance of Bcc in samples collected during "Treatment" compared to "Stable", antibiotic treatment appears to have less of an effect on this bacterium which may be due to increased antibiotic resistance (see Chapter 4) or other unidentified factors.

The percent of *Pseudomonas* in each of the sputum samples using qPCR could not be determined. In some samples, we unexpectedly observed a higher copy number of *Pseudomonas* than the copy number using primers to target the 16S gene. We speculate there may be a difference in the efficiency of these two reactions due to a difference in amplicon size or to stochastic effects generated by primers targeting different regions of genomic DNA. For the purpose of our study, we could use both data sets since separate standards were generated for each primer set using the same genomic DNA for each reaction.

4.2 Change in Bacterial Diversity and Richness Over Time

A barcoded strategy for Illumina sequencing was used to determine the change in bacterial diversity and bacterial richness in a subset of the sputum samples collected during our study. Next generation sequencing has previously shown that little change in

diversity occurs over time in sputum samples collected from CF patients. A decade-long bacterial diversity study by Zhao et al. examined the diversity in sputum samples collected from six CF patients, three with moderate lung disease and three with advanced lung disease. Similar to our results, they detected no change in diversity between samples collected during periods of stability and periods before and after antibiotic treatment [18]. The authors did detect a significant decrease in diversity during antibiotic treatment which was not observed in samples collected from our patient. Similar to their conclusions and based on a lack of significant change in diversity between samples collected during our defined treatment categories; we determined that changes in diversity are unlikely to be involved in the occurrence of a PE.

Our observation of changes in bacterial richness over time and during treatment indicates that specific bacteria may be involved in initiating a PE (Table 1). Compared to "Stable", we observed a decreased richness in the samples collected prior to and after antibiotic treatment. A further decrease in richness, which was significant, was seen during antibiotic treatment. We were not surprised to see a decrease in richness during treatment since other studies have reported the same results from serially collected sputum samples [20]. A study by Daniels et al. suggested an inverse relationship between the mean relative abundance of *Pseudomonas* and bacterial richness during antibiotic treatment. Our qPCR results did not reflect that inverse relationship since we showed a significant decrease in both the absolute abundance of *Pseudomonas* and bacterial richness. This result along with the qPCR result above that indicates antibiotics are having an effect on *Pseudomonas* and an effect on the less abundant taxa, but to what degree is unknown. Antibiotics have been previously shown to have a greater effect on

other CF-associated bacteria than on *Pseudomonas*, which is likely attributed to the phenotypic diversity and increased antibiotic tolerance of *Pseudomonas* strains from the chronic CF infections [21].

Observing stability in the relative abundance of the bacterial community has been shown before with sputum samples collected over time from CF patients [22]. Similar to diversity in our samples, we detected little change in relative abundance over time. Our findings on diversity and relative abundance in a CF patient are similar to those seen by Goddard et al. in lung explants from CF subjects undergoing lung transplantation [22]. In the Goddard study, the lungs were dominated by few CF-associated pathogens including *P. aeruginosa*, *B. cepacia*, and *Achromobacter xylosoxidans*. That study also examined throat and sputum samples from patients collected just prior to transplantation and found a high discordance between the throat and lung explant samples. And while the sputum samples that were obtained did identify the dominant pathogen identified in the lung explant; a mixture of other, non-typical pathogens were also identified. These results and the results from a 2012 study by Fodor et al. using mouthwash samples, suggest normal oral flora may contaminate sputum samples to a degree in which their detection may be misinterpreted as part of the low abundant microbiota in the CF lung [23]. While we recognize the potential influence the oral microbiota may have on the changes in diversity, relative abundance, and richness in our sputum samples, the degree to which they are affected has not been fully determined. However, we must note that sputum is an accepted method for pulmonary sampling [24].

Ours is the first study to examine the diversity and abundance of bacteria in frequently collected sputum samples from a single CF patient over a multi-year period.

While we recognize the statistical limitations of using a single patient, we were able to identify changes over time which have yet to be documented in other quantitative and metagenomic studies of the CF lung microbiota. Our use of genus-specific primers along with qPCR to target both *Pseudomonas* and Bcc in sputum revealed a significant, positive relationship over time. Clinical measurements of lung function over this time period did not indicate a rapid decline in lung function. Perhaps the use of antibiotics, which had a significant effect on reducing the bacterial load of *Pseudomonas*, helped to maintain a relatively stable health condition. Interestingly, the abundance of *Pseudomonas* appeared to "level off" during the second half of our sampling period thus reaching a virtual threshold of abundance. The use of antibiotics, however, did not appear to affect the persistent increase in Bcc which may also be driving the onset of a PE. Because of the seemingly rapid changes in abundance between samples, we may not have observed this long-term change had we not chosen to collect sputum samples twice-weekly from the same patient. Based on the qPCR results alone, we can speculate that given enough time, Bcc might become the dominant pathogen, especially if the recovery of Bcc after antibiotic treatment is more rapid than that of *Pseudomonas*.

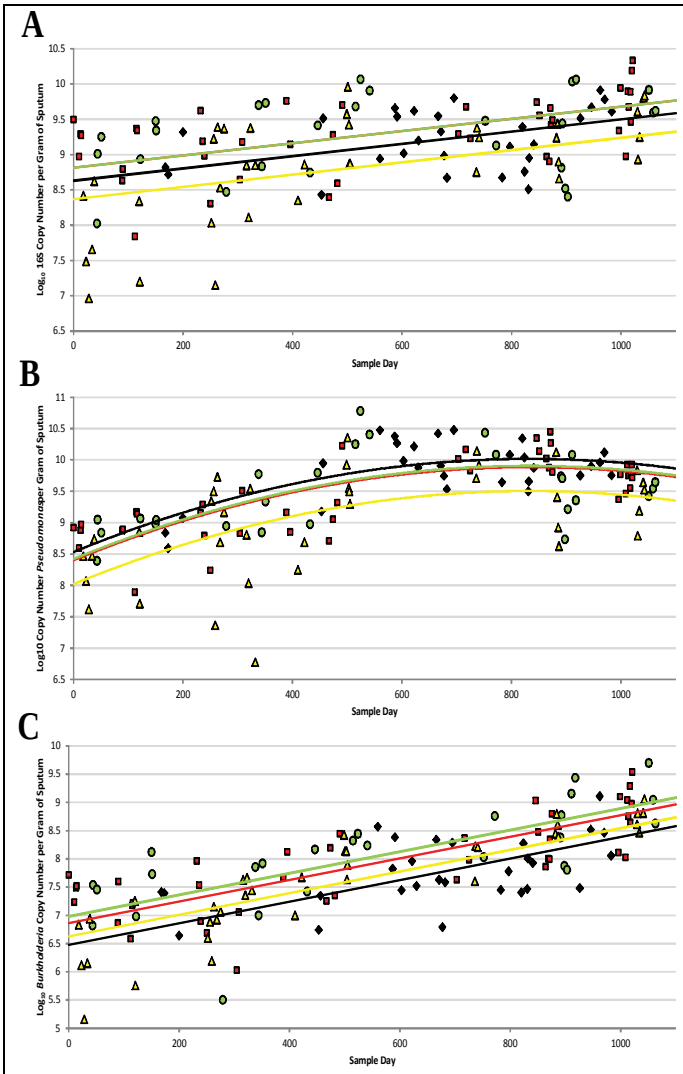


Figure 5.1. Variation in abundance of (A) all bacteria, (B) *Pseudomonas*, and (C) Bcc in sputum samples collected over a 35-month period during which nine exacerbations occurred. Treatment was considered to be during or within 48 hours of termination of antibiotics given to treat an exacerbation. Time points are plotted as ● "Before Treatment" (≤ 30 days before an exacerbation); ▲ "Treatment" (samples collected during and within 48 hours of termination of treatment); ■ "Recovery" (≤ 30 days after an exacerbation); and ◆ "Stable" (> 30 days prior to or after an exacerbation). Universal primers targeting the 16S rRNA gene, *Pseudomonas*-specific primers, and *Burkholderia*-specific primers were used to measure abundance which is expressed as $\log_{10}$ DNA copy number per gram of sputum (y-axis) over the collection period (x-axis).

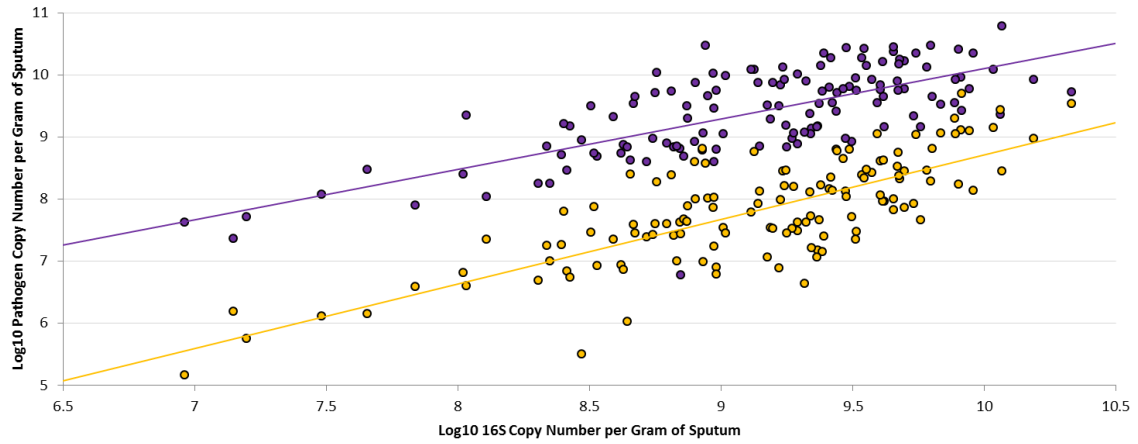
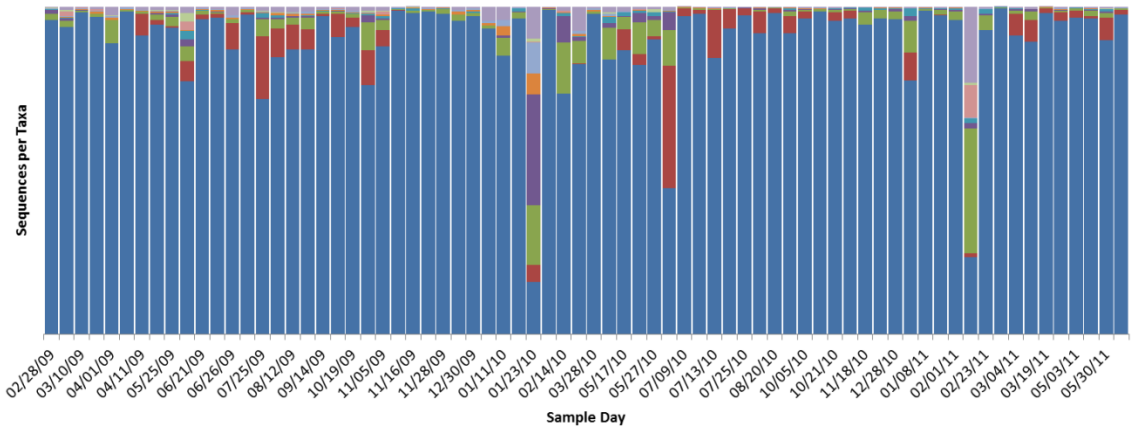


Figure 5.2. Comparison of the absolute abundance of primary pathogens, ● *Pseudomonas* and ● Bcc, to the abundance all bacteria. The abundance of both, *Pseudomonas* and Bcc, have a highly significant relationship ($p < .0001$) with an increase in the abundance of all bacteria.



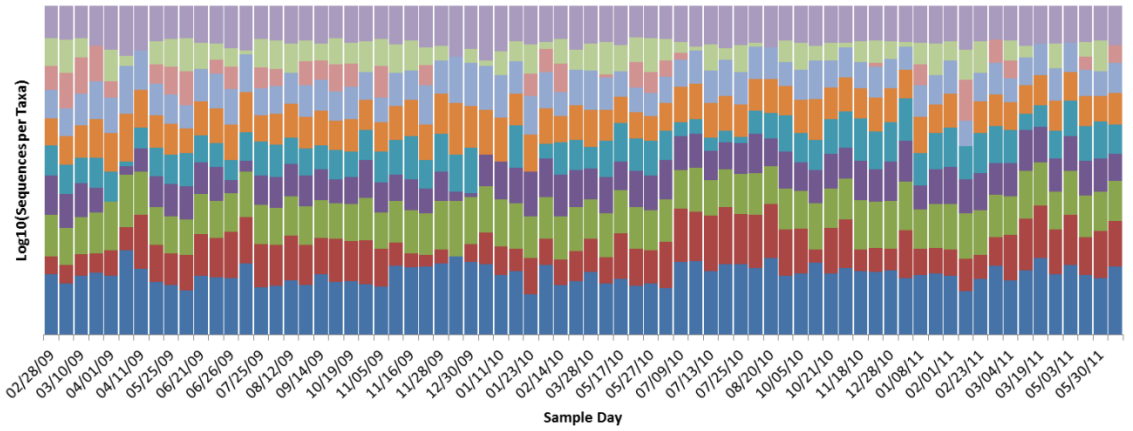
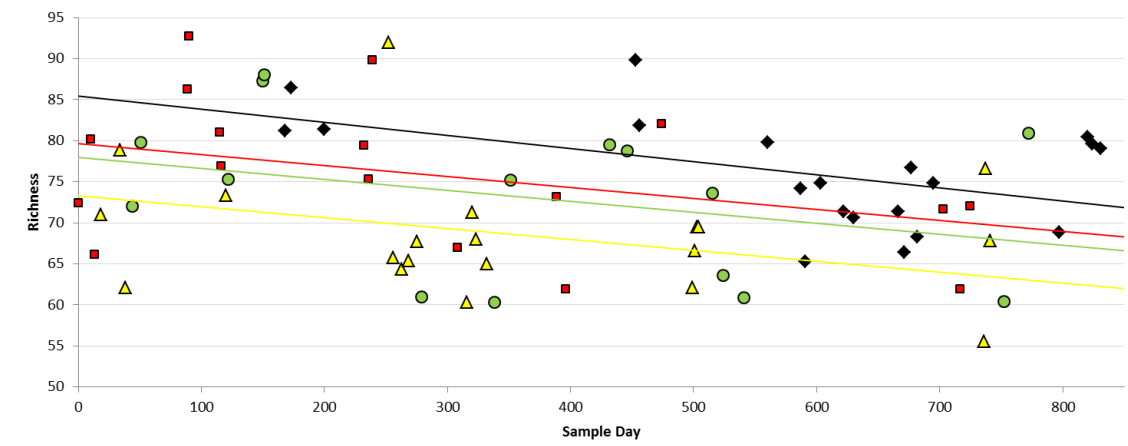


Figure 5.3. Relative abundance of taxa classified to the genus level. The top nine most abundant genera are shown (■ *Pseudomonas*, ■ *Burkholderia*, ■ *Streptococcus*, ■ *Rothia*, ■ *Veillonella*, ■ *Fructobacillus*, ■ uncultured bacterium, ■ *Haemophilus*, and ■ *Prevotella*). The relative abundances of all other genera, each representing <1% of the community, are grouped in the "■ Other" category. The colored bars represent the proportion of (unlogged (A), $\log_{10}$ (B)) reads mapped to each genus.



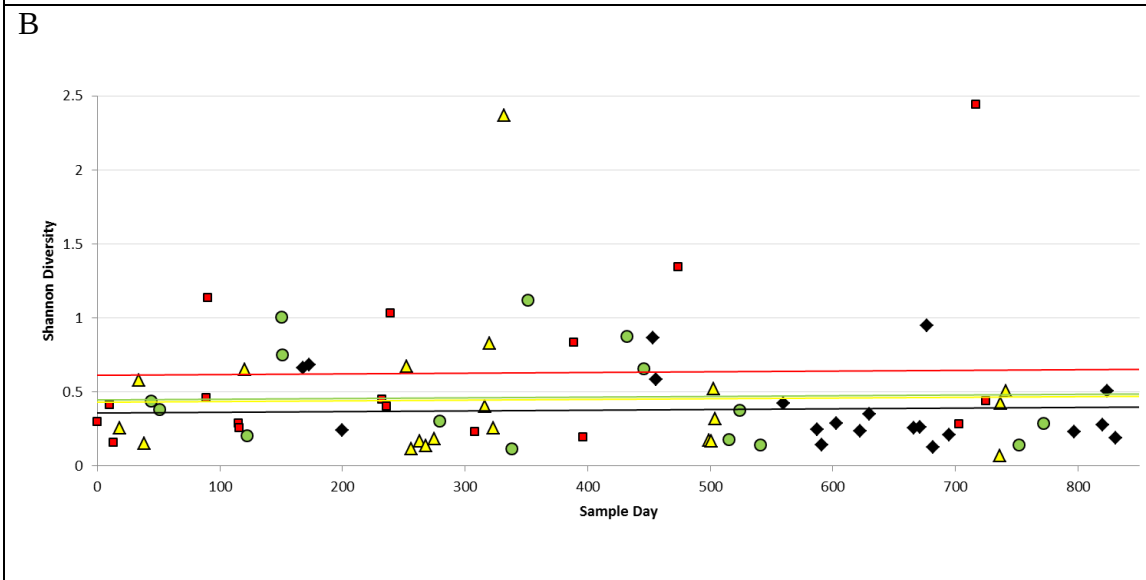


Figure 5.4. The change in bacterial richness (A) and bacterial diversity (B) from 72 sputum samples are shown over time. Time points are plotted as ● "Before Treatment" (≤ 30 days before an exacerbation); ▲ "Treatment" (samples collected during and within 48 hours of termination of treatment); ■ "Recovery" (≤ 30 days after an exacerbation); and ◆ "Stable" (> 30 days prior to or after an exacerbation)

Table 5.1

List of taxa classified to the genus level which significantly decreased or increased in relative abundance over time.

Genus	Adjusted p-value[1]	Original p-value	Slope	Intercept	Correlation coefficient	Percent of total sequences
Enhydrobacter	1.15E-07	7.68E-10	0.000877	-0.0998	0.44	0.024%
uncultured bacterium	1.22E-04	4.08E-06	-0.000660	1.1392	0.30	0.151%
Moryella	1.22E-04	2.05E-06	-0.000823	0.4934	0.31	0.043%
uncultured bacterium	1.22E-04	4.08E-06	-0.000660	1.1392	0.30	0.018%
uncultured bacterium	1.22E-04	4.08E-06	-0.000660	1.1392	0.30	0.006%
Fructobacillus	4.59E-04	1.83E-05	-0.000781	1.4114	0.26	0.233%
Oribacterium	1.07E-03	1.00E-04	0.000747	-0.0073	0.22	0.038%
Bacillus	1.07E-03	8.76E-05	-0.000509	0.8137	0.23	0.034%
Bacillus	1.07E-03	8.76E-05	-0.000509	0.8137	0.23	0.011%
Bacillus	1.07E-03	8.76E-05	-0.000509	0.8137	0.23	0.004%
Bacillus	1.07E-03	8.76E-05	-0.000509	0.8137	0.23	0.003%
Capnocytophaga	3.43E-03	3.43E-04	-0.000270	0.2092	0.23	0.004%
Deinococcus	2.45E-02	2.78E-03	-0.000275	0.2639	0.19	0.007%
Haemophilus	2.68E-02	3.22E-03	-0.000601	0.4822	0.21	0.131%
Escherichia-Shigella	4.50E-02	6.00E-03	-0.000230	0.3404	0.14	0.023%
uncultured Intrasporangiaceae bacterium	5.90E-02	8.26E-03	-0.000367	0.6620	0.12	0.047%

[1] Adjusted to a normal distribution using the Benjamini-hochberg method

Table 5.2. Antibiotic schedule for each PE

Antibiotic Category	Antibiotic	Sampling Period																																		
Oral	Ciprofloxacin	■	■	■	■	■	■	■	■	■	■	■			■	■	■	■	■										■	■	■					
	Bactrim	■	■	■	■	■	■	■				■	■	■				■	■																	
	Minocycline														■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
I.V.	Ceftazidime														■	■	■											■	■	■	■					
Exacerbation		1					2		3						4			5		6					7			8				9				

Table 5.3. Illumina primer sequences

	Sequencing primer	Barcode	Amplification primer
Forward			
1	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ATAGCG	CAACGCGARGAACCTTACC
2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	AGGGT	CAACGCGARGAACCTTACC
3	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TTCAT	CAACGCGARGAACCTTACC
4	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GATCGT	CAACGCGARGAACCTTACC
5	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GCCCGT	CAACGCGARGAACCTTACC
6	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTGTC	CAACGCGARGAACCTTACC
7	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CACGT	CAACGCGARGAACCTTACC
8	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CGTACG	CAACGCGARGAACCTTACC
9	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GGAC	CAACGCGARGAACCTTACC
10	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TAGA	CAACGCGARGAACCTTACC
11	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TCAT	CAACGCGARGAACCTTACC
12	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACTT	CAACGCGARGAACCTTACC
Reverse			
1	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	ATAGCG	ACAACACGAGCTGACGAC
2	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	AGGGT	ACAACACGAGCTGACGAC
3	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TTCAT	ACAACACGAGCTGACGAC
4	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GATCGT	ACAACACGAGCTGACGAC
5	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GCCCGT	ACAACACGAGCTGACGAC
6	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CTGTC	ACAACACGAGCTGACGAC
7	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CACGT	ACAACACGAGCTGACGAC
8	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CGTACG	ACAACACGAGCTGACGAC
9	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GGAC	ACAACACGAGCTGACGAC
10	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TAGA	ACAACACGAGCTGACGAC
11	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TCAT	ACAACACGAGCTGACGAC
12	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	ACTT	ACAACACGAGCTGACGAC

CHAPTER 6: ILLUMINA SEQUENCING ANALYSIS IS ENHANCED WITH QUANTITATIVE PCR WHEN EXAMINING CHANGES IN ABUNDANCE OVER TIME OF BACTERIA IN A CYSTIC FIBROSIS PATIENT

1. Introduction

DNA-based analyses of microbial communities from environmental samples have expanded our knowledge of the known number of bacteria in the environment. High-throughput sequencing (HTS) is a tool that is now used to examine microbial communities from various environment samples such as those associated with the Human Microbiome Project (HMP). This technology has also been used to examine bacterial infections associated with chronic diseases such as Cystic Fibrosis (CF). Bacterial infections in CF were once thought to be limited to few bacterial species but with the development of DNA-based sequencing analyses, an increasing number of taxa have been shown to inhabit the lungs and contribute to the progression of lung disease.

While metagenomic studies of mixed microbial communities have revealed taxa not previously associated with a particular environment [1], they are limited in quantifying relationships between organisms. As stated by Faust et al., "Since microbial counts are not known and measurements depend on sampling and sequencing depth, an increase in one relative abundance must be accompanied by a compositional decrease in another…" [2]. In Cystic Fibrosis, this limitation is more pronounced due to the typical dominance of one or two pathogens, which in adults with CF, is often *Pseudomonas aeruginosa*. Also, since PCR is often performed during library prep for HTS platforms

such as 454-FLX pyrosequencing and Illumina, the amount of DNA input is at best semi-quantitative and may be saturated towards the PCR reaction endpoint by the dominant organism [3, 4].

A quantitative analysis such as quantitative PCR (qPCR) can be performed to obtain absolute abundance measurements of these microbial communities. qPCR is a real-time quantitative assay which can be used to target one or more organisms in the microbial environment. Universal primers, targeting the 16S rRNA gene, have been previously used to determine the bacterial load in sputum samples [5]. Genus-specific primers can also be used to measure the abundance of a particular bacterium such as *P. aeruginosa* in the lungs of CF patients [5, 6].

The focus of many recent studies examining the microbial environment in CF has included using HTS to identify the organisms present and qPCR to quantify the total bacterial load [7-9]. These studies examine sputum samples collected from patients at various time points which provide a snapshot of microbial community composition and abundance. Few studies have used these methods in parallel to monitor the changes in the microbial community over time or in response to antibiotics. Using the same patient cohort, Stressman et al. and Daniels et al. used qPCR and T-RFLP to examine changes in the absolute abundance, relative abundance, and richness of both, all bacteria and of *P. aeruginosa* prior to an exacerbation [5, 6]. A significant increase the relative abundance of *P. aeruginosa* along with a significant decrease in taxa richness was observed during treatment when compared to the no treatment time periods [6]. However, no significant change in absolute abundance was observed prior to or after antibiotic treatment. A decade-long study of six individuals, with varying stages of lung disease, by Zhao et al,

used pyrosequencing and qPCR to show that while diversity decreases in CF over time, the total bacterial abundance remains stable [7]. Each of these studies provides an example of the advantage of using quantitative and sequencing analysis to characterize changes in the microbial community in CF.

Our study followed a single, adult CF patient over three years while collecting sputum samples twice weekly. The aim of our study was to examine long-term changes in the microbial community over time and in response to antibiotics used to treat pulmonary exacerbations, which are periods of acute illness marked by an increase in signs or symptoms of the disease. As part of the study, we used genus-specific primers and qPCR to measure the absolute abundance of *P. aeruginosa* and *Burkholderia multivorans*. We also used a bar-coded Illumina sequencing strategy to examine the changes in relative abundance of *P. aeruginosa* and *B. multivorans*. Using both methods on the same set of samples, we were able to compare the computational analysis of sequencing data used to generate the relative abundance of each taxa with the absolute quantification generated through qPCR. We found no correlation between relative abundance and absolute abundance of *P. aeruginosa* or *B. multivorans*. We did, however, see a difference in the output from each method which would lead to a possibility of two logical conclusions: 1. the abundance of the primary pathogens do not change over time or in response to antibiotics 2. the abundance of both of the primary pathogens increase gradually over time and decrease significantly in abundance in response to antibiotic use. Here, we highlight the differences in conclusions that may be drawn from each method separately and the characteristics of complex microbial communities that suggest when it is important to use both methods.

2. Methods (the same methods used here were used in Chapter 5)

3. Results

3.1 Sequencing

From the DNA sequencing data, we examined the changes in relative of abundance of the primary pathogens, classified as *Pseudomonas* and *Burkholderia*, for each of the PE categories above. No pattern of change was measured over time, with the occurrence of a PE, or in response to antibiotic treatment for *Pseudomonas* (Figure 1A) or *Burkholderia* (Figure 1B). The difference in relative abundance of *Pseudomonas* was not significant at any one point with a range of 2.99 to 6.67 $\log_{10}$ sequences. We observed similar results for the difference in relative abundance of *Burkholderia* which had a range of 0 to 5.57 $\log_{10}$ sequences.

3.2 qPCR

A significant decrease in the abundance of *Pseudomonas* was measured when we compared the samples collected during "Treatment" to those collected during the "Stable" ($p = 0.00020$), "Before Treatment" ($p = 0.00163$), and "Recovery" ($p = 0.00279$) periods (Figure 2A).

When compared to "Stable", the abundance of *Burkholderia* showed a significant increase in samples collected "Before Treatment" ($p = 0.00598$) and during "Recovery" ($p = 0.00101$) (Figure 2B). Little difference was seen in the abundance of *Burkholderia* in samples collected during "Treatment" ($p = 0.30715$) when compared to samples collected during the "Stable" category (Figure 2B).

3.3 Sequencing and qPCR

Using a linear regression model, we compared the relative abundance from Illumina sequencing and the absolute abundance from qPCR within each time point for both *Pseudomonas* and *Burkholderia* (Figure 3). No significant relationship was observed for *Pseudomonas* within any of the time periods. For *Burkholderia*, we observed a significant relationship in those time points collected during the "Recovery" and "Treatment" time periods.

4. Discussion

Examining the effect of antibiotics and changes in time of bacteria in the lungs of CF patients is challenging due to the nature of the polymicrobial environment [10]. Classifying bacteria from sputum using culture-dependent methods may lead to misidentification of bacteria such as anaerobes which have specific growth requirements [11]. DNA-based tools such as sequencing and qPCR have shown to be useful in both identifying the presence of different bacterial species and determining their abundance [12, 13]. Various studies have used these methods to examine spatial distribution of bacteria in the CF lung [14], identify changes in bacterial abundance and richness prior to a PE [5, 6], compare bacteria in expectorated sputum to samples of the CF lung from transplant patients as well as mouthwash samples [9, 15], and to characterize the changes in the microbial community over long-term sampling periods [7]. Each of these studies has used both sequencing analysis and quantitative analysis to some degree to characterize bacteria in the CF lung. The limitations of each of these techniques have also been demonstrated within each study.

The study performed here combined Illumina sequencing and qPCR from frequently-collected sputum samples from a single CF patient. The aim was to determine if the conclusions from either technique alone might change when performing both techniques on the same sample set. Our method is different from previous studies mentioned above in that we collected samples twice weekly over a long-term period. To our knowledge, no other study has combined both techniques along with our frequency of sampling to examine the changes in the microbiota in the CF lung over time and in response to antibiotic treatment for a PE.

From our sequencing data, we observed no pattern of short-term changes or long-term changes over time for those sequences which were identified as *Pseudomonas* or *Burkholderia*. We observed the same lack of changes for both taxa in response to antibiotics. This observation was surprising since our patient eventually recovered from a PE after treatment after being prescribed antibiotics which were either *Pseudomonas*-specific or *Burkholderia*-specific. Contrary to our results, Daniels et al., observed a significant increase in the relative abundance of *Pseudomonas* during antibiotic treatment in their patient population [6]. The authors attributed this change to a decrease in the non-pseudomonal species with, due the nature of sequencing analysis, increases the relative abundance of *Pseudomonas*.

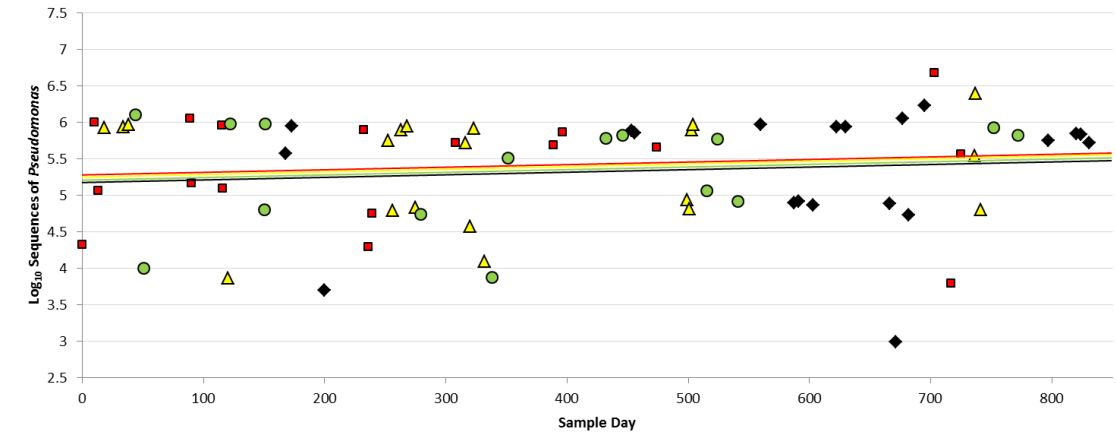
Using qPCR analysis we observed a gradual increase in absolute abundance of each genus over time. This observation was not shown in our sequencing data where each genus appeared to remain stable over time. With qPCR, we were also able to measure a significant decrease in absolute abundance in response to antibiotics. Again, we were unable to make this same observation with the sequencing data. Perhaps not

surprisingly, qPCR provided more insight into the short term and long-term changes of these individual taxa. The regression analysis between the sequencing and qPCR data did not change our view of the data since no significant correlation could be measured that would explain the changes taking place in the microbial community.

HTS sequencing analyses are informative of the changes or potential long-term effects of antibiotics but are limited to measuring changes in relative abundance of bacteria within the environment [16]. HTS, such as the Illumina platform, of microbial communities typically relies on PCR amplification of a target gene. As a result, the enumeration of taxa within a sample is co-dependent on the number of reads obtained from sequencing and the PCR amplification which is not set to a quantitative endpoint. Both computational effects and biological effects can limit the output of information from sequencing. Computational effects may include the necessary method of normalizing sequencing data between samples [17]. Biological effects include the saturation in the later stages of PCR especially if a dominant organism is present in the sample [4]. This effect makes identifying changes in the rare taxa challenging to due to the potential for rare taxa to be overshadowed by "saturation" of the sample from the dominant taxa. The phenomenon described here is not limited to CF. We would expect that any complex microbial sample that contains predominant taxa might generate sequencing saturation effects which could be addressed via qPCR.

Although the issue of quantification using sequencing data is not new, this study demonstrates the current limitation of using sequencing alone to quantify changes in abundance of an individual taxa within the microbial community in Cystic Fibrosis. Computational methods are currently being developed to overcome the challenges of

quantitative analysis of taxa from sequencing data [2]. Here, we've demonstrated that metagenomic data gathered from HTS methods would benefit from the use of absolute quantification methods such as qPCR to provide a more descriptive analysis of changes in a diverse microbial environment.



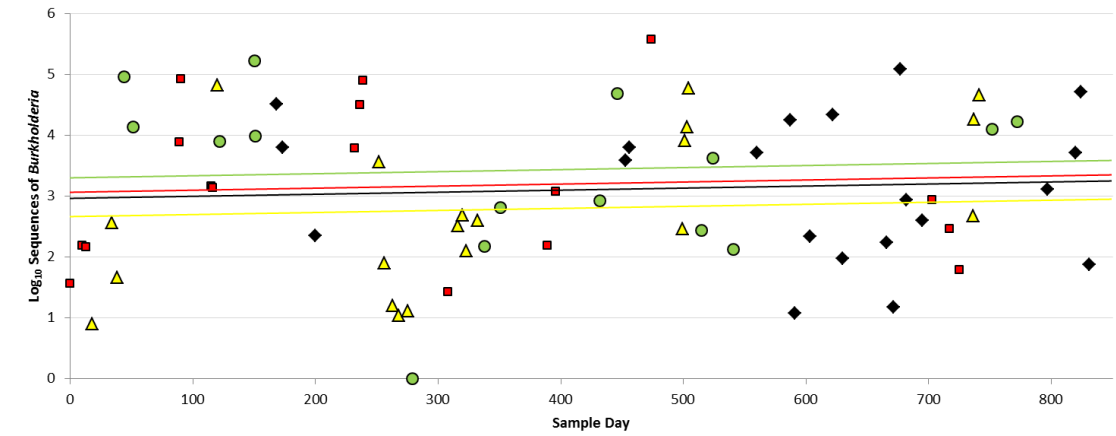


Figure 6.1. Relative abundance of the proportion of reads from *Pseudomonas* (A) and *Burkholderia* (B) from 72 sputum samples. Time points are plotted as ● "Before Treatment" (≤ 30 days before an exacerbation); ▲ "Treatment" (samples collected during and within 48 hours of termination of treatment); ■ "Recovery" (≤ 30 days after an exacerbation); and ◆ "Stable" (> 30 days prior to or after an exacerbation)

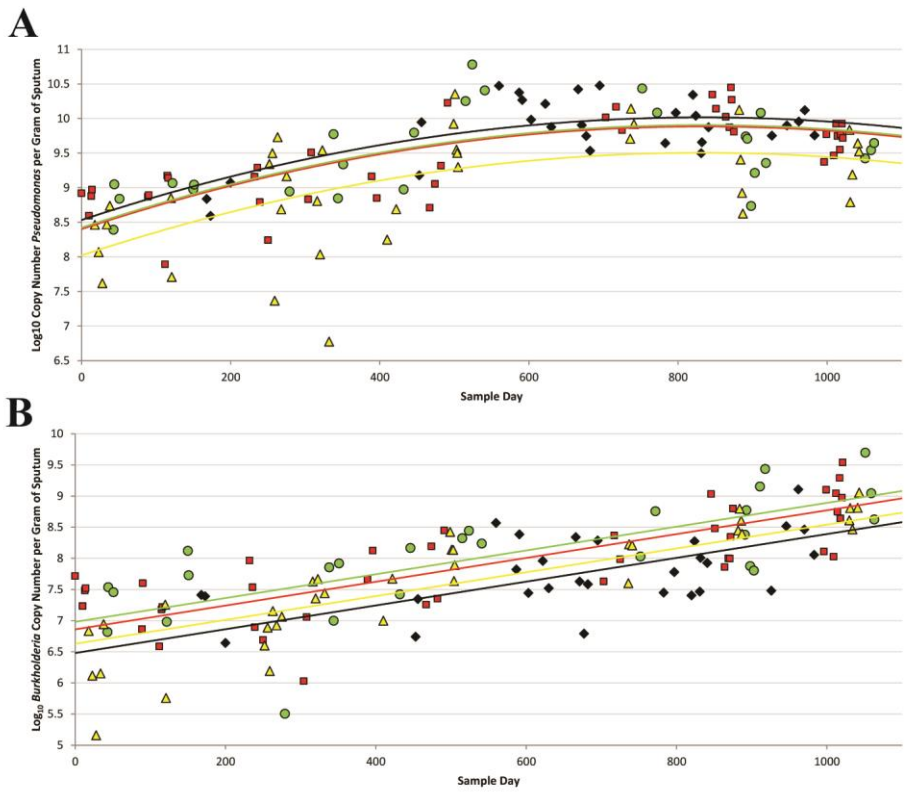


Figure 6.2. Variation in abundance of (A) *P. aeruginosa* and (B) *B. multivorans* in sputum samples collected over a 35-month period during which nine exacerbations occurred. Treatment was considered to be during or within 48 hours of termination of antibiotics given to treat an exacerbation. Time points are plotted as ● "Before Treatment" (≤ 30 days before an exacerbation); ▲ "Treatment" (samples collected during and within 48 hours of termination of treatment); ■ "Recovery" (≤ 30 days after an exacerbation); and ◆ "Stable" (> 30 days prior to or after an exacerbation). *Pseudomonas*-specific primers and *Burkholderia*-specific primers were used to measure abundance which is expressed as $\log_{10}$ DNA copy number per gram of sputum (y-axis) over the collection period (x-axis).

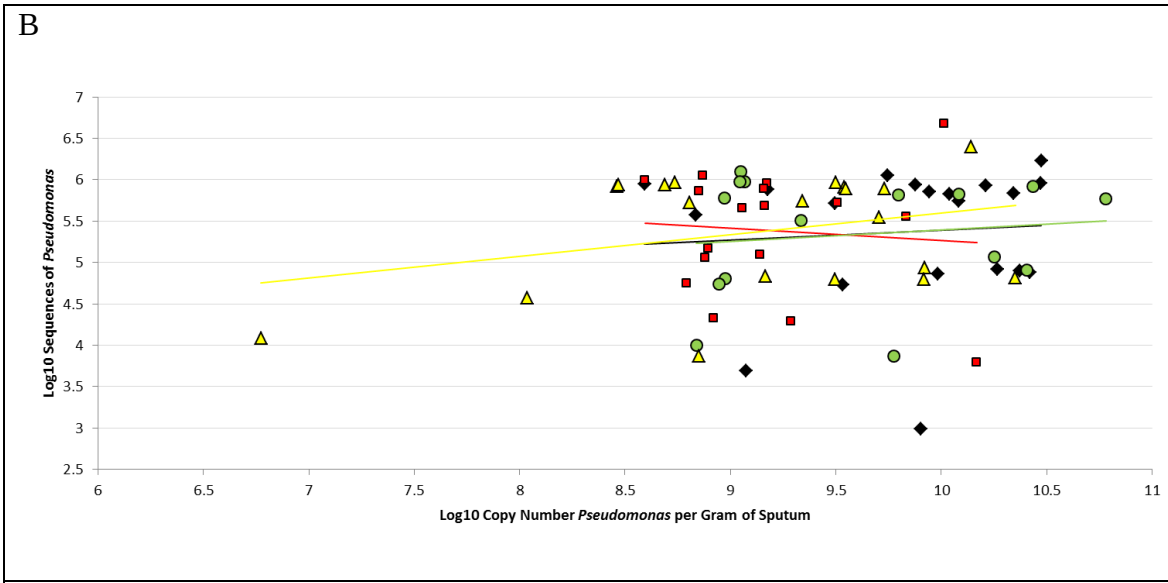


Figure 6.3: Comparison of the number of the $\log_{10}$ sequences from Illumina (y-axis) to the $\log_{10}$ copy number from qPCR (x-axis) for *Pseudomonas* (A) and *Burkholderia* (B). A significant relationship was found for the time points within the "Recovery" ($p = .041$) and "Treatment" ($p = .024$) categories in *Burkholderia* based on a regression analysis for each treatment status. No other significant relationships were observed. Time points are plotted as ● "Before Treatment" (≤ 30 days before an exacerbation); △ "Treatment" (samples collected during and within 48 hours of termination of treatment); ■ "Recovery" (≤ 30 days after an exacerbation); and ◆ "Stable" (> 30 days prior to or after an exacerbation)

REFERENCES

Abstract

1. Harris, J.K., et al., Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proc Natl Acad Sci U S A, 2007. 104(51): p. 20529-33.

2. Rogers, G.B., 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): p. 2601-4.

3. van Belkum, A., et al., Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. FEMS Immunol Med Microbiol, 2000. 27(1): p. 51-57.

4. Bittar, F., et al., Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One, 2008. 3(8): p. e2908.

5. Armougom, F., et al., Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. Eur J Clin Microbiol Infect Dis, 2009. 28(9): p. 1151-4.

6. Kolak, M., et al., Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. Int J Med Microbiol, 2003. 293(4): p. 309-317.

7. Rogers, G.B., et al., 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): p. 5176-5183.

8. Rogers, G.B., et al., 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): p. 3548-3558.

9. Sibley, C.D., H. Rabin, and M.G. Surette, Cystic fibrosis: a polymicrobial infectious disease. Future Microbiol, 2006. 1: p. 53-61.

10. Bragonzi, A., et al., *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am J Respir Crit Care Med, 2009. 180(2): p. 138-45.

11. Hoboth, C., et al., Dynamics of adaptive microevolution of hypermutable *Pseudomonas aeruginosa* during chronic pulmonary infection in patients with cystic fibrosis. J Infect Dis, 2009. 200(1): p. 118-30.

12. Starkey, M., et al., *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J Bacteriol, 2009. 191(11): p. 3492-503.

13. Kohler, T., A. Buckling, and C. van Delden, Cooperation and virulence of clinical *Pseudomonas aeruginosa* populations. Proc Natl Acad Sci U S A, 2009. 106(15): p. 6339-6344.

14. Sibley, C.D., et al., A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. Proc Natl Acad Sci U S A, 2008. 105(39): p. 15070-5.

15. Duan, K.M., et al., Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Molecular Microbiology, 2003. 50(5): p. 1477-1491.

16. McLaughlin, F.J., et al., Clinical and bacteriological responses to 3 antibiotic regimens for acute exacerbations of cystic fibrosis – ticarcillin-tobramycin, azlocillin-tobramycin, and azlocillin-placebo. J Infect Dis, 1983. 147(3): p. 559-567.

17. Smith, A.L., et al., Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration - Lack of association in cystic fibrosis. Chest, 2003. 123(5): p. 1495-1502.

18. Lyczak, J.B., C.L. Cannon, and G.B. Pier, Lung infections associated with cystic fibrosis. Clin Microbiol Rev, 2002. 15(2): p. 194-222.

19. Smith, E.E., et al., Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A, 2006. 103(22): p. 8487-92.

20. Nguyen, D. and P.K. Singh, Evolving stealth: Genetic adaptation of *Pseudomonas aeruginosa* during cystic fibrosis infections. Proc Natl Acad Sci, 2006. 103(22): p. 8305-8306.

21. Mathee, K., et al., Dynamics of *Pseudomonas aeruginosa* genome evolution. Proc Natl Acad Sci U S A, 2008. 105(8): p. 3100-5.

22. Sibley, C.D., et al., The relevance of the polymicrobial nature of airway infection in the acute and chronic management of patients with cystic fibrosis. Curr Opin Investig Drugs, 2009. 10(8): p. 787-94.

23. Bittar, F. and J.M. Rolain, Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients. Clin Microbiol Infect, 2010. 16(7): p. 809-20.
24. Hauser, A.R., et al., Clinical Significance of Microbial Infection and Adaptation in Cystic Fibrosis. Clin Microbiol Rev, 2011. 24(1): p. 29-70.

25. Spencer, M.D., et al., Association Between Composition of the Human Gastrointestinal Microbiome and Development of Fatty Liver With Choline Deficiency. Gastroenterology, 2011. 140(3): p. 976-986.

26. Rogers, G.B., et al., 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): p. 78-86.

Foreword

1. Stokell, J.R. and T.R. Steck, Viable but Nonculturable Bacteria, in eLS. 2012, John Wiley & Sons, Ltd.

2. Stokell, J.R., R.Z. Gharaibeh, and T.R. Steck, Rapid emergence of a ceftazidime-resistant *Burkholderia multivorans* strain in a Cystic Fibrosis patient. J Cyst Fibros, 2013.

Chapter 1

1. Cystic Fibrosis Foundation. About Cystic Fibrosis: What You Need to Know. Available from: http://www.cff.org/AboutCF/.

2. Kidd, T.J., et al., *Burkholderia cepacia* complex epidemiology in persons with cystic fibrosis from Australia and New Zealand. Res Microbiol, 2008. 159(3): p. 194-9.

3. Feliziani, S., et al., Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa* from cystic fibrosis chronic airways infections. PLoS One, 2010. 5(9).

4. Goss, C.H. and J.L. Burns, Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. Thorax, 2007. 62(4): p. 360-7.

5. Amadori, A., et al., Recurrent exacerbations affect FEV(1) decline in adult patients with cystic fibrosis. Respir Med, 2009. 103(3): p. 407-13.

6. Rosenfeld, M., et al., Defining a pulmonary exacerbation in cystic fibrosis. J Pediatr, 2001. 139(3): p. 359-65.

7. Ferkol, T., M. Rosenfeld, and C.E. Milla, Cystic fibrosis pulmonary exacerbations. J Pediatr, 2006. 148(2): p. 259-64.

8. Fuchs, H.J., et al., Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med, 1994. 331(10): p. 637-42.

9. Mena, A., et al., Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol, 2008. 190(24): p. 7910-7.

10. Sibley, C.D. and M.G. Surette, The polymicrobial nature of airway infections in cystic fibrosis: Cangene Gold Medal Lecture. Can J Microbiol, 2011. 57(2): p. 69-77.

11. Bittar, F. and J.M. Rolain, Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients. Clin Microbiol Infect, 2010. 16(7): p. 809-20.

12. Tunney, M.M., et al., Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med, 2008. 177(9): p. 995-1001.

13. Sibley, C.D., et al., Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. PLoS pathogens, 2008. 4(10): p. e1000184.

14. Ulrich, M., et al., Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis. Thorax, 2010. 65(11): p. 978-84.

15. Field, T.R., et al., The genus *Prevotella* in cystic fibrosis airways. Anaerobe, 2010. 16(4): p. 337-44.

16. Johnson, C., et al., Factors influencing outcomes in cystic fibrosis: a center-based analysis. Chest, 2003. 123(1): p. 20-7.

17. Hauser, A.R., et al., Clinical significance of microbial infection and adaptation in cystic fibrosis. Clin Microbiol Rev, 2011. 24(1): p. 29-70.

18. Stressmann, F.A., 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): p. 281-91.

19. van der Gast, C.J., et al., Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. ISME J, 2011. 5(5): p. 780-91.

20. Bittar, F., et al., Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One, 2008. 3(8): p. e2908.

21. Armougom, F., et al., Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. Eur J Clin Microbiol Infect Dis, 2009. 28(9): p. 1151-4.

22. Sibley, C.D., et al., A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. Proc Natl Acad Sci U S A, 2008. 105(39): p. 15070-5.

23. Duan, K., et al., Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol Microbiol, 2003. 50(5): p. 1477-91.

24. Potvin, E., et al., In vivo functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. Environ Microbiol, 2003. 5(12): p. 1294-308.

25. Rogers, G.B., et al., Using bacterial biomarkers to identify early indicators of cystic fibrosis pulmonary exacerbation onset. Expert Rev Mol Diagn, 2011. 11(2): p. 197-206.

26. Marshall, B.C., Pulmonary exacerbations in cystic fibrosis: it's time to be explicit! Am J Respir Crit Care Med, 2004. 169(7): p. 781-2.

27. Mayer-Hamblett, N., et al., Association between pulmonary function and sputum biomarkers in cystic fibrosis. Am J Respir Crit Care Med, 2007. 175(8): p. 822-8.

28. Blasi, F., D. Stolz, and F. Piffer, Biomarkers in lower respiratory tract infections. Pulm Pharmacol Ther, 2010. 23(6): p. 501-7.

29. Christ-Crain, M. and B. Muller, Biomarkers in respiratory tract infections: diagnostic guides to antibiotic prescription, prognostic markers and mediators. Eur Respir J, 2007. 30(3): p. 556-73.

30. van Ewijk, B.E., et al., Viral respiratory infections in cystic fibrosis. J Cyst Fibros, 2005. 4 Suppl 2: p. 31-6.

31. Fierer, N., et al., The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proc Natl Acad Sci U S A, 2008. 105(46): p. 17994-9.

32. Hamady, M., et al., Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat Meth, 2008. 5(3): p. 235-237.

33. Hamp, T.J., W.J. Jones, and A.A. Fodor, The effects of experimental choices and analysis noise on surveys of the rare biosphere. Appl Environ Microbiol, 2009.

Chapter 2

1. Xu, H.S., et al., Survival and Viability of Nonculturable *Escherichia coli* and *Vibrio cholerae* in the Estuarine and Marine-Environment. Microbial Ecology, 1982. 8(4): p. 313-323.

2. Pinto, D., et al., Resuscitation of *Escherichia coli* VBNC cells depends on a variety of environmental or chemical stimuli. J Appl Microbiol, 2011. 110(6): p. 1601-11.

3. Stokell, J.R. and T.R. Steck, Viable but Nonculturable Bacteria, in eLS. 2012, John Wiley & Sons, Ltd.

4. Trevors, J.T., Viable but non-culturable (VBNC) bacteria: Gene expression in planktonic and biofilm cells. J Microbiol Methods, 2011. 86(2): p. 266-73.

5. Bogosian, G. and E.V. Bourneuf, A matter of bacterial life and death. EMBO reports, 2001. 2(9): p. 770-4.

6. Nyström, T., Not quite dead enough: on bacterial life, culturability, senescence, and death. Archives of Microbiology, 2001. 176(3): p. 159-164.

7. Oliver, J.D., Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiology Reviews, 2010. 34(4): p. 415-25.

8. Barcina, I. and I. Arana, The viable but nonculturable phenotype: a crossroads in the life-cycle of non-differentiating bacteria? Reviews in Environmental Science and Biotechnology, 2009. 8(3): p. 245-255.

9. Nyström, T., Bacterial senescence, programmed death, and premeditated sterility. ASM News, 2005. 71: p. 363-369.

10. Khan, M.M., B.H. Pyle, and A.K. Camper, Specific and rapid enumeration of viable but nonculturable and viable-culturable gram-negative bacteria by using flow cytometry. Appl Environ Microbiol, 2010. 76(15): p. 5088-96.

11. Coutard, F., et al., Real-time reverse transcription-PCR for transcriptional expression analysis of virulence and housekeeping genes in viable but nonculturable *Vibrio parahaemolyticus* after recovery of culturability. Appl Environ Microbiol, 2007. 73(16): p. 5183-9.

12. Kogure, K., U. Simidu, and N. Taga, A tentative direct microscopic method for counting living marine bacteria. Can J Microbiol, 1979. 25(3): p. 415-20.

13. Keer, J.T. and L. Birch, Molecular methods for the assessment of bacterial viability. J Microbiol Methods, 2003. 53(2): p. 175-83.

14. Trevors, J.T., Can dead bacterial cells be defined and are genes expressed after cell death? J Microbiol Methods, 2012. 90(1): p. 25-8.

15. Anderson, M., et al., Viable but nonculturable bacteria are present in mouse and human urine specimens. J Clin Microbiol, 2004. 42(2): p. 753-8.

16. Liu, Y., et al., Quantification of viable but nonculturable *Escherichia coli* O157:H7 by targeting the *rpoS* mRNA. Anal Chem, 2010. 82(7): p. 2612-5.

17. Nocker, A., et al., Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol, 2007. 73(16): p. 5111-7.

18. Mishra, A., N. Taneja, and M. Sharma, Viability kinetics, induction, resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable *Vibrio cholerae* O1 in freshwater microcosm. J Appl Microbiol, 2012. 112(5): p. 945-53.

19. Shleeva, M.O., et al., Formation and resuscitation of "non-culturable" cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. Microbiology, 2002. 148(Pt 5): p. 1581-91.

20. Whitesides, M.D. and J.D. Oliver, Resuscitation of *Vibrio vulnificus* from the Viable but Nonculturable State. Appl Environ Microbiol, 1997. 63(3): p. 1002-5.

21. Hayes, C.S. and D.A. Low, Signals of growth regulation in bacteria. Curr Opin Microbiol, 2009. 12(6): p. 667-73.

22. Hayes, C.S., S.K. Aoki, and D.A. Low, Bacterial contact-dependent delivery systems. Annu Rev Genet, 2010. 44: p. 71-90.

23. Lewis, K., Persister cells. Annu Rev Microbiol, 2010. 64(1): p. 357-72.

24. Fauvart, M., V.N. De Groote, and J. Michiels, Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol, 2011. 60(Pt 6): p. 699-709.

25. Bogosian, G., et al., Recovery of hydrogen peroxide-sensitive culturable cells of *Vibrio vulnificus* gives the appearance of resuscitation from a viable but nonculturable state. J Bacteriol, 2000. 182(18): p. 5070-5.

26. Kong, I.S., et al., Role of catalase and *oxyR* in the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiol Ecol, 2004. 50(3): p. 133-42.

27. Signoretto, C., M. Lleo, and P. Canepari, Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. Curr Microbiol, 2002. 44(2): p. 125-31.

28. Lleo, M.M., et al., Inhibition of the resuscitation from the viable but non-culturable state in *Enterococcus faecalis*. Environ Microbiol, 2007. 9(9): p. 2313-20.

29. Heim, S., et al., The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. Journal of Bacteriology, 2002. 184(23): p. 6739-6745.

30. Chiu, S.W., S.Y. Chen, and H.C. Wong, Localization and expression of MreB in *Vibrio parahaemolyticus* under different stresses. Appl Environ Microbiol, 2008. 74(22): p. 7016-22.

31. Boaretti, M., et al., Involvement of *rpoS* in the survival of *Escherichia coli* in the viable but non-culturable state. Environ Microbiol, 2003. 5(10): p. 986-96.

32. Smith, B. and J.D. Oliver, *In situ* and *in vitro* gene expression by *Vibrio vulnificus* during entry into, persistence within, and resuscitation from the viable but nonculturable state. Appl Environ Microbiol, 2006. 72(2): p. 1445-51.

33. Mukamolova, G.V., et al., Muralytic activity of *Micrococcus luteus* Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation. Mol Microbiol, 2006. 59(1): p. 84-98.

34. Vora, G.J., et al., Microarray-based detection of genetic heterogeneity, antimicrobial resistance, and the viable but nonculturable state in human pathogenic *Vibrio* spp. Proc Natl Acad Sci U S A, 2005. 102(52): p. 19109-14.

35. Trevors, J.T. and L. Masson, DNA technologies: what's next applied to microbiology research? Antonie Van Leeuwenhoek, 2010. 98(3): p. 249-62.

36. Rahman, I., et al., Potential virulence of viable but nonculturable *Shigella dysenteriae* type 1. Applied and Environmental Microbiology, 1996. 62(1): p. 115-20.

37. Rivers, B. and T.R. Steck, Viable but nonculturable uropathogenic bacteria are present in the mouse urinary tract following urinary tract infection and antibiotic therapy. Urol Res, 2001. 29(1): p. 60-6.

38. Liu, Y., et al., Production of Shiga-like toxins in viable but nonculturable *Escherichia coli* O157:H7. Water Res, 2010. 44(3): p. 711-8.

39. Dinu, L.D. and S. Bach, Induction of viable but nonculturable *Escherichia coli* O157:H7 in the phyllosphere of lettuce: a food safety risk factor. Appl Environ Microbiol, 2011. 77(23): p. 8295-302.

40. Saha, S.K., S. Saha, and S.C. Sanyal, Recovery of injured *Campylobacter jejuni* cells after animal passage. Appl Environ Microbiol, 1991. 57(11): p. 3388-9.

41. Rahman, M.H., S. Suzuki, and K. Kawai, Formation of viable but non-culturable state (VBNC) of *Aeromonas hydrophila* and its virulence in goldfish, *Carassius auratus*. Microbiol Res, 2001. 156(1): p. 103-6.

42. Oliver, J.D. and R. Bockian, In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl Environ Microbiol, 1995. 61(7): p. 2620-3.

43. Colwell, R.R., et al., Viable but nonculturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. World Journal of Microbiology and Biotechnology, 1996. 12(1): p. 28-31.

44. Binsztein, N., et al., Viable but nonculturable *Vibrio cholerae* O1 in the aquatic environment of Argentina. Appl Environ Microbiol, 2004. 70(12): p. 7481-6.

45. Asakura, H., et al., Viable but nonculturable *Salmonella* species recovery and systemic infection in morphine-treated mice. J Infect Dis, 2002. 186(10): p. 1526-9.

46. Aurass, P., R. Prager, and A. Flieger, EHEC/EAEC O104:H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environ Microbiol, 2011. 13(12): p. 3139-48.

47. Senoh, M., et al., Conversion of viable but nonculturable enteric bacteria to culturable by co-culture with eukaryotic cells. Microbiol Immunol, 2012. 56(5): p. 342-5.

48. Grey, B.E. and T.R. Steck, The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. Appl Environ Microbiol, 2001. 67(9): p. 3866-3872.

49. Santander, R.D., et al., *In planta* recovery of *Erwinia amylovora* viable but nonculturable cells. Trees, 2012. 26(1): p. 75-82.

50. Muresu, R., et al., Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes. FEMS Microbiol Ecol, 2008. 63(3): p. 383-400.

Chapter 3

1. Cleland, W.W., Dithiothreitol, a New Protective Reagent for Sh Groups. Biochemistry, 1964. 3: p. 480-2.

2. May, J.R., The bacteriology of chronic bronchitis. Lancet, 1953. 265(6785): p. 534-7.

3. Nelson, A., et al., Assessment of sample handling practices on microbial activity in sputum samples from patients with cystic fibrosis. Lett Appl Microbiol, 2010. 51(3): p. 272-7.

4. Zhao, J., et al., Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A, 2012. 109(15): p. 5809-14.

5. Fodor, A.A., 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): p. e45001.

6. Rogers, G.B., et al., Reducing bias in bacterial community analysis of lower respiratory infections. ISME J, 2013. 7(4): p. 697-706.

7. Cohen, J., Statistical Power Analysis for the Behavioral Sciences (2nd Edition). 1988: Routledge Academic.

8. Rogers, G.B., et al., 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): p. 3548-58.

9. Blainey, P.C., et al., Quantitative analysis of the human airway microbial ecology reveals a pervasive signature for cystic fibrosis. Sci Transl Med, 2012. 4(153): p. 153ra130.

10. Rawlins, G.A., A method of liquefying sputum for the culture of organisms other than *M. tuberculosis*. J Med Lab Technol, 1955. 13(3): p. 133-43.

11. Stressmann, F.A., 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): p. 281-91.

Chapter 4

1. Drevinek, P. and E. Mahenthiralingam, *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect, 2010. 16(7): p. 821-30.

2. Coutinho, C.P., et al., Long-term colonization of the Cystic Fibrosis lung by *Burkholderia cepacia* complex bacteria: epidemiology, clonal variation and genome-wide expression alterations. Front Cell Inf Microbio, 2011. 1(12).

3. Foundation, C.F. Cystic Fibrosis foundation annual patient registry. 2010; Available from: http://www.cff.org/UploadedFiles/LivingWithCF/CareCenterNetwork/PatientRegistry/2010-Patient-Registry-Report.pdf.

4. Zahariadis, G., M.H. Levy, and J.L. Burns, Cepacia-like syndrome caused by *Burkholderia multivorans*. Can J Infect Dis, 2003. 14(2): p. 123-5.

5. Mowat, E., et al., *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. Am J Respir Crit Care Med, 2011. 183(12): p. 1674-1679.

6. Henry, D.A., et al., Identification of *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of a simple new selective medium. J Clin Microbiol, 1997. 35(3): p. 614-9.

7. CLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard - 7th Edition. 2006. 26(2): M7-A7.

8. Nocker, A., et al., Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol, 2007. 73(16): p. 5111-7.

9. Horz, H.P., et al., Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: general implications and practical use in endodontic antimicrobial therapy. J Clin Microbiol, 2005. 43(10): p. 5332-7.

10. Nadkarni, M.A., et al., Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology, 2002. 148(1): p. 257-266.

11. Ho, C.C., et al., Novel pan-genomic analysis approach in target selection for multiplex PCR identification and detection of *Burkholderia pseudomallei*, *Burkholderia thailandensis*, and *Burkholderia cepacia* complex species: a proof-of-concept study. J Clin Microbiol, 2011. 49(3): p. 814-21.

12. Baldwin, A., et al., Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. J Clin Microbiol, 2005. 43(9): p. 4665-4673.

13. Larkin, M.A., et al., Clustal W and Clustal X version 2.0. Bioinformatics, 2007. 23(21): p. 2947-2948.

14. Altschul, S.F., et al., Basic local alignment search tool. J Mol Biol, 1990. 215(3): p. 403-410.

15. Coutinho, C.P., et al., *Burkholderia cenocepacia* phenotypic clonal variation during a 3.5-year colonization in the lungs of a cystic fibrosis patient. Infect Immun, 2011. 79(7): p. 2950-60.

16. Daniels, T.W.V., et al., Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. J Cyst Fibros, 2012. Epub.

17. Willner, D., et al., Spatial distribution of microbial communities in the cystic fibrosis lung. ISME J, 2012. 6(2): p. 471-4.

18. Aaron, S., et al., Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with *Burkholderia cepacia*. Am J Respir Crit Care Med, 2000. 161(4): p. 1206-1212.

19. Zhou, J., et al., Antimicrobial susceptibility and synergy studies of *Burkholderia cepacia* complex isolated from patients with cystic fibrosis. Antimicrob Agents Chemother, 2007. 51(3): p. 1085-8.

20. Leitão, J., et al., Variation of the antimicrobial susceptibility profiles of *Burkholderia cepacia* complex clonal isolates obtained from chronically infected cystic fibrosis patients: a five-year survey in the major Portuguese treatment center. Eur J Clin Microbiol Infect Dis, 2008. 27(11): p. 1101-1111.

Chapter 5

1. Cystic Fibrosis Foundation. About Cystic Fibrosis: What You Need to Know. [cited 2011 August 10]; Available from: http://www.cff.org/AboutCF/.

2. Amadori, A., et al., Recurrent exacerbations affect FEV(1) decline in adult patients with cystic fibrosis. Respir Med, 2009. 103(3): p. 407-13.

3. Goss, C.H. and J.L. Burns, Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. Thorax, 2007. 62(4): p. 360-7.

4. Marshall, B.C., Pulmonary exacerbations in cystic fibrosis: it's time to be explicit! Am J Respir Crit Care Med, 2004. 169(7): p. 781-2.

5. Rogers, G.B., et al., Using bacterial biomarkers to identify early indicators of cystic fibrosis pulmonary exacerbation onset. Expert Rev Mol Diagn, 2011. 11(2): p. 197-206.

6. Stressmann, F.A., 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): p. 281-91.

7. van der Gast, C.J., et al., Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. ISME J, 2011. 5(5): p. 780-91.

8. Daniels, T.W.V., et al., Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. J Cyst Fibros, 2012. Epub.

9. Stressmann, F.A., et al., Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? J Cyst Fibros, 2011. 10(5): p. 357-65.

10. Nocker, A., et al., Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol, 2007. 73(16): p. 5111-7.

11. Nadkarni, M.A., et al., Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology, 2002. 148(1): p. 257-266.

12. Ho, C.C., et al., Novel pan-genomic analysis approach in target selection for multiplex PCR identification and detection of *Burkholderia pseudomallei*, *Burkholderia thailandensis,* and *Burkholderia cepacia* complex species: a proof-of-concept study. J Clin Microbiol, 2011. 49(3): p. 814-21.

13. Volkmann, H., et al., Evaluation of inhibition and cross-reaction effects on real-time PCR applied to the total DNA of wastewater samples for the quantification of bacterial antibiotic resistance genes and taxon-specific targets. Mol Cell Probes, 2007. 21(2): p. 125-33.

14. Arthur, J.C., et al., Intestinal inflammation targets cancer-inducing activity of the microbiota. Science, 2012. 338(6103): p. 120-3.

15. Wang, Q., et al., Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol, 2007. 73(16): p. 5261-7.

16. Bittar, F. and J.M. Rolain, Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients. Clin Microbiol Infect, 2010. 16(7): p. 809-20.

17. Eickmeier, O., et al., Sputum biomarker profiles in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and association between pulmonary function. Cytokine, 2010. 50(2): p. 152-7.

18. Zhao, J., et al., Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A, 2012. 109(15): p. 5809-14.

19. Guss, A.M., et al., Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. ISME J, 2011. 5(1): p. 20-9.

20. Daniels, T.W., et al., Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. J Cyst Fibros, 2013. 12(1): p. 22-8.

21. Mowat, E., et al., *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. Am J Respir Crit Care Med, 2011. 183(12): p. 1674-1679.

22. Goddard, A.F., et al., Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proc Natl Acad Sci U S A, 2012. 109(34): p. 13769-74.

23. Fodor, A.A., 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): p. e45001.

24. Albertini-Yagi, C.S., et al., Sputum induction as a research tool for the study of human respiratory mucus. Respir Physiol Neurobiol, 2005. 145(1): p. 101-10.

Chapter 6

1. Rogers, G.B., et al., Lung infections in cystic fibrosis: deriving clinical insight from microbial complexity. Expert Rev Mol Diagn, 2010. 10(2): p. 187-96.

2. Faust, K., et al., Microbial co-occurrence relationships in the human microbiome. PLoS Comput Biol, 2012. 8(7): p. e1002606.

3. Schloss, P.D., D. Gevers, and S.L. Westcott, Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One, 2011. 6(12): p. e27310.

4. Gonzalez, J.M., et al., Amplification by PCR artificially reduces the proportion of the rare biosphere in microbial communities. PLoS One, 2012. 7(1): p. e29973.

5. Stressmann, F.A., et al., Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? J Cyst Fibros, 2011. 10(5): p. 357-65.

6. Daniels, T.W., et al., Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. J Cyst Fibros, 2013. 12(1): p. 22-8.

7. Zhao, J., et al., Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A, 2012. 109(15): p. 5809-14.

8. Blainey, P.C., et al., Quantitative analysis of the human airway microbial ecology reveals a pervasive signature for cystic fibrosis. Sci Transl Med, 2012. 4(153): p. 153ra130.

9. Fodor, A.A., 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): p. e45001.

10. Sibley, C.D., et al., A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. Proc Natl Acad Sci U S A, 2008. 105(39): p. 15070-5.

11. Rogers, G.B., M.P. Carroll, and K.D. Bruce, Studying bacterial infections through culture-independent approaches. J Med Microbiol, 2009. 58(Pt 11): p. 1401-18.

12. Maughan, H., et al., Analysis of the cystic fibrosis lung microbiota via serial Illumina sequencing of bacterial 16S rRNA hypervariable regions. PLoS One, 2012. 7(10): p. e45791.

13. Murray, D.C., et al., DNA-based faecal dietary analysis: a comparison of qPCR and high throughput sequencing approaches. PLoS One, 2011. 6(10): p. e25776.

14. Willner, D., et al., Spatial distribution of microbial communities in the cystic fibrosis lung. ISME J, 2012. 6(2): p. 471-4.

15. Goddard, A.F., et al., Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proc Natl Acad Sci U S A, 2012. 109(34): p. 13769-74.

16. Carroll, I.M., et al., Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. PLoS One, 2012. 7(10): p. e46953.

17. Friedman, J. and E.J. Alm, Inferring correlation networks from genomic survey data. PLoS Comput Biol, 2012. 8(9): p. e1002687.