PSEUDOMONAS AERUGINOSA METABOLISM IN THE CYSTIC FIBROSIS LUNG

By

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A DISSERTATION

Submitted to Michigan State University in partial fulfillment of the requirements for the degree of

Cell and Molecular Biology - Doctor of Philosophy

2016

ABSTRACT

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Cystic fibrosis of the lung is an autosomal recessive disease caused by defects in the Cystic Fibrosis Transmembrane conductance Regulator. While current treatments and interventions have greatly reduced morbidity and increased life expectancy in this population, cystic fibrosis patients face chronic infection of the lung. These chronic infections cause a chronic inflammatory state of the lung to develop, leading to slow degradation of the lung parenchyma and expansion of the conductive pathways, a disease known as bronchiectasis. Treatment at the late stages of this pulmonary disease is by heart and lung transplantation, a surgical intervention that carries significant risk as well as limited by the total availability of such organs. A potential intervention in this disease process that may significantly reduce the progression of bronchiectasis is in the effective removal of pathogenic organisms from the cystic fibrosis lung. Of the various organisms that cause chronic infection of the cystic fibrosis lung, *Pseudomonas aeruginosa* demonstrates the highest prevalence of infection and greatest correlated risk of morbidity and mortality in this population.

Pseudomonas aeruginosa is a Gram negative bacterial species know for a wide range of opportunistic infections (e.g. burn wounds, urinary tract infections secondary to catheterization, and ventilator-associated pneumonia) and a diverse range of environments that it inhabits. Effective treatment of *P. aeruginosa* infection is greatly complicated due to its intrinsic resistance to many different classes of antibiotics as well as the rapid development of resistance to treatment during chronic cystic fibrosis lung infection. A greater understanding of the metabolic phenotypes of *P. aeruginosa* may lead to the development of both better clinical tests monitoring the state of infection of the lung as well as the intelligent design of pharmaceutical interventions that target metabolic pathways important to growth in this organism during infection.

In this dissertation, I present the quantified carbon metabolism of six cystic fibrosis *P*. *aeruginosa* isolates and the laboratory reference strain PA01. In these studies, I demonstrate the difference of metabolism under glucose only minimal media growth (M9) and in a media formulated to mimic the cystic fibrosis lung under aerobic and anoxic growth (Simplified Synthetic Cystic Fibrosis sputum Media). These studies reveal shared and divergent metabolic phenotypes across several isolates from different geographical as well as genetic backgrounds. These studies also identify the importance of the glyoxylate cycle and polyamine metabolism during the growth of *P*. *aeruginosa* and to demonstrate the variation in utilization of other metabolic sub-systems of central carbon metabolism. Finally, these studies demonstrate the suboptimal biomass production of *P*. *aeruginosa* and identify overproduction of reductant as a key product of the central metabolism of *P*. *aeruginosa*. Overall, this research makes substantial progress in understanding not only the metabolism of *P*. *aeruginosa* in the cystic fibrosis lung but identifying key metabolic pathways for future investigation that may have a significant impact in the understanding of biofilm formation and pathogenic infection of other bacteria. Copyright by MICHAEL JAY OPPERMAN 2016

ACKNOWLEDGMENTS

I wish to thank the current and former members of the Shachar-Hill lab during my time as a student for both their technical as well as their emotional support during my PhD years at MSU. I wish to specifically thank Lisa Carey, Dr. Wilson Chen, and Dr. Rahul Deshpande for their technical support as well as their mentorship and guidance during the early years of my research career. I greatly thank Teresa Clark for her technical support and guidance, especially with the MFA process on the HPCC. I also wish to thank Nathan Praschan and Raven Batshon both for their culturing work during the start of this research as well as the experience of mentorship. Finally, I wish to thank Matthew Jeurgens for their technical expertise and friendship during these last, stressful years, of my research.

I wish to warmly thank Dr. Cindy Arvidson and Dr. Chris Waters for their allowance of laboratory space for my experiments. I wish to also thank Dr. Martha Mulks for her gift of the strain PA01 as well as guidance during my early experiments. I also greatly thank my guidance committee, Dr. Cindy Arvidson, Dr. Claire Vieille, Dr. Robert Worden, Dr. Sheng-Yang He, and Dr. Yair Shachar-Hill both for their time as well as for their appreciated role as mentors. Finally, I wish to thank my ever-loving wife and companion of many years, Amanda Opperman, whom I could write many words but have so, so little space left. I wish to thank my parents, Roderick "Dabba" Opperman and Nancy Opperman, for their constant love and support. I cannot forget to thank my bother Nathaniel Opperman, my lifelong friend, and his wife Dr. Lindsey VanSchoiack. I finally wish to thank my Grandparents: Earl and Lodema Harmon; and Herb and Marjorie Opperman for their lifelong love and support as well as the rest of my extended family, too numerous for this page, who have helped me to become the man I am today.

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KEY TO ABBREVIATIONS

- $\alpha KG \alpha$ -ketoglutarate
- $^{13}C Carbon-13$
- 6PGC 6-phospho-gluconate
- ABC ATP-binding cassette protein
- Ac-CoA acetyl-CoA
- ARG arginine
- ASP-aspartate
- CCE Carbon Conversion Efficiency
- CIT-citrate
- CITR citrulline
- CI confidence interval
- CF-cystic fibrosis
- CFTR Cystic Fibrosis Transmembrane conductance Regulator
- EMP Embden–Meyerhof–Parnas pathway
- EDP Entner-Doudoroff pathway
- E4P erythrose 4-phosphate
- FBA Constraints-based Flux Balance Analysis
- FVA Flux Variability Analysis
- FUM-fumarate
- GLU glutamate
- G5SH glutamate-5-semialdehyde
- G3P glyceraldehyde 3-phosphate

- GLX- glyoxylate or glyoxylate cycle
- H6P hexose-6-phosphate
- HCA Hierarchical Clustering Analysis
- ICIT-isocitrate
- LPS lipopolysaccharide
- MAL malate
- MFA Metabolic Flux Analysis
- ORN-ornithine
- OAA-oxaloacetate
- OPPP Oxidative Pentose Phosphate Pathway
- P5P pentose-5-phosphate
- PEP phosphoenolpyruvate
- PGN polygalacturan
- PCA Principal Component Analysis
- PKA Protein Kinase A
- PYR pyruvate
- S7P sedoheptulose 7-phosphate
- SUCC succinate
- SS_{res} sum of squared residuals
- TCA tricarboxylic acid cycle
- T2SS Type II Secretion System
- T3SS Type III secretion system

CHAPTER 1 -

Pathogenesis of Cystic Fibrosis and Pseudomonas aeruginosa Infection:

A Literature Review

The work described in this thesis concerns metabolic changes during adaptation and pathogenesis of the bacteria *Pseudomonas aeruginosa* in chronic infection of the cystic fibrosis lung. *Pseudomonas aeruginosa* is a significant factor in the morbidity and mortality of cystic fibrosis (CF). Cystic fibrosis is an autosomal recessive disease caused by a defect in the Cystic Fibrosis Transmembrane Conductance Regulator, which causes pathological changes in bicarbonate secretion and mucus function. The research described in this thesis uses Metabolic Flux Analysis and Flux Balance Analysis to quantify central carbon metabolism in cystic fibrosis isolates of *P. aeruginosa*. These techniques yield characteristic flux maps that define the metabolic phenotypes of the strains under environmental and lung-like conditions. Such maps yield a better systemic understanding of the metabolic evolution of the organism in the cystic fibrosis lung. The metabolic phenotypes can also aid in identifying potential metabolic drug targets.

This introductory chapter describes the medical history, pathogenesis, molecular basis, and current treatment of cystic fibrosis. Also included is a description of the taxonomy and the metabolic characteristics of the genus *Pseudomonas*. The virulence characteristics of *P*. *aeruginosa*, its pathogenesis during pulmonary infection in cystic fibrosis, and the current complications in the treatment of lung infections of cystic fibrosis are presented next. Lastly, an introduction is given to the technical aspects of Metabolic Flux Analysis and Flux Balance Analysis, the methods used in the studies presented in subsequent chapters on the metabolism of *P. aeruginosa*.

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The Medical History of Cystic Fibrosis

Cystic fibrosis of the pancreas, also known as mucoviscidosis but more commonly as cystic fibrosis, was named for the observation of pancreatic cystic and fibrotic lesions observed during autopsy [1]. Modern investigation of the disease can be traced to the published case study of 49 patients with steatorrhea (excessive fat in feces) caused by pancreatic insufficiency who were subsequently shown to have cyst formation and fibrotic lesions in the pancreas at autopsy by Dr. Dorothy Andersen [1]. While early descriptions of the disease focused on the Celiac-like symptoms of the disease, steatorrhea and failure to thrive, a frequent note was the associated chronic respiratory infections in patients that survived the neonatal period [1,2]. Patients with the disease seemed to fall into two groups, one that would die due to obstruction of the digestive system, meconium ileus, within the neonatal period and those that would live past infancy but succumb to chronic respiratory infection [1,2]. Early work to understand the pulmonary complications of cystic fibrosis showed infection principally by Staphylococcus aureus and treatment was diet supplementation with vitamins, limiting fat intake, and ingestion of pancreatic extracts with sulfadiazine or other sulfa drugs used as antibacterial therapy [3]. During this time, 50 percent of those with meconium ileus would die within the first two weeks of life, and 50 percent of those without meconium ileus would die within the first 6 months of life [4].

While early observations included the discussion of many bodily glands possibly being affected, it was not until the 1940s that abnormal mucus gland function was investigated as the basis for the clinical presentation of this disease [5,6]. The study conducted by Farber *et al.* demonstrated that unlike Celiac disease, cystic fibrosis patient secretions were lower in viscosity and were greatly reduced in the secretion of the pancreatic enzymes trypsin and lipase [6]. A second paper by the Farber gave the disease the name mucoviscidosis to describe the viscous mucus secretions that provided the mechanical basis for the decreased enzyme secretion of the

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pancreas as well as the increased viscosity of the fluids seen [7]. This conclusion of thickened mucus being a central symptom of the disease would soon be used to explain the strong association between meconium ileus and cystic fibrosis [8,9].

Realization that the disorder involved all exocrine glands would come in the late 1940s. A heat wave in New York City in August 1948 led to the discovery of sweat gland involvement, with an obvious bias toward cystic fibrosis children being hospitalized for heat exhaustion and further investigation showing examples of patients moved to warmer climates to protect against respiratory infections dying quickly as a result of heat exhaustion complications [10]. This observation led to a case-control study and subsequent direct experimental measurement of sweat and blood salt concentration in patients with suspected cystic fibrosis in 1953 [11]. This study provided evidence of abnormal salt loss in cystic fibrosis with increased secretion of sodium, chloride, and potassium by sweat and salivary glands, even under salt-limited intake [11].These results would lead to the development of sweat testing to quickly and reliably determine cystic fibrosis in patient populations [12].

The next decades of cystic fibrosis research, while providing more effective treatments to prolong life, did not yield the true cause of the disease. Treatment of the severest of disease symptoms, meconium ileus, did not begin until 1948, leading to increased survival in some patients [13,14]. Antibiotic treatment, new to medical science, also increased survival of cystic fibrosis patients, but soon led to cases of infection by gram negative organisms, chiefly *Pseudomonas aeruginosa*, in the 1950s [14–17]. In the 1960s and 1970s, research into proposals that cystic fibrosis is an inborn error of metabolism or is caused by abnormal production of mucus proteins would prove these ideas to be wrong, while the discovery of abnormal ciliary motility and subsequent proposal of a cilliary-toxic factor defining cystic fibrosis also failed to

yield a clear understanding of the disease [18–21]. Ultimately, the lack of another organism with similar presentation and difficulty in culturing cells from cystic fibrosis patients hindered understanding the molecular basis of the disease well into the 1980s [19,22]. Abnormalities in salt content of pulmonary secretions would finally be demonstrated in lung epithelial tissue in 1981, directly showing excessive sodium uptake and demonstrating a likely defect in chloride ion regulation on the apical membrane in cystic fibrosis patients [23]. This theory was further supported by work in cultured cells from the tracheal mucosa of a cystic fibrosis patient [24].

While evidence of chloride ion dysfunction in epithelial tissue was beginning to be established, the genetics of the disease were also beginning to be untangled. Early work by Andersen et al. demonstrated the likelihood that the disease was an autosomal recessive genetic disease [5]. By the 1970s, it had become clear this was a genetic disease predominately affecting Caucasian populations [25–27] and was rarely seen in non-Caucasian populations [26,28–30]. In the US, rates of gene prevalence were shown to be approximately 2%-5%, but multiple studies failed to show if this was a multi-loci or multi-allelic disease [31–33].

The molecular basis for the disease and its genetic quirks would finally be revealed with the development and application of modern DNA technology. Development of genetic linkage analysis using restriction fragment length polymorphisms [34] would lead to the localization of the cystic fibrosis gene to band q31 of chromosome 7 [35,36]. In 1989, Collins, Riordan and Tsui cloned and named the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) [37–39]. This work would establish the CFTR as being an ATP-binding cassette (ABC) protein, as well as describe what would be known as the most common mutation, F508del [37,40]. Confirmation of the chloride channel activity of the CFTR would come from work on CFTR point mutations and direct purification and testing of bound CFTR in the early 1990s [41–43]. These techniques have

also led to a much clearer understanding of the world-wide prevalence of the disease, with those of Northern European descent having the highest risk of cystic fibrosis in the United States with a birth rate of 1 in 3000 live births and worldwide prevalence of the disease varying from 1 in 900 live births in some populations in Canada to 1 in 10,500 live births in Native American populations [44]. With a relatively high allelic rate in northern European populations, it has been proposed that there may be a protective effect for heterozygotes against certain pulmonary infections or conditions, but the true cause for the selective pressure retaining the genetic defect remains an open scientific question [45,46].

The Molecular Basis for Cystic Fibrosis

Much is now know about the protein responsible for cystic fibrosis, the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). The CFTR is a cAMP-regulated ABC transporter with the unique property among such proteins of transporting several ions, preferentially chloride ions [47–49]. These properties help the protein regulate epithelial sodium channels, regulate ATP-sensitive outwardly rectifying chloride channels, directly transport ATP, regulate potassium channels, and aid in functioning of the bicarbonate/chloride exchange in the pancreas [50–53]. CFTR resides in the apical membrane surface with two membrane spanning domains that make up the pore that gates chloride ion transfer [54]. It also contains two cytoplasmic domains that define the protein as a member of the ATP-binding cassette transporter family, an ATP binding domain and an ATP hydrolyzing domain [55]. Regulation of CFTR activity is primarily through phosphorylation by Protein Kinase A (PKA), which is activated by cAMP, but it can also be activated by other proteins (e.g. PKGII) [56–58]. Presently under investigation is the role of the CFTR in bicarbonate transport outside of pancreatic exocrine

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secretion, as acidic secretions are a prominent feature of cystic fibrosis that best explain the broad pathological features of the disease [59–61].

With these advances in the molecular understanding of CFTR, the more than 2000 known genetic defects in the CFTR have been classified as pathologies of protein quantity or quality[44]. Defects in protein quantity can involve correct mRNA production, which can be caused by abnormal gene transcription as a result of missense (39.9% of reported CFTR variants), nonsense (8.3%), frameshift (15.7%), or splicing (11.5%) mutations, which lead to premature stop codons or accelerated mRNA degradation and subsequent lack of sufficient CFTR in the cell membrane [62,63]. Mutations causing these transcription defects are more commonly seen in Mediterranean countries (G542X) and in Ashkenazi Jewish populations (W1282X) [55,64]. Additional mutations in the gene promoter region or alternative gene splicing of the CFTR, seen in A455E and P574H defects, can further lead to decreased transcript production and thus decreased amounts of CFTR in the cell membrane [64]. Protein manufacturing defects also reduce protein quantity, such as the endoplasmic reticulum quality control degradation of the CFTR in F508del, which leads to few successful insertions of the CFTR into the cell membrane [65]. Finally, the CFTR itself can be intrinsically unstable, with the Q1412X leading to a CFTR protein with a reduced half-life, caused by the loss of the last 70 amino acids of the protein [64].

Cystic fibrosis pathologies are also caused by a second class of CFTR defects, which are due to intrinsic issues with protein function [44,56,64]. CFTR activity can be affected by genetic mutations that allow for proper protein insertion and levels to be present in the cell, but cause incorrect opening of the channel or limited ion conductance when open [44,56,66]. The 3rd most common cystic fibrosis defect, G551D, is due to dysfunction of the ATP-binding site, which

causes obstruction of the open conformation of the protein [55,64]. The conductance of the CFTR can also be affected, as seen in the R117H genetic defect, changing the current-voltage relationship from linear to strongly inwardly rectifying [56,62,64].

The Pathophysiology of Cystic Fibrosis

Ultimately, the defective bicarbonate ion localization due to a CFTR defect leads to the central pathology of thickened secretions as a result of improper mucin expansion on epithelial surfaces. The pathophysiology of cystic fibrosis is that chronically dehydrated mucus leads to thickened and sticky secretions, causing the disease pathology [60,61]. While previous theories have explained the lack of proper mucus hydration on the lack of correct chloride transport, a unified theory on cystic fibrosis based on chloride transport does not fully explain the dehydrated mucus seen in the alimentary tract [61,67]. Instead, recent work has established the importance of calcium ions and bicarbonate; calcium ions and bicarbonate regulate charge shielding and alkaline pH, which is critical for the proper unpacking and unfolding of mucin polymers to form a high viscosity hydrated gel [67–70]. This explanation of how defective CFTR function affects bicarbonate secretion and consequently mucus function connects the molecular changes to the organ-level dysfunction and symptom presentation in cystic fibrosis.

The accumulation of mucus in the digestive system, in particular the duodenum, gives rise to the earliest clinical presentation of the disease. The duodenum, the most proximal section of the small intestine contains the anatomical channels for both the pancreas and the liver, and is where bicarbonate and mucus secretions from the pancreas and duodenum neutralize, and protect the organ from, the acidic stomach contents [71]. This creates the proper environment for micelle formation of lipids and activation of digestive enzymes from the pancreas [72,73]. In about 15% of infants with cystic fibrosis, the first indication of a mucus-plugged small intestine appears

with the failure to pass the first stool within the first few days of life, a condition called meconium ileus [44,74]. The CFTR in the pancreatic ducts is central to the high rate of bicarbonate secretion, and the degree of loss of bicarbonate transport in the expressed CFTR allele determines whether pancreatic insufficiency is seen in a patient, which manifests as the pancreatic duct clogged with thickened mucus and accumulating proenzymes beginning in the neonatal period [75–78]. The closed pancreatic duct allows the digestive enzymes to activate within the pancreas itself, causing enzymatic digestion, pancreatitis and fibrosis [79,80]. In time, the lack of secretion of digestive enzymes by the pancreas begins to manifest in fatty stools (steatorrhea) and failure to thrive in infants and toddlers due to fat soluble vitamin and lipid absorption failure [80]. Current treatment for malnutrition caused by cystic fibrosis is to increase caloric intake and supplement pancreatic enzymes and fat soluble vitamins [81].

Additional symptoms of cystic fibrosis present as patients enter adulthood. Without sufficient bicarbonate secretion in the duodenum, the small intestine remains acidic, producing an environment hospitable to bacterial overgrowth, further complicating the malnutrition symptoms of the disease [82]. Continuous failure to neutralize gastric contents leads to increased gastric emptying times and decreased lower esophageal tone, causing gastroesophageal reflux disease and peptic ulcers [83–85]. In time, chronic production of thickened mucus in the digestive tract can obstruct the small bowel, presenting in Distal Intestinal Obstruction Syndrome, Meconium Plug Syndrome, constipation, intussusception, and, in extreme cases, Pneumatosis Intestinalis, where an intussuscepted bowel ruptures and leads to bowel contents emptying into the abdominal cavity [84]. 97% of males with cystic fibrosis are sterile due to congenital bilateral absence of vas deferens, an obstructive azoospermia likely related to the lack of necessary CFTR expression for proper male reproductive tract development [86,87]. (It should

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be noted that there is no relationship between sterility in women and cystic fibrosis, and the pregnancies of those with a normal range of lung function and BMI are regarded as low-risk [44,81].) Pancreatic enzyme secretion obstruction due to mucus plugs in the secreting lobes causes cellular metaplasia in mucus secreting cells and, finally, fibrosis of the organ, leading to episodes of pancreatitis[80,84,88]. The endocrine function of the pancreas is affected in 50% of cystic fibrosis patients who are 30 or older, although it is unclear if this is due to direct destruction of the beta cells of the pancreas or dysfunction related to decreased CFTR expression [89,90].

However, pathology in the pulmonary system is the greatest cause of mortality in cystic fibrosis patients. Current understanding is that the pseudostratified ciliated columnar epithelium provides innate bacterial defense by using cilia to move a thin mucus layer on the cell surface towards the larynx, pulling inhaled pathogens away from the lower airways of the lung towards the destructive environment of the stomach [91]. To have sufficient water to allow for proper fluidity of this mucus layer, the proper bicarbonate and chloride transport by the CFTR and sodium by the epithelial sodium channel (ENaC) must be in place to form a pericillary layer below the normal mucus layer [92,93]. In cystic fibrosis, this mucus layer is disturbed, although it is currently unknown if the liquid layer height or viscosity of the mucus is the primary basis of the dysfunction [94,95]. Ultimately, the thickened mucus cannot be moved by the ciliated cells, causing pooled mucus to form and providing a non-hostile environment for bacteria and non-bacterial species to grow [93]. Commonly seen infectious species in this population are *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Hemophilus influenza*, although *Streptoccocus* sp., *Burkholderia* sp., *Streptoccocus pneumoniae*, and *Aspergillus* sp. are also of concern [96]. With this increase in susceptibility to infection, cystic fibrosis patients face a

pathological cycle of lung destruction due to infection, inflammation, lung tissue destruction due to the immune response, infection clearance, and, followed by another infection [97–99]. This prolonged multiple infection leads to a chronic inflammatory state of the lung causes repeated cycles of neutrophil elastase digestion of the lung parenchyma, which causes the obstructive lung disease Bronchiectasis, with a majority of cystic fibrosis patients demonstrating the disease by three years of age [100–102].

While treatment of the pulmonary conditions of cystic fibrosis is possible, many issues do arise. Chronic inflammation caused by infection is treated with high-dose ibuprofen, but such treatment can cause Analgesic nephropathy [103]. While alternative anti-inflammatory treatments are under investigation, including Nitric Oxide modulators such as Riociguat or GSNOR inhibitors, ibuprofen remains the most common current treatment [104]. Acute and chronic infection is treated with antibiotics, most commonly aminoglycosides such as tobramycin, vaccination, or monoclonal antibodies against specific bacteria or viruses, but the ever-present issue of antibacterial resistance greatly complicates chronic infection treatment[96,105,106]. DNAses are also used to help reduce thickened mucus secretions [106]. Perhaps the most hopeful treatments involve direct treatment of the defective CFTR, such as the CFTR potentiator, ivacaftor, and CFTR modulators such as lumacaftor, but these cannot treat all of the known defects in the CFTR [104,107]. Epidemiological studies of cystic fibrosis patients with mild and severe disease both show a strong decline in lung function starting at 40 years of age, with no difference seen in pulmonary disease severity [108]. Final treatment for decreased lung function caused by bronchiectasis due to chronic infection is lung transplantation, though the lack of sufficient organs for transplant makes this option unavailable to most [109,110]. Because the leading cause of morbidity and mortality in cystic fibrosis patients is chronic lung

infection, understanding *Pseudomonas aeruginosa* infection in cystic fibrosis patients is of crucial importance [96,105].

Current prevalence rates of *P. aeruginosa* infection of has been shown to be 74% of all cystic fibrosis patients 26 years and older, with over 50% of patients over the age of 18 demonstrating infection [111]. The current theory of infection of the lung by *P. aeruginosa* begins with a clinically independent exposure to the bacteria, due to the bacterium's environmental ubiquity [112]. Inhaled *P. aeruginosa* begins to establish persistent biofilm communities within the lower respiratory tract, beginning a cycle of innate immune system response, inflammation, decreased *P. aeruginosa* concentration and then re-infection due to a failure of clearance of the biofilm [113,114]. This chronic cycle of inflammation causes the scarring and widening of the airways of the lower respiratory tract known as Bronchiectasis, a condition that can only be treated with lung transplantation in the late stages of the disease [102]. *Pseudomonas aeruginosa* treatment using current antibacterial therapy is insufficient, as biofilm increases microbial survival and recolonization after treatment, eventually leading to a state of nearly complete antibiotic resistance in chronic infection [115]. The long range goal of the research described in this thesis is to identify metabolic features of *P. aeruginosa* during cystic fibrosis infections which can be used to aid in treatment.

The Taxonomy and Metabolism of Pseudomonas aeruginosa

The Pseudomonadaceae are a Family of organisms that have been observed since the beginning of the study of bacteria, with the first description and use of the name *Pseudomonas* occurring in 1885 [116,117]. With the establishment of ribosomal RNA-based taxonomy the present genera of bacteria in this family consists primarily of *Pseudomonas* sp., *Azotobacter* sp., *Azotobacter* sp., *Azotobacter* sp., and *Cellvibrio* sp.[118]. *Pseudomonas aeruginosa* belongs to

the genus *Pseudomonas*, the Type Genus of the family[118,119]. While initially classified as most genetically similar to *P. fluorescens* and *P. putida*, inclusion of several house-keeping gene sequences to establish higher resolution inter-species genetic lineages has demonstrated *P. oleovorans* and *P. stutzeri* as belonging to the *P. aeruginosa* subgroup and *P. fluorescens* and *P. putida* belonging to separate subgroups of *Pseudomonas* [119,120].

Members of *Pseudomonas* share several physically and chemically definable characteristics. *Pseudomonas* sp. are Gram negative bacilli with polar flagella found ubiquitously in water and land ecosystems between 4°C-42°C and a pH range of 4-8, making many environments potential reservoirs [118,121]. They are common opportunistic pathogens of animals and plants [118,119]. A defining characteristic of some members of the genus, though not expressed by all members, is the production of fluorescent colored compounds, such as phenazine pigments, toxins that can act as siderophores [122,123]. The metabolic characteristics of the genus *Pseudomonas* help further define the group. *Pseudomonas* are chemoorganotrophic, nonphotosynthetic bacteria that require only simple carbon compounds to survive, with only three known species of the genus requiring more complex carbon compounds to grow [118]. While all genes for tricarboxylic acid cycle (TCA) and oxidative pentose phosphate pathway (OPPP) enzymes have been found through genome sequencing of several *Pseudomonas* species [124–127], the general experimental finding in the genus is the unique lack of an active 6phosphofructokinase[128–131], causing a loss of a fully functional Embden–Meyerhof–Parnas (EMP) pathway and the reliance on the Entner-Doudoroff (ED) pathway[118].

The Entner-Doudoroff pathway is an enzymatic pathway that allows for the cleavage of 6-phospho-gluconate to one pyruvate and one glyceraldehyde-3-phosphate using the enzymes 6-phosphogluconate dehydratase and 2keto-3-deoxyphosphogluconate aldolase [132]. This

pathway is seen in many bacterial species, and may be a more primitive form of carbohydrate catabolism than the EMP pathway [133]. This pathway only produces one ATP, one NADPH, and one NADH compared to the EMP pathway's net production of 2 ATP and 2 NADH, but the ED pathway has been proposed to decrease total protein need for glycolytic metabolism, allowing for a total net increase in energy with respect to metabolic need for enzyme production [134]. Anaerobic metabolism and fermentation pathways do exist in *Pseudomonas*, which are often generally mis-described as a genus of obligate aerobes. Arginine can be deaminated to ornithine to directly produce ATP in several *Pseudomonas* species, with growth only observed under rich media conditions [135–137]. Pyruvate fermentation is known to occur under anaerobic growth in only two members of the genus, *Pseudomonas extremaustralis* and *Pseudomonas aeruginosa*, with *P. aeruginosa* producing lactate, acetate, acetaldehyde, or ethanol [138,139].

A striking metabolic characteristic of the genus *Pseudomonas* is the ability to use nitrogen compounds as alternative electron acceptors[140–143]. Using the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, *Pseudomonas* are able to reduce nitrate to nitrite, nitric oxide, nitrous oxide, and finally to nitrogen gas [144]. These enzymes receive electrons from the same electron donors to oxygen oxidases, ubiquinols and the cytochrome bc_1 complex that are employed in aerobic respiration. This allows for the electron transfer chain of oxidative phosphorylation to continue functioning in anoxia with only a slight decrease in energy transfer efficiency [144,145].

Members of Note of the Pseudomonas genus

Members of the *Pseudomonas* genus have been studied for various scientific and industrial properties. *Pseudomonas putida* has been studied in the potential "green" production of

food flavoring additives, (e.g. cinnamic acid, cis-cis-muconate, p-hydroxy- benzoate), important aromatic chemical precursors (e.g. p-cuomarate), and pharmaceutical chemical precursors (e.g.myxochromide) [146–148]. *Pseudomonas putida* strain KT2440 is noteworthy, as it is a well-studied, sequenced organism that is a Generally Recognized as Safe certified strain [146,149]. *Pseudomonas fluorescens*is a well-known rhizosphere bacteria, known for its commensal nature with plant roots in helping obtain nutrients while producing anti-fungal compounds [150].

The *Pseudomonas* genus members most similar to *P. aeruginosa*, *P. oleovorans* and *P. stutzeri* are also of scientific and practical interest. *P. oleovorans*, a bacteria that can directly oxidize petroleum derived hydrocarbons, is under continued study for use in the "green" production of biodegradable thermoplastics[151–153]. *P. stutzeri* has been of interest for its bioremediation potential because of its ability to digest aromatic hydrocarbons and its resistance to various heavy metals [154].

Pseudomonas syringae is a well-known plant pathogen of model and agricultural plants such as kiwifruit producing *Actinidia deliciosa* and hazelnut producing *Corylus avellana*; the bacterium is used as a model system for plant-pathogen interactions [155,156]. The pathogenesis of *P. syringae* in plants involves entering a plant at sites of injury or openings such as the stomata[157,158]. Once inside, the bacterium uses a Type III secretion system (T3SS) to directly inject the plant cell with virulence factors to suppress plant cell recognition as well as developing a biofilm to sustain infection[157,159].

The Human Pathogen Pseudomonas aeruginosa

Of the *Pseudomonads*, *P. aeruginosa* is the most significant for human health. The name *aeruginosa*, literally Latin for "copper rust", is a reference to the similarity of the pigmented

organism's colonies to that of the color of oxidized copper (verdigris) [119]. The first recorded isolation and description of *P. aeruginosa* was in 1872 [119]. It is an opportunistic pathogen and, in addition to humans, can infect amoebas, fungi, plants, nematodes and other mammals [160–163]. Opportunistic infections of *P. aeruginosa* occur as a result of the breakdown or weakening of anatomical barriers of the host; this makes the bacterium a common cause of nosocomial infection, principally from contaminated medical equipment that bypasses physical barriers: catheterization-related Urinary Tract Infections, ventilator-associated pneumonia, and a general infection risk of wounds and burns in a hospital setting [124,164–166]. *P. aeruginosa*, in a non-medical setting, is more commonly seen in "hot tub folliculitis", an infection of hair follicles seen post exposure to a water source that has not been properly treated against bacterial growth [166].

The study of the organism as a causative agent of human disease has led to a close examination of the many factors responsible for its pathogenicity. *P. aeruginosa* use Type IV pili and fimbriae for motility and attachment to epithelial cell membranes and other materials [167]. *P. aeruginosa* cells that have entered the respiratory tract have been shown to bind to the sialic acid structures present on epithelial cells and mucin proteins [168,169]. Sialic acid structures on cells are believed to be sources of self-recognition by the immune system and have been shown to increase during inflammation [170,171]. In the consistently pro-inflammatory state with thickened and static pools of mucin in the pulmonary system of cystic fibrosis, *P. aeruginosa* quickly establishes residency [172].

After attaching, *P. aeruginosa* begins to secrete various compounds to begin digestion of the host environment. *P. aeruginosa* has a well described Type III secretion system (T3SS) allowing for direct injection of host cells with virulence factors [114]. Expression of this

virulence factor alone is associated with a six-fold increase in the relative risk of mortality in *P. aeruginosa* associated pneumonia [173]. *P. aeruginosa* secretes several proteins through a Type II Secretion System (T2SS), such as Exotoxin A, a diptheria-toxin like toxin that causes ADP-ribosylation of EF-2 that may help in breakdown of tissue barriers during infection [174,175]. Various proteases are also secreted via the T2SS, importantly: the elastase LasB, which cleaves elastin to cause the breakdown of the mechanical barriers that hold tissues together; the lysine-specific zinc metallo-endopeptidase LasA that enhances elastase activity and cleaves *Staphylococcus aeures* peptidoglycan; and phospholipases to cleave the phospholipid cell membrane of the host cells [115,175–179].

With sufficient concentration of *P. aeruginosa*, formation of the virulence factor with the greatest scientific and medical interest, biofilm, begins. Biofilm production by bacteria is widely reported, and is seen as a protective mechanism of bacteria against their environment and especially against antibacterial agents [164,180–182]. Biofilm production is a complex and coordinated communal behavior that relies on group signaling, known as quorum signaling, to produce coordinated production of biofilm precursor products at the correct bacteria density to form a mature biofilm [183–185]. In *P. aeruginosa* production of sufficient quantities of the quorum signaling molecules belonging to the family of N-acyl homoserine lactone, 3-oxo-C12-HSL and C4–HSL, leads to activation of the *lasRI* and the *rhlRI* gene regulation systems [184]. This regulation system causes metabolic and cellular changes to shift the bacteria into production of biofilm niche living [186]. Important changes to respiration are likely to occur as well, as low oxygen tension within cystic fibrosis mucus is further reduced within biofilm [187]. The biofilm community also fosters the growth of a small population of highly resistant cells known as

persister cells, capable of replenishing the biofilm with bacteria after exposure to an antibiotic [188,189].

P. aeruginosa expresses a diverse array of antibiotic resistance beside biofilm production. *P. aeruginosa* has an inducible protein AmpC, a cephalosporinase that can hydrolyze the β -lactam ring found in the penicillin drug family of antibiotics to cause inactivation [190]. The bacteria also contains several efflux pump proteins, capable of removing the antibacterial drug families of fluoroquinolones and aminoglycosides from the cell's cytosol [191]. Disturbingly, strains of *P. aeruginosa* have been reported, that are resistant to colistin, a last-resort antibiotic [192].

Pseudomonas aeruginosa and the Cystic Fibrosis Lung

Many studies of *P. aeruginosa* as a cystic fibrosis pathogen have focused on identifying genetic changes during chronic infection and the production of biofilms and virulence factors, but much less is known about the system-wide metabolic phenotypes of this and other pathogenic microbes or their physiological adaptations during chronic infections [112,124,164,193–202]. Omic studies of pathogenic *P. aeruginosa* strains have described changes at the genomic [203–212] transcriptomic [206,213–217] and proteomic [181,213,218] level that point to numerous changes in adaptation to the cystic fibrosis lung environment. Evidence in these and other studies indicates that this organism grows in a microaerobic to anaerobic environment within the lung; nitrate is available for respiration and available primary carbon sources include amino acids, glucose, and lactic acid [219,220]. Direct measures of *P. aeruginosa* cystic fibrosis isolates found that the overproduction of the biofilm component alginate is commonly associated with increased morbidity and mortality in the patient population. This produces a mucoid colony phenotype in culture [176,221–223]. Chronic

infection isolates of *P. aeruginosa* also demonstrate decreased production of other known virulence factors [197].

However, these identified changes, have not led to a clear understanding of the metabolic adaptations that the organism undergoes for two distinct reasons. First, chronic infection strains of *P. aeruginosa* often lack mismatch repair enzymes, creating the hypermutable phenotype, allowing for increased niche specialization in the pulmonary system [224]. This causes most chronic infection strain isolates *P. aeruginosa* to have significant genomic instability, introducing significant uncertainty in the direct genomic measurements due to the very high genomic diversity observed [217,224–226]. Second, different Omic datasets do not correlate well (Pearson's correlation coefficients (r²) values are generally around 0.4-0.5) within data sets or with comparisons to other Omic measures of the same samples, giving uncertainty to the true meaning of the differences recorded with these techniques [227–229]. To identify which changes are actually significant for pathogenesis and to measure and predict functional changes in metabolism, a toolbox of network based computational and experimental methods is available.

Metabolic Network Analysis

Metabolic Network Analysis is the application of topological analysis and computational theory to biological measurements to better understand the interplay between genetics and biochemistry to produce metabolism [230]. The methods for describing metabolism mathematically and modeling it computationally were developed for the purpose of directed genetic engineering of microbes to produce desirable biomolecules [231,232]. The major approaches to analyzing and modeling metabolic fluxes can be divided into enzyme kinetics based representation of chemical reactions or steady-state assumption based approaches

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[233].The work in this thesis focuses on two steady-state based modeling techniques, Metabolic Flux Analysis and Flux Balance Analysis.

Metabolic Flux Analysis

Metabolic Flux Analysis (MFA) centers on the use of isotopically labeled substrates (usually ¹³C) in experiments allowed to reach a stable pattern of isotopic distribution (end point labeling) [234]. The labeling patterns in metabolic products or intermediates are used to deduce internal metabolic state and pattern of carbon fluxes through the cellular metabolic system: a flux map[235]. To produce such a map measurements are made of extracellular input and output fluxes such as biomass production, gas exchange, and carbon inputs[235]. Second, metabolite pools must also accurately represent subcompartmentalization of a cell such that measured mass isomers (isotopomers) accurately represent labeling in different parts of the cells [236]. Third, the major biochemical pathways need to be known and the stoichiometry and carbon positional transformations for each reaction are needed for each reaction step [237]. Fourth it is assumed (based on past metabolic studies) that there are no changes in enzyme kinetics due to increases in mass due to isotopic labeling [234].

With these assumptions in mind, the computational modeling and fitting of ¹³C labeling data can be discussed. Metabolism of carbon-containing compounds by enzymatic processes occurs at defined carbon positions within the molecule due to the biochemical specificity of enzymatic catalysis; this fundamental property of enzymatic reactions allows for characteristic labeling patterns to develop for the carbon in the molecule [238]. To describe the re-shuffling of label for a reaction, an atom mapping matrix is constructed for each reaction in the network [239]. Since each carbon atom in a metabolite is either labeled or unlabeled, there are a total of 2ⁿ isotopomers [239]. Using mapping matrices and isotopomer matrix representations matrix

algebra can be used to predict the labeling pattern at steady state in all metabolites by assuming reaction rates (the values of the metabolic fluxes) [235]. The computed results are then compared to the measured ¹³C labeled measurements and the flux values are iteratively changed to optimize the fit of the modeled to the measured labeling patterns [235]. Computational software such as ¹³C-Flux is available for this stage of MFA [240]. This results in MFA quantifying carbon fluxes through metabolism.

To date, most MFA studies of microbial systems have focused on questions related to biotechnology and metabolic engineering, microbial physiology, and gene function [241–245]. Several recent studies have analyzed the metabolic interactions of pathologically relevant bacteria within their host environment [246,247] and metabolic differences between mutant strains [248]. A recent study of pathogenesis related metabolism in *P. aeruginosa* employed MFA to compare 17 uropathogenic strains [249]. A more detailed discussion with a methodologically improved study directed specifically at cystic fibrosis isolates can be found in the next chapter of this dissertation.

Flux Balance Analysis

In addition to the use of MFA to quantify metabolism, Constraints-based Flux Balance Analysis (FBA) is a biochemically-based mathematical modeling approach to the steady state analysis of metabolism that incorporates carbon and non-carbon elements in a large-scale mass balance analysis; energy and redox co-factors are also balanced and no labeling experimental data are needed [250–252]. FBA can extend the metabolic scope beyond carbon and beyond central metabolism to a complete genomic scale coverage of metabolism [251,253]. The biochemical equations that make up the individual steps of metabolism are converted into a matrix equation in which the stoichiometry of each reaction (how many of which metabolites are consumed and produced) is described in a Stoichiometric or S matrix and this acts on a vector containing the carbon flux values for each reaction to yield a vector containing the change of metabolite concentrations over time (Equation 1).

(1)
$$\frac{dx}{dt} = Sv$$

Where:

 $\frac{dx}{dt}$ is change of concentration over time S is the stoichometrixmatrix y is the flux vector

Equation 1 can be constrained to reduce the possible mathematical solution space using assumptions, such as the steady state assumption of no net accumulation of internal metabolite pools, and through measurements like proteomic or transcriptomic data to restrict the reactions that are potentially active in the model [251]. Additionally, optimization algorithms can be used to maximize or minimize selected individual fluxes or combinations of fluxes (called objective functions) in order to produce the most likely carbon flux of the metabolic network being tested [251]. The COBRA (Constraints Based Reconstruction Analysis) toolbox in MATLAB is an FBA computational system with access to these functions as described above were used for FBA in this research project [254].

FBA investigations of pathogenic organisms have been used to search for novel drug targets and have identified potential metabolic targets not affected by current therapeutics, such as amino acid production or fatty acid metabolism [255–262]. Recent FBA research on *P. aeruginosa* includes a genome-based metabolic and transport model by Oberhardt and colleagues [263], and used Flux Balance Analysis (FBA) with transcript data from two cystic fibrosis clinical strains isolated 44 months apart to investigate *P. aeruginosa*'s metabolic capabilities and

potential metabolic changes during prolonged infection [264]. FBA has also been used to identify potential metabolic drug targets during biofilm growth of *P. aeruginosa*[265].

The combination of both FBA and MFA allows for investigation of suboptimal growth and testing of alternative objective functions that may be optimized besides maximal biomass production. However, there have been surprisingly few studies in which these complementary network flux analysis approaches have been combined [146,227,241,266–271], let alone in direct application to understand bacterial pathogenicity. In the following chapters, the investigation of *Pseudomonas aeruginosa* metabolism and its changes due to exposure to and growth in the cystic fibrosis lung environment will be described. REFERENCES
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CHAPTER 2 –

Metabolic Flux Analyses of *Pseudomonas aeruginosa* Cystic Fibrosis Isolates^{*}

^{*} This manuscript was submitted to Metabolic Engineering Journal in March 2016 (Opperman and Shachar-Hill); it is currently under review.

ABSTRACT

Pseudomonas aeruginosa is a metabolically versatile wide-ranging opportunistic pathogen. In humans *P. aeruginosa* causes infections of the skin, urinary tract, blood, and the lungs of Cystic Fibrosis patients. In addition, *P. aeruginosa*'s broad environmental distribution, relatedness to biotechnologically useful species, and ability to form biofilms have made it the focus of considerable interest. We used ¹³C Metabolic Flux Analysis and Flux Balance Analysis to understand energy and redox production and consumption and to explore the metabolic phenotypes of one reference strain and five strains isolated from the lungs of Cystic Fibrosis patients. Our results highlight the importance of the Oxidative Pentose Phosphate and Entner-Doudoroff pathways in *P. aeruginosa* growth. Among clinical strains, we report two divergent metabolic strategies and identify changes between genetically related strains that have emerged during a chronic infection of the same patient. MFA revealed that the magnitude of fluxes through the glyoxylate cycle correlates with growth rates.

INTRODUCTION

Pseudomonas aeruginosa is a gram negative, environmentally widespread, opportunistically pathogenic bacterium; it causes infections, growth inhibition and death in organisms as diverse as amoebas, fungi, plants, nematodes and mammals [1–4]. In humans this organism causes infections of the skin, urinary tract, blood, and lung; it is among the most frequent and harmful causes of hospital-acquired infections [5–7]. *P. aeruginosa*, which forms biofilms in many environmental and pathogenic situations, is metabolically versatile [8] and intrinsically resistant to many antibiotics; it develops further resistance during chronic infections resulting in treatment failure [9–11]. *P. aeruginosa* infections of the lungs of Cystic Fibrosis (CF) patients are of particular concern.

CF is a genetic disease involving defects in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein that affects an estimated 70,000 individuals worldwide [12–17]. Defective membrane transport leads to pancreatic insufficiency, diabetes mellitus, bronchiectasis, and chronic bacterial infection of the pulmonary system in adulthood [18,19]. While new treatments have begun to change how some CF sub-types are treated (e.g. ivacaftor; [20]), most adults with CF still face chronic bacterial infection, especially by *P. aeruginosa*, leading to the development of highly resistant strains and to the clinical failure of pulmonary treatment, chronic inflammation and progressive damage to the lung, pulmonary failure, and eventual lung transplantation or death in mid-adulthood [5,9,11,12,21,22].

While many studies of *P. aeruginosa* as a pathogen have focused on identifying genetic changes during chronic infection, and on the production of biofilms and virulence factors, much less is known about the system-wide metabolic phenotypes of this and other pathogenic microbes or their physiological adaptations during chronic infections [5,7,23–32]. Omic studies of

pathogenic *P. aeruginosa* strains have described changes at the genomic [33–41], transcriptomic [41–46], and proteomic [47,43,48] levels. Such studies have demonstrated numerous changes in the cellular inventory during evolution in the lung environment. To identify which of these many changes are significant for pathogenesis and to measure and predict functional changes in metabolism, a toolbox of network based computational and experimental methods is available. In recent years, these tools have begun to be applied to pathogenic microbes, including *P. aeruginosa*.

Constraints-based Flux Balance Analysis (FBA) uses the structure of the metabolic network and the stoichiometries of the reactions of which they are composed to build computational models of metabolism (see [49–51] for recent reviews). Such models are used to investigate the potential flows of carbon and other elements as well as cofactor balances and can extend to genome-wide coverage [50,52]. In addition to identifying reactions and conditions essential for growth and improving gene annotations, FBA and related tools allow the prediction of maximal growth rates and the exploration of predicted metabolic flux distributions under the assumption of different optimization strategies ("objective functions", most commonly maximal growth efficiency). FBA investigations of pathogenic organisms have been used to search for novel drug targets and have pointed to potential metabolic targets not affected by current therapeutics, such as amino acid production or fatty acid metabolism [53-60]. Oberhardt and colleagues have constructed a genome-based model of metabolism and transport in P. aeruginosa [61], and used Flux Balance Analysis (FBA) with transcript data from two CF clinical strains to investigate P. aeruginosa's metabolic capabilities and potential metabolic changes during prolonged infection [62]. FBA has also been used to identify potential metabolic drug targets during biofilm growth of P. aeruginosa [63]. To map network-wide metabolic

fluxes without assumptions about the strategies or objectives being pursued during cellular metabolism, isotopic labeling experiments are used together with metabolic network models [64].

Metabolic Flux Analysis (MFA) combines ¹³C labeling results with the growth and uptake measurements used for FBA to yield estimates of carbon fluxes through central metabolism [65–68]. Most MFA studies of microbial systems to date have focused on questions related to biotechnology and metabolic engineering, microbial physiology, and gene function [69–73]. Several recent studies have analyzed the metabolic interactions of pathologically relevant bacteria within their host environment [74,75] and metabolic differences between mutant strains [76]. A recent study of pathogenesis related metabolism in *P. aeruginosa* employed MFA to describe 17 uropathogenic strains [77].

The combination of FBA and MFA allows the testing of alternative objective functions, and can identify the origins of sub-maximal growth rates. However there have been surprisingly few studies in which these complementary network flux analysis approaches have been combined [70,78–85]. Here we performed FBA analysis together with ¹³C MFA of one reference and five selected CF pathogenic strains of *P. aeruginosa* to understand energy and redox production and consumption processes and to explore metabolic (in)efficiencies and metabolic phenotypes. Our results highlight the importance of the Oxidative Pentose Phosphate Pathway (OPPP) and Entner-Doudoroff Pathway (EDP) in *P. aeruginosa* growth and point to a substantially lower flux around the tricarboxylic acid pathway than has been previously reported for the same reference strain under similar conditions. The differences were explained by the smaller experimental dataset previously used. Among the clinical strains we report two metabolic strategies and identify changes between genetically related strains that have emerged

during the course of a chronic infection of the same patient. MFA identified fluxes through the glyoxylate cycle, whose magnitude correlated with growth rates across strains, although this pathway is not predicted by FBA to increase growth efficiency. Finally, we compared the degree of relatedness of metabolism among strains obtained in two ways: in the first approach ¹³C labeling data alone was used, and in the second the fluxes obtained by MFA were compared. While both approaches clearly discriminated among the strains, the patterns of relatedness revealed by labeling data did not correspond to those apparent from the flux analyses.

MATERIALS AND METHODS

Strain selection and culture

Pseudomonas aeruginosa strains are described in Table 2.1. Strains were chosen that have sequenced genomes, clinical and epidemiological importance, and to include an example of an ancestral and descendant strains [86]. Strains AA2, AA43, AA44, and LES 400 were obtained from the Belgian Co-ordinated Collections of Micro-organisms (Ghent, Belgium; LMG numbers: 27630, 27631, 27632, and 27623 respectively). *Pseudomonas aeruginosa* strain AMT 0023-30 was obtained from Cystic Fibrosis Isolate Core (Seattle, Washington). *Pseudomonas aeruginosa* strain PA01 was obtained from Dr. M. Mulks at Michigan State University. M9 defined minimal media with 22 mmol glucose as the sole carbon source was used as the culture media for all experiments [87]. All cultures were pre-cultured from -80°C frozen stock samples onto M9 media solidified with 1.5% agarose for 24 hours at 37°C. Single colonies were transferred into 30ml of M9 liquid media in 250 ml Bellco triple baffled shake flasks, incubated at 37°C and shaken at 120 RPM until stationary phase (12 to 14 hours) before spectrophotometric measurement of cell density as absorbance at 600nm (OD₆₀₀) and inoculation of experimental cultures to initial densities of 0.01 OD_{600} . For ¹³C-labeling experiments cells were cultured using either 99.9% (mol/mol) [1-¹³C] glucose or 20% (mol/mol) [U-¹³C] glucose.

Cell and media sample harvest

Cell pellets were collected by centrifugation of cultures at mid-log phase ($OD_{600} \approx 0.5$). 1 ml of culture was centrifuged at 15000 g for 5 minutes. The supernatant was removed and 100 µl of 6N HCl was added to the pellet, which was stored at -20°C. Cell pellet sample preparation for amino acid analysis was based on previously reported methods [78,88].

Determination of substrate uptake rates and product secretion

Glucose uptake rates for each strain were determined from 1mL culture supernatants using at least three biological replicate samples for each of three log phase time points taken at 15 minute intervals. After lyophilization, samples were resuspended in 600 μ L of 99% D₂O, and then lyophilized and resuspended in 600 μ L of 99.9% D₂O. 1H-NMR was performed on an Agilent *DirectDrive2* 500MHz instrument using an Agilent *OneNMR* Probe with *Protune* for auto-tuning. Spectra were obtained at 500 MHz with a pulse angle of <45°, acquisition time of2.05 s, and recycle delay of 2 s. Glucose concentrations relative to the initial 22mM were determined with reference to 10 mM methylphosphamide added as internal standard. No secreted products were detected at significant levels in the media after culture growth for any of the strains. Calculation of glucose uptake rates during exponential growth used the following equation:

$$v = \frac{\mu}{X_i} \frac{C - C_0}{e^{-\mu t} - 1}$$

Where μ is intrinsic growth rate (hr⁻¹); C is the concentration of glucose (mM) at time t; X_i is the initial density (g·DW/L) of cells; t is time (hours); and v is uptake flux (mmol/g·DW/hr).

Strain	Origin	Related Isolates	Relevant Characteristics	References
PA01	Australia Wound Isolate (1955)	N/A	Standard Reference Strain.	[7,30,32]
AMT 0023- 30	U.S.A. CF - Pediatric Clinical Isolate (~1998)	AMT 0023-34	Persister Cells Present	[31,39]
LES 400	U.K. CF - Adult Clinical Isolate (2003)	Liverpool Epidemic Strains (LES)	Increased Acute Morbidity Risk Decreased Life Expectancy with Infection Pyrocyanin Overproduction Alginate Overproduction CF Transmissible	[24,25,27,37,89]
AA2	Germany CF – Adult Clinical Sequential Isolates (~1998 & ~2003)	AA43, AA44	Parental strain of AA43, AA44	
AA43		AA2, AA44	7.5 years total lung colonization time Increased Acute Morbidity	[23,29,33]
AA44		AA2, AA43	risk Motility defect Protease reduction LPS and PGN changes	

 Table 2.1. Pseudomonas aeruginosa strains analyzed. LPS, Lipopolysaccharide; PGN, polygalacturan. N/A, not applicable.

Determination of CO₂ efflux rates

Cultures were grown, as described above, to early log phase that were sealed with gastight caps and incubated for two hours. Due to the high sensitivity and low maximum range of CO_2 detection of the LICOR CO_2 measurement, *P. aeruginosa* growth was limited to a low range of optical density for this measurement. The cell density of the culture was measured and growth was stopped by adding 1 mL 6N HCl to the culture, which also converted dissolved bicarbonate to CO_2 . Total CO_2 was measured using a LICOR LI-6400with dry CO_2 free air as input into the culture flask at a rate of 500µl/s. Readings of total CO_2 were recorded over 5 minutes (1s time resolution) to allow for removal of CO_2 from the flask. Total CO_2 evolved was measured in at least three replicate cultures with CO_2 levels integrated for total CO_2 efflux. Due to the very small change in OD during the CO_2 measurement period, CO_2 efflux was calculated from total CO_2 evolved per change in time per total change in dry-weight in grams.

Analysis of Amino Acid labeling

Cell pellets were suspended in 1 mL of 6N HCl and incubated at 100°C for 24 hours, and dried at 60°C under a stream of N₂. Amino acids were derivatized using N-Methyl-N-[tert-butyldimethyl-silyl] trifluoroacetimide (MTBSTFA, SIGMA-ALDRICH) in a 25µl pyrimidine and 25µl 1% MTBSTFA solution incubated at 40°C for 1hour as previously described [78,88,90]. GC-MS analyses of derivatized amino acids were performed on an Agilent 5973GC/quadrupole MS. GC-MS signals were corrected for natural isotope abundance [88]. Amino acid fragments used in ¹³C MFA were based on reported reliability [90]. Unlabeled amino acid samples collected were used to confirm the accuracy of natural abundance correction. GC-MS amino acid data was corrected for natural abundance isotopic contents with average values and standard deviation calculated for biological replicates used as model inputs.

Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA)

GC-MS measured mass fragment mass abundance data for amino acids of strains harvested after growth in 100% 1-¹³C glucose or 20% uniformly labeled (1,2,3,4,5,6-¹³C) glucose was collected. Amino acid fragments that were measureable in three biological replicates were then processed in MATLAB (2010a, Mathworks, Natick MA, U.S.A.) using the PCA function to produce a principal component analysis [91]. HCA was performed on this data set as well as on fluxes determined by MFA that were normalized to each strain's glucose uptake rate. HCA was implemented in the programming language R using complete linkage criteria using Euclidian distance [92].

¹³C Metabolic Flux Analysis

A carbon isotopic network of *P. aeruginosa* central metabolism was constructed by an approach similar to that previously used for *E. coli* [78]. The iM01056 FBA model [61] reaction network was simplified by only including carbon atoms of reactants and products, by introducing both net and exchange fluxes for reversible fluxes, by combining linear reaction sequences that do not alter carbon positions, and by condensing secondary metabolic pathways into a growth equation that consumes central metabolic intermediates as precursors to produce cellular biomass as previously defined [61,93]. By preserving the architecture of the FBA model, results of FBA and ¹³C MFA could be directly compared, and ¹³C MFA results examined by FBA [78].

Measured rates of glucose uptake, CO_2 efflux, and ¹³C labeling patterns in amino acids together with growth rates were used to estimate internal fluxes of central carbon metabolism by fitting flux values to the experimental data using the ¹³C-FLUX software as previously described [67,68,78]. For most labeling data, variation among biological replicates was very small (see Supplemental 2.1); since experimental standard deviations (SDs) are not a reliable estimate of true population SDs when 5 or fewer replicates are analyzed, experimentally observed SD's with low SD's were increased to 2% of mean values for MFA modeling as previously described [78]. This also avoids excessively constraining modeling results to labeling data at the expense of direct flux measurements and reduces the potential for distortions due to precise but inaccurate mass isomer quantification [78]. To allow for uncertainties in *P. aeruginosa* biomass compositions, production rates were constrained to be within 50% of the *E. coli* biomass values [78].

To minimize the risk that solutions represented local rather than global optimization minima, multiple randomly generated initial fluxes constrained by sampling the feasible solution space were used. First, at least 100 randomly generated feasible starting points that produced optimized fits by the ¹³C-FLUX program were found. Next, the 10 starting points that yielded the lowest final residuum values were used in the second stage to generate 1000 more starting points by randomly perturbing these starting points to yield 100 new points each. The final, lowest residuum optimized flux values that resulted in the best fit to the data were used. Confidence intervals for flux values were estimated using a Monte Carlo approach to randomly generate values of: biomass, glucose uptake rate, CO_2 efflux rate, and amino acid labeling data for each strain [94] based on the experimentally determined standard deviations. At least 20 such datasets were then fitted, as described above to yield best-fit flux maps for each strain. These 20 flux value sets were then used to calculate 90% confidence intervals for each flux modeled for each strain.

All MFA computations were performed using the High Performance Computer Center, Michigan State University, using a parallel 1536 core cluster of 192 nodes (two four-core Intel

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Xeon E5620s at 2.4 GHz with 24 GB of RAM and 250 GB local disk space per node). Global sum of squared residuals (SS_{res}) for each strain is listed in Supplemental 2.2.

Flux Balance Analysis

The genome-derived stoichiometric *P. aeruginosa* model iM01056 developed by Oberhardt *et al.* was used for FBA [61]. The results of PA01are representative of predicted central metabolism for all the strains studied here since their genomes do not appear to lack any functional central metabolic fluxes [23,39,41]. The model was modified to better account for lipid production (see Supplemental 2.3) resulting in a total of 1013 reactions and 875 metabolites. The COBRA Toolbox 2.0 in MATLAB (Mathworks, Natick MA, U.S.A.) implementing the Gurobi 6 optimizer was used for FBA using the objective function of maximal biomass production [52]. FBA simulations used measured glucose uptake rates by strain PA01. To determine the range of fluxes that allow 99% or 90% of the maximal growth rates, the Flux Variability Analysis (FVA) function of the COBRA Toolbox was used [95].

RESULTS

The physiology, growth and yield of the reference and clinical strains were compared during growth in defined medium to characterize any intrinsic or evolved differences among them. Specific growth rates, final culture density (as a measure of carbon conversion efficiency over the full growth cycle), glucose uptake rates, and CO_2 efflux rates were measured as described in Materials and Methods and are shown in Figure 2.1.

The growth rates show a 40% range of values, and both glucose uptake and CO_2 efflux rates show a twofold range across strains. Compared to those reported for uropathogenic *P*. *aeruginosa* strains grown in an artificial urine medium [77], strain LES 400 showed a growth rate as high as or higher than the highest previously reported (0.96 hr⁻¹ vs 0.91 hr⁻¹) and strain

AA44 showed a glucose uptake rate as low as or lower than the lowest rate reported in uropathogenic strains (4.92 mmol gDW⁻¹ hr⁻¹ vs 5.37 mmol gDW⁻¹ hr⁻¹). *P. aeruginosa* pathogenic strains display a large range of growth parameters under defined growth conditions pointing to divergent physiological phenotypes. By contrast, the maximum optical densities attained at stationary phase showed no statistically significant differences among strains, indicating that the growth yields over the culture period are similar.

To explore whether final yields are reflected in substrate use efficiencies during growth, Carbon Conversion Efficiency (CCE) was calculated during log phase growth using biomass production rates compared to: (a) glucose uptake rates; and (b) CO_2 production rates (Figure 2.2). CCE values for both methods are not significantly different for any strain, as seen from the 95% confidence intervals and confirmed by a heteroscedastic two sided t-test (p>0.05).

These results are consistent with the absence of detected secreted products in culture media (closed carbon balance). Previous studies of *P. aeruginosa* and *P. fluorescens* also found no evidence for significant metabolite export during growth in defined simple media [77,96]. The values of CCE% found for these strains (59%-72%) are higher than the 52% reported for *E. coli* growing under the same conditions [97]. In that case the CCE is lowered by acetate secretion, but the range here is lower than the estimated 86% for *P. fluorescens* (as calculated from reported MFA results [96]).

To assess whether the diverse pathogenic strains have evolved divergent metabolic phenotypes, ¹³C labeling data for amino acids from cultures grown to steady state with labeled glucose, were analyzed by Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA). Both analyses (Figure 2.3) demonstrate clear differences between strains, with 70% of the variation of the amino acid label data contained in the first three principal

components of the PCA. Sample labeled amino acid data used in Figure 2.3 can be found in Supplemental 2.1. Both PCA and HCA show two groups of strains, one containing AMT 0023-30 and PA01 and the other consisting of AA43, AA2, AA44, and LES 400.

The ¹³C MFA flux map of *P. aeruginosa* reference strain (PA01) is shown in Figure 2.4. The MFA model, experimental data, and flux tables are given in Supplemental 2.2. The map shows that the flux through the Entner-Doudoroff Pathway (EDP) is equivalent to 60% of the glucose uptake rate. Flux through the decarboxylation step of the Oxidative Pentose Phosphate Pathway is equivalent to 85% of the glucose uptake. Published values for aerobic E. coli grown under similar conditions show a flux of 25%-27% through the OPPP compared to glucose uptake with glycolysis carrying close to 80% of the glucose uptake flux [78,98]. A substantial flux recycles carbon from the OPPP to hexose-monophosphates so that the sum of these fluxes (the total flux from hexose-6-phophate to 6-phosphogluconate) is higher than the glucose uptake rate. Escherichia coli studies have also reported cyclic OPPP fluxes under these conditions [78,98]. Little or no net flux was estimated to occur from triose-phosphate to hexose-6-phosphate via the reversible steps of glycolysis. Also noteworthy is the low tri-carboxylic acid (TCA) cycle flux under these aerobic conditions, with significant fluxes estimated though the glyoxylate cycle and anapleurosis from phosphoenolpyruvate carboxylase. Confidence interval (CI) calculations of the MFA model indicate that the fluxes are well estimated by the data, with 90% CIs for net fluxes limited to +/-10% of flux values in the EDP and OPPP and close to +/-20% of fluxes in the TCA cycle.


Figure 2.1. Growth physiology. (A) Specific growth rates (n=5, AA43 n=4); (B) Final cell densities (OD600, n=3); (C) Glucose uptake rates (AA44 n=7, LES400 n=3, AMT0023-3 n=6, others n=5), and (D) CO₂ efflux rates (AMT0023-30 n=4, AA2 n=5, others n=3). Error bars represent 95% confidence intervals. Horizontal bars in (A), (C), and (D) connect strains that do not show significant difference ($\alpha \le 0.05$) under two-sided t-tests, [no significant differences among strains in (B)].



Figure 2.2. Carbon conversion efficiency during growth. Filled bars show values calculated from glucose uptake and biomass production; Empty bars show values calculated from CO_2 efflux and biomass production. Error bars represent 95% confidence intervals. (CCE% Glucose for LES400 n=4; CCE % CO_2 AA2 n=5, AMT0023-30 n=4; others n=3)



Figure 2.3. Discrimination among strains using ¹³**C labeling fingerprinting.** (Figure 2.3A - Left) Principal Component Analysis of the isotopomers of proteinogenic amino acids and their fragments. Steady state labeling was measured in three biological replicates from 100% 1^{-13} C and 20% U_{6}^{-13} C Glucose experiments (see materials and methods). Strains: AMT0023-30 (circles), AA2 (triangles), AA43 (squares), AA44 (diamonds), LES 400 (pentagrams), and PA01 (hexagrams).(Figure 2.3B - Right) Hierarchical Clustering Analysis (HCA, see materials and methods) of the same data used in Figure 2.3A.

Flux Balance Analysis of *Pseudomonas aeruginosa* strain PA01 was performed using a modified genome-derived model (see methods) together with the glucose uptake rate measured for PA01. A predicted flux map based on maximum growth efficiency (maximal biomass production for the glucose uptake rate) as the objective function is shown in Figure 2.5. A listing of the predicted net fluxes for optimal growth is in the supplemental materials (Supplemental 2.3).

The FBA flux map shows several differences from the MFA experimentally based map for PA01. Cyclic flux in the oxidative pentose phosphate pathway is not seen in the FBA map; instead the majority of the carbon flows through the Entner-Doudoroff pathway and the OPPP decarboxylation flux is predicted to be lower than estimated by MFA. FBA predicts a robust tricarboxylic acid cycle flux that is not consistent with the fluxes estimated by MFA. FBA predicts maximal growth efficiency (CCE) to be 70%, compared to the 60-65% observed. To assess whether particular fluxes estimated by MFA are likely to be responsible for the submaximal CCE observed for PA01, flux variability analysis (FVA) was used to determine the ranges of fluxes consistent with 99-100% of maximal predicted. The results are shown in Figure 2.5 and Table 2.2 and predict that near-maximal growth could be sustained with a large range of individual fluxes. For example the first committed step of the OPPP (decarboxylation of6phosphogluconate) can have 0-130% of the predicted optimal value. The MFA map for PA01 shows several net fluxes outside the range defined by FVA for near-optimal growth: (A) G3P to PEP flux is estimated by MFA at 20% of glucose uptake with a range of 49%-81% calculated by FVA;(B) pyruvate to Ac-CoA and CO₂ (19% of glucose uptake in MFA vs 51-133% from VFA); and (C) PEP to pyruvate (6% vs. 49-81%).



Figure 2.4. ¹³**C MFA flux map of strain PA01.** Strain PA01 was grown in defined minimal media with either 100% 1^{-13} C or 20% U₆-¹³C Glucose (n=3 replicates for each substrate) to steady state labeling during exponential growth. Labeling and external fluxes were measured and modeled as described in materials and methods. Arrow thicknesses are proportional to net fluxes. Numbers represent net carbon fluxes in units of c-mmol flux/(gDW•hr); values are given +/-90% confidence intervals (CI). The rage of fluxes catalyzed by Aldolase and alpha-ketogluarate dehydrogenase show the optimized model results paired with the upper range of the calculated CI90% as these fluxes are limited to 0 (irreversible). Non-standard or potentially ambiguous abbreviations: 6PGC, 6-phospho-gluconate; G3P, Glyceraldehyde 3-phosphate; H6P, hexose-6-phosphate; P5P, Pentose-5-Phosphate.



Figure 2.5. Flux map from FBA analysis of strain PA01. Uptake of glucose, production of CO_2 and growth rate were used to constrain a stoichiometric genome-derived metabolic model (see materials and methods) with the objective function being maximal growth (corresponding to maximal growth yield on glucose). Numbers are net fluxes as in Figure 2.4. Ranges are from flux variability analysis (range of each flux consistent with 99-100% of maximal growth).

To quantify the metabolic differences among strains isolated from the cystic fibrosis lung environment, Metabolic Flux Analysis was conducted on the clinical strains (Table 2.1). Flux values estimated by MFA are listed in Table 2.2 (full flux lists can be found in Supplemental 2.2) and net fluxes are displayed relative to the PA01 flux values in Figure 2.6A while Figure 2.6B shows flux values relative to the uptake rate of glucose for each strain. This figure demonstrates that CO_2 efflux and phosphoenolpyruvate carboxylase flux rates across all strains are within 30% of the reference strain flux even though glucose uptake rates show a variation of 49-106%. The estimated EDP and TCA cycle fluxes vary widely across strains with 2-fold and 3.5-fold ranges, respectively. The α -ketoglutarate dehydrogenase flux is very low in reference strain PA01, and is highly variable across the other strains (Figure 2.6A). Finally, it was observed that the flux rate of isocitrate lyase plotted against the measured intrinsic growth rate of all strains showed the greatest linear correlation (0.599 R²) of flux to growth rate outside of the biomass related synthesis fluxes. This value increased to a R² value of 0.982 with the removal of strain AA2's MFA derived flux from this data set (Figure 2.7).

Hierarchical Clustering Analysis (HCA) of MFA flux values normalized to measured glucose uptake rates (Figure 2.8) separated strains into two groups with distinct metabolic strategies. LES 400, AA44, and PA01 were in one group and AA2, AA43, and AMT 0023-30 were in the other. The group containing LES 400, PA01, and AA44 shows little or no decarboxylation of 2-oxoglutarate (α-ketoglutarate, in the TCA cycle) and much higher OPPP fluxes in comparison to the AA2, AMT0023-30, and AA43 group. To investigate the differences that underlie these groupings, the ratios of fluxes through key pathways are shown in Table 2.3.

Strain	PA01		LES 400		AMT 0023-30		AA2		AA43		AA44	
Flux	Net Flux	Xch Flux	Net Flux	Xch Flux	Net Flux	Xch Flux	Net Flux	Xch Flux	Net Flux	Xch Flux	Net Flux	Xch Flux
Glucose Uptake	$9.45{\pm}0.00$	N/A	10.05±0.22	N/A	9.25 ± 0.24	N/A	6.96 ± 0.23	N/A	5.79 ± 0.23	N/A	4.80±0.11	N/A
H6P→6PGC	13.71±0.47	N/A	14.42±0.52	N/A	12.69±0.42	N/A	6.34 ± 0.28	N/A	5.34±0.43	N/A	5.75±0.20	N/A
6PGC→G3P +PYR	5.67 ± 0.57	N/A	10.13±0.65	N/A	11.35±0.55	N/A	5.96±0.21	N/A	4.97 ± 0.25	N/A	3.26±0.11	N/A
G3P→PEP	3.69 ± 0.32	0.84 ± 0.02	2.87 ± 0.37	0.86 ± 0.03	2.19 ± 0.43	0.86 ± 0.04	4.36 ± 0.20	0.81±0.03	3.62 ± 0.22	0.95 ± 0.04	2.31±0.07	0.78 ± 0.04
PEP→PYR	1.14 ± 0.31	0.56 ± 0.03	0.75 ± 0.29	0.57±0.03	-0.24 ± 0.44	0.58 ± 0.02	$2.19{\pm}0.19$	0.53 ± 0.02	1.64 ± 0.17	0.44 ± 0.03	0.97±0.10	0.45 ± 0.02
2 G3P→H6P	0.00 ± 0.45	N/A	2.85 ± 0.54	N/A	3.44 ± 0.51	N/A	0.00 ± 0.00	N/A	0.00 ± 0.00	N/A	0.00 ± 0.04	N/A
$6PGC \rightarrow 2R5P$	8.04 ± 0.63	N/A	$4.29{\pm}0.72$	N/A	1.34 ± 0.46	N/A	0.38 ± 0.23	N/A	0.36 ± 0.37	N/A	2.48 ± 0.18	N/A
$2 \text{ R5P} \rightarrow \text{S7P} + \text{G3P}$	2.50 ± 0.21	0.51 ± 0.09	1.20 ± 0.23	0.47±0.03	0.31 ± 0.15	0.29 ± 0.05	-0.01 ± 0.08	0.35 ± 0.02	0.02 ± 0.12	0.28 ± 0.04	0.72 ± 0.06	0.20 ± 0.05
$S7P + G3P \rightarrow R5P + E4P$	2.50 ± 0.21	0.95 ± 0.05	1.20±0.23	0.95 ± 0.00	0.31 ± 0.15	0.95 ± 0.00	-0.01±0.08	0.95 ± 0.00	0.02 ± 0.12	0.95 ± 0.00	0.72±0.06	0.95 ± 0.00
$R5P + E4P \rightarrow H6P + G3P$	2.13±0.21	0.17 ± 0.07	0.77 ± 0.22	0.00 ± 0.01	$0.00{\pm}0.14$	0.00 ± 0.04	-0.28±0.09	0.28 ± 0.06	-0.22±0.12	0.14 ± 0.06	0.50 ± 0.05	0.14 ± 0.04
$PYR \rightarrow CO_2 + AcCoA$	$3.54{\pm}0.52$	N/A	$6.84{\pm}0.45$	N/A	8.14 ± 0.38	N/A	5.41 ± 0.28	N/A	4.31±0.31	N/A	2.17±0.16	N/A
$OAA + AcCoA \rightarrow CIT$	1.93 ± 0.37	N/A	3.76 ± 0.28	N/A	6.86 ± 0.56	N/A	4.14 ± 0.30	N/A	$3.49{\pm}0.31$	N/A	1.13±0.13	N/A
CIT→ICIT	1.93 ± 0.37	N/A	3.76 ± 0.28	N/A	6.86 ± 0.56	N/A	4.14 ± 0.30	N/A	3.49±0.31	N/A	1.13±0.13	N/A
$ICIT \rightarrow \alpha KG + CO_2$	$1.07{\pm}0.25$	0.00 ± 0.11	140±0.16	0.21±0.09	$6.20{\pm}0.72$	0.00 ± 0.06	3.62 ± 0.35	0.05 ± 0.09	3.30 ± 0.38	0.00 ± 0.10	0.48 ± 0.14	0.00 ± 0.06
$\alpha KG \rightarrow SUCC + CO_2$	0.09 ± 0.27	N/A	$0.19{\pm}0.15$	N/A	5.32 ± 0.71	N/A	2.85 ± 0.41	N/A	2.68 ± 0.37	N/A	0.01±0.12	N/A
$SUCC \rightarrow FUM$	0.96 ± 0.38	0.01 ± 0.00	2.55 ± 0.23	0.01 ± 0.00	5.98 ± 0.55	0.01	3.37 ± 0.32	0.01 ± 0.00	2.88 ± 0.30	0.01 ± 0.00	0.66 ± 0.09	0.01 ± 0.00
$FUM \rightarrow MAL$	$0.97{\pm}0.20$	0.95 ± 0.15	1.89 ± 0.11	0.00 ± 0.15	$3.39{\pm}0.28$	0.16±0.13	$2.03{\pm}0.16$	0.69 ± 0.15	1.72 ± 0.16	0.67 ± 0.11	0.60 ± 0.05	0.01 ± 0.13
$FUM \rightarrow MAL$	$0.97{\pm}0.20$	0.95±0.15	1.89 ± 0.11	0.00 ± 0.15	$3.39{\pm}0.28$	0.16±0.13	2.03 ± 0.16	0.69±0.15	1.72±0.16	0.67 ± 0.11	0.60 ± 0.05	0.01±0.13
$MAL \rightarrow OAA$	2.81 ± 0.54	0.95±0.15	6.13±0.39	0.95 ± 0.00	7.43 ± 0.43	0.95 ± 0.06	4.58 ± 0.29	0.95 ± 0.06	3.63 ± 0.31	0.95 ± 0.08	1.86±0.15	0.95±0.06
$ICIT \rightarrow GLX + SUCC$	0.86 ± 0.16	N/A	2.36 ± 0.20	N/A	0.66 ± 0.24	N/A	$0.52{\pm}0.18$	N/A	$0.20{\pm}0.16$	N/A	0.65 ± 0.08	N/A
$GLX + AcCoA \rightarrow MAL$	0.86 ± 0.16	N/A	2.36 ± 0.20	N/A	0.65 ± 0.25	N/A	0.52 ± 0.19	N/A	$0.20{\pm}0.17$	N/A	0.65 ± 0.09	N/A
$MAL \rightarrow PYR + CO_2$	0.00 ± 0.00	0.00 ± 0.10	0.00 ± 0.00	0.76 ± 0.08	0.00 ± 0.00	0.92 ± 0.06	0.00 ± 0.01	0.79 ± 0.04	0.00 ± 0.00	0.82 ± 0.08	0.00 ± 0.00	0.40 ± 0.06
$PEP + CO_2 \rightarrow OAA$	1.81 ± 0.19	0.00 ± 0.05	1.25 ± 0.16	0.00 ± 0.02	1.82 ± 0.28	0.00 ± 0.04	1.62 ± 0.17	0.17 ± 0.07	1.50 ± 0.16	0.00 ± 0.08	0.89 ± 0.06	0.00 ± 0.04
$2 \text{ GLX} \rightarrow \text{PEP}$	0.00 ± 0.00	0.00 ± 0.03	0.00 ± 0.00	0.00 ± 0.02	$0.00{\pm}0.05$	0.00 ± 0.01	0.00 ± 0.06	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.01
CO ₂ Efflux	13.62±0.95	N/A	14.13±0.77	N/A	21.67±1.76	N/A	13.29±0.61	N/A	11.58±0.78	N/A	7.82±0.55	N/A
Final Residuum	Final Residuum 182.27		142.11		99.88		73.72		72.75		86.43	

 Table 2.2. Net and exchange (Xch) fluxes determined by ¹³C MFA with 90% confidence intervals. (See materials and methods)

 Residuum is the minimal sum-of-squares optimized value found using ¹³C-Flux. Abbreviations as for Figure 2.4.



Figure 2.6. ¹³**C MFA flux maps of** *P. aeruginosa* **cystic fibrosis clinical isolates.** (A) Net fluxes relative to those of the reference strain, PA01. (B) MFA determined fluxes (moles of C) in experimental strains relative to the carbon molar flux of glucose uptake. Each flux is expressed with its +/- 90% confidence intervals. Several arrows were removed in comparison to Figure 2.3 due to either low flux in the reference strain (ED7 in A) or zero fluxes seen in reaction for all strains. From top to bottom: AA2, AA44, AA43, LES400, AMT0023-30, and PA01 (B only). Arrow weights of figure do not quantitatively correspond to flux sizes. The reactions catalyzed by Aldolase and alpha-ketogluarate dehydrogenase (strain PA01) show the optimized model results paired with the upper range of the calculated CI 90% as modeled confidence interval results are limited to a flux of zero and notation used for other reactions would imply a reversible flux.



Figure 2.7 Comparison of select metabolic fluxes determined by ¹³**C MFA with growth rates of** *P. aeruginosa* **strains.** MFA derived fluxes are plotted against measured intrinsic growth rate with coefficient of determination of linear regression of fluxes for glyceraldehyde phosphate dehydrogenase (diamond), isocitrate lyase (filled squares; the value for strain AA2 is represented with and empty square), and aspartate synthesis flux (filled circles). The R² value for isocitrate lyase with the inclusion of strain AA2 is 0.599. Net flux values are as in Table 2.2.



Figure 2.8. Discrimination of metabolic phenotypes among *P. aeruginosa* **strains.** Hierarchical Clustering Analysis using MFA derived flux values was employed to identify two

main groupings as discussed in the text.

	Growth Rate (μ)	GLX/TCA	ED/TCA	OPPP/ED	OPPP/TCA	NAD(P)H	ATP	FADH2	Respiratory ATP Synthesis
PA01 FBA	0.85	0.00	1.87	0.07	0.14	38.71	-2.82	3.42	99.11
LES 400	0.96±0.12	0.63±0.11	2.69±0.10	0.20±0.20	0.53±0.21	39.35±2.54	-9.12±0.67	2.55±0.23	93.09±0.97
PA01	0.78±0.05	0.45±0.27	2.93±0.22	0.74±0.13	2.16±0.21	38.86±2.48	-8.21±0.58	0.96±0.38	90.36±1.34
AA44	0.73±0.03	0.57±0.17	2.89±0.12	0.37±0.09	1.06±0.14	17.69±0.98	-3.81±0.33	0.66±0.09	41.40±0.66
AA2	0.89 ± 0.08	0.13±0.36	1.44±0.08	0.00±9.79	0.00±9.79	29.10±1.99	-1.91±0.83	3.37±0.32	75.88±1.15
AMT 0023-30	0.71±0.07	0.10±0.38	1.65±0.09	0.05 ± 0.50	0.07±0.51	44.41±3.41	-4.17±1.39	5.98±0.55	115.82±1.72
AA43	0.67±0.03	0.06±0.82	1.42±0.10	0.01 ± 5.81	0.01±5.81	24.71±2.38	-1.47±0.83	2.88±0.30	64.62±1.23
P. fluorescens SBW25	0.04	0	0.59±0.00	0.35±40.61	0.21±40.61	15.03±1.56	0.20±2.04	3.06±100.00	42.36±102
P. fluorescens mucA- ∆algC	0.04	0.11±0.0	0.44±0.00	1.34±0.00	0.59±0.00	9.19±1.86	2.45±3.80	1.47±100.20	27.63±212
<i>E. coli</i> aerobic	0.58±0.01	0.14±0.79	5.14±4.05 [#]	0.33±0.03 [#]	1.68±0.29	31.89±2.32	10.77±1.93	0.27±0.33	90.90±3.98

Table 2.3. Flux ratios and cofactor production rates for *P. aeruginosa* **strains.** The ratios of fluxes at major central metabolic branch points, as discussed in the text, separate the strains into the two groupings shown in Figure 2.8. Estimated cofactor production rates are from MFA determined flux maps normalized to the glucose uptake rate for each strain. ATP, substrate level phosphorylation; NADP(H) total cellular NADH + NADPH production. Strains LES 400, PA01, and AA44 show higher flux ratios but lower relative ATP generation rates than strains AA2, AMT 0023-30, and AA43. Flux ratios calculated using: GLX/TCA =

 $V_{(ICIT_GLX+SUCC)}/V_{(OAA+AC-COA_CIT)}$; ED/TCA = $V_{(6PG_G3P+PYR)}/V_{(OAA+AC-COA_CIT)}$; ED/OPPP = $V_{(6PG_G3P+PYR)}/V_{(P5P+P5P_S7P+G3P)}$; OPPP/TCA = $V_{(P5P+P5P_S7P+G3P)}/V_{(OAA+AC-COA_CIT)}$. These results show the differences in energy turnover, with strain AMT0023-30 being the highest in energy produced per glucose uptake and ATP production per unit of growth. NADPH consumption related to biosynthesis estimated from FBA values were removed from the total NAD(P)H pool before ATP equivalent calculation.

These ratios were based on major branch points such as between the TCA and Glyoxylate cycles and EDP:OPPP as well as on alternate sources of co-factors. Total NAD(P)H and ATP production rates are also shown. This analysis points to the ratios GLX/TCA, ED/TCA, OPPP/ED and OPPP/TCA being markedly different between the two flux phenotypes discriminated by HCA (Figure 2.8). A calculation of flux ratios derived from FBA using a maximized biomass production objective function is included, showing that with the exception of the GLX/TCA flux, maximal growth fluxes fall within the variation across strains while none of the strains shows flux partitioning predicted by maximal growth. Flux ratios derived from MFA aerobic *E. coli* measures [78] also are included, with EMPP used in place of EDP, for comparison to another gram-negative bacteria grown under similar conditions. While the E. coli is closer in its flux ratios to strains LES 400, PA01, and AA44, Table 2.3 clearly demonstrates very different energy utilization strategies for glucose consumption, with a net negative total direct ATP production in *P. aeruginosa* compared to a positive ATP production in *E. coli. P.* aeruginosa growth is also significantly higher than E. coli under similar conditions. A related species, P. fluorescens, MFA[96] under similar conditions is also included in this table, demonstrating metabolic differences between alginate non-producing wild-type (SBW25) and an alginate operon induced but non-alginate producing mutant ($mucA-\Delta algC$; [99]).

DISCUSSION

Many studies of *Pseudomonas aeruginosa* have focused on identifying genetic changes [33,37,39], production of biofilm [30,100–102], variation in biofilm components [28,31,103], biofilm dispersal [32,104], virulence factor production [23,24,27,29,41], understanding antibiotic resistance [22], and identifying pathogenic and phenotypic differences between environmental isolates and pathogenic isolates [105,106]. Quantifying metabolic fluxes across strains can show

whether the genetic and physiological differences correspond to metabolic flux patterns. This study provides the first direct evidence of distinct metabolic phenotypes among clinical isolates from cystic fibrosis infections by *P. aeruginosa*.

An earlier MFA study of uropathogenic *P. aeruginosa* strains reported a significantly different map of central metabolic fluxes for the PA01 reference strain to the one we observed (Table 2.2). These include a much smaller carbon flux through the OPPP (1.07 mmol $gDW^{-1} hr^{-1}$ vs our finding of 8.03 mmol gDW⁻¹hr⁻¹), a larger portion of carbon passing through the EDP (8.24 mmol gDW⁻¹hr⁻¹ vs 5.67 mmol gDW⁻¹hr⁻¹), a substantial TCA cyclic flux (6.42 mmol gDW⁻¹hr⁻¹ vs1.93 mmol gDW⁻¹hr⁻¹), and import of carbon into the TCA cycle from pyruvate and its metabolites with an output flux from the TCA cycle from oxaloacetate to phosphoenolpyruvate [77]. If this divergence were due to the effects of the media (moderately higher salt content in the earlier study), it would highlight metabolic plasticity in *P. aeruginosa*. The alternative is that experimental/analytical differences account for the flux map discrepancies. Since Berger et al., in keeping with common practice, used a single labeling scheme (100% 1^{-13} C glucose), we repeated our MFA analysis using only the 100% 1-¹³C glucose labeled amino acid data obtained in this study. This yielded fluxes closer to that reported by Berger et al., with a decreased OPPP decarboxylation flux, and increased fluxes in the TCA cycle and EDP (see supplemental 2.4). We also tested the effect of removing the measurements of CO_2 efflux rate (which limited this study's MFA CO₂ efflux rate to be between 23-39% of glucose uptake rates and which is not measured in most MFA studies). This additional reduction in constraining data resulted in estimated fluxes that are very similar to the one reported by Berger et al. (see supplemental 2.4). Crown et al. [98] recently reported that multiple glucose labeling experiments yield better resolution and confidence in ¹³C MFA of *E. coli* than one labeled isotopomer or one

combination of isotopomers. This is consistent with previous label design studies (reviewed in [107]), and our observations here and previously [78,97,108,109], which also support the value of gas exchange measurements in ¹³C MFA.

Due to the generally low throughput of MFA, there have been efforts both to speed up flux analyses [72,110], to use ¹³C labeling patterns in metabolite profile datasets without flux mapping to identify pathway activities [111] and to correlate differences in steady state labeling of biomass (protein amino acids) with alterations in particular fluxes among knockout mutants or different substrate use [112]. Here, we examined whether relatedness of strains in their overall labeling patterns was linked to relatedness in flux maps, which would be particularly valuable in flux phenotype screening of multiple strains. Our results show that while strains can be reliably separated using amino acid labeling patterns, the nature and degree of metabolic differences cannot be straightforwardly inferred.

Previous sequencing work creates the potential to assess genotype-phenotype relationships. Strain LES 400 was genomically compared to strain PA01, identifying multiple potentially significant genetic differences between these strains [37]. Under these conditions, the genomic differences did not cause large changes in the flux patterns. Previous study of the closely related strains AA2, AA43, and AA44, identified AA44 as being different in its nonmucoid character [23]. Here we observed substantial flux pattern differences between AA2 and AA43 on the one hand and AA44 on the other under conditions where no significant exopolysaccharide production was detected. Strain AMT0023-30, which was characterized as having persister cells [31] segregated with these mucoid strains. Persister cells are often associated with biofilm formation [113] so the flux differences observed here may be functionally related to differences between strains that for biofilms versus non-biofilm formers. A MFA study of a wild-type strain of *P. fluorescens* and a mutant that has induced alginate production signaling with corresponding deletion of a necessary enzyme for alginate production strain reported flux map differences that resemble the differences seen in this study between the two phenotypic groups: high TCA, low OPPP flux in one; and low TCA and high OPPP flux in the other [96]. An FBA study of *Neisseria meningitidis*, another species lacking a complete EMPP, also predicted that these two extremes in TCA and OPPP flux can exist [114]. We note that the flux phenotypes of strains AA43 and AA44 show divergent metabolic adaptations compared to their shared ancestor: strain AA2. Thus different basal metabolic strategies can be observed in related strains after prolonged survival in the cystic fibrosis lung [29].

Several studies have investigated the importance and interplay between metabolism and pathogenicity. The Entner-Doudoroff Pathway (EDP) is a well conserved pathway in the *Pseudomonas* genus[115,116], whose members lack a complete EMPP, and has been shown to be preferentially utilized by glucose consuming marine bacteria and to correlate with oxidative stress tolerance [117]. The EDP was postulated to have selective advantages based on the observation of lowered fitness when Fructose-bisphosphate aldolase was introduced in a related species, *Pseudomonas putida* [118]. The EDP has also been argued to have advantages due to lower protein expression requirements and increased NADPH production in comparison to the EMPP [118,119]. A previous transcriptomics study on sequential CF isolates of *P. aeruginosa* from three separate patients demonstrated no change in expression levels of EDP enzymes, which contrasts with the variability in the carbon flux among the strains investigated here [43]. This study also demonstrates through MFA findings that efficient growth relies on utilization of EDP over the OPPP. Glyceraldehyde 3 phosphate cycling from the EDP has also been discussed

in the literature of *Pseudomonas*, with evidence of this occurring in mutants [115] and alginate label studies in *Pseudomonas mendocina* [120].

Decreases in TCA utilization in pathogenic organisms has also been shown to correspond to changes in virulence. These metabolic changes are associated with increases in survivability and growth within the oxidative environment of activated macrophages in *Salmonella typhimurium* [121,122], attenuation of virulence in *Salmonella enterica* and *Yersinia pseudotuberculosis* [76,123], and decrease in type III secretion system expression in *Y*. *pseudotuberculosis* and *P. aeruginosa* [76,124,125]. Finally, the glyoxylate shunt has been implicated in pathogenesis of organisms in human disease [126]. In addition to upregulation of isocitrate lyase in CF infections [45], direct evidence of the importance of the glyoxylate shunt in *P. aeruginosa* pathogenicity was shown in a mutant screen, where the knockout of isocitrate lyase prevented infection of alfalfa seedlings and substantially reduced lung infection [127]. Here we observed small-to-moderate fluxes through this shunt in all strains, and a significant correlation between this flux and intrinsic growth rate across strains. This pathway activity under conditions when neither fatty acids nor acetate were provided, points to a possible anapleurotic role.

Oxidative stress during chronic infection may explain some of the flux patterns observed. Oxidative bursts are seen in many different organisms' response to bacterial infection [128–131], and high oxidative stress in cystic fibrosis patients has been well documented [132,133]. A correlation has been established between oxidative stress and strain diversity in *P. aeruginosa* [134]. Glucose catabolism via the EDP and OPPP pathways produces NADPH, which is needed in antioxidant production/regeneration. The ratio of OPPP to EDP fluxes would regulate the NADPH production rate, since the first produces two, and the second one NADPH per glucose

equivalent. Immune cell oxidative attack has been shown to result in inhibition of glycolytic metabolism beyond glyceraldehyde 3 phosphate [135], perhaps favoring utilization of the EDP to bypass the lower steps of glycolysis. The gene producing glucose-6-phosphate dehydrogenase, *zwf*, normally under repression when non-glucose carbon sources are available, becomes dysregulated in some cystic fibrosis isolates [136,137]. It will be necessary to extend the present approach to characterizing clinical strains to determine whether the flux phenotypes and their correlates observed here also hold under conditions more closely resembling those in the CF lung.

ACKNOWLEDGMENTS

We wish to thank Matthew Juergens for performing the Hierarchical Clustering Analysis and for the preliminary work and culturing for this project by Nathan Praschan and Raven Batshon. We also wish to thank Seattle Children's Hospital Cystic Fibrosis Isolate Core (NIH P30 DK089507) for their donation of strain AMT0023-30 and the RTSF Mass Spectrometry and Metabolomics Core for at Michigan State University for their analytical support. Michael Opperman was supported in part by funds from the Cellular and Molecular Biology Graduate Program and College of Human Medicine at Michigan State University and by a MD-PhD Fellowship grant by Spectrum Health.

APPENDICES

APPENDIX A - Electronic Supplemental Descriptions

Descriptions of the supplemental material uploaded as an electronic supplement of this dissertation.

Supplemental 2.1: ¹³C labeling data of amino acids used in Principal Component Analysis and Metabolic Flux Analysis models of this study.

Supplemental 2.2: Complete list of reactions in Metabolic Flux Analysis model with complete flux table results and list of biomass values set for each strain.

Supplemental 2.3: Complete flux results for Flux Balance Analysis of biomass optimized growth of strain PA01and Flux Variability Analysis results for 99% and 90% maximally optimized biomass flux rate. Alterations to the biomass equation and additional reactions added to Oberhardt *et al.* FBA model are also described.

Supplemental 2.4: Metabolic Flux Analysis flux results for strain PA01 model with removal of 20% 1,2,3,4,5, 6^{-13} C Glucose AA label data and CO₂ efflux data.

Supplemental 2.5: The version of the formatted Flux Balance Analysis model used in this study (.xls format).



APPENDIX B - Additional Figure

Figure S2.1. Averaged carbon-uptake normalized flux map from ¹³**C MFA analysis of** *P. aeruginosa* **cystic fibrosis isolates grown in M9.** MFA determined c-mol fluxes of individual experimental strains normalized to total carbon uptake averaged with standard deviation determined. Arrow thicknesses are proportional to averaged % of total carbon uptake. Arrows are colorized to demonstrate variability of total uptake carbon through experimental strains.

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CHAPTER 3 –

Quantification of *Pseudomonas aeruginosa* Metabolism in Cystic Fibrosis

Lung Like Conditions

ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that infects a wide range of species and of significant importance to human health. Known for causing nosocomial infections of burns and of the urinary tract, as well as those related to ventilator-associated pneumonia, P. aeruginosa is a major factor in the morbidity and mortality of cystic fibrosis (CF) patients. With intrinsic abilities to resist antibacterial agents and quick adaptation against new antibiotics, P. aeruginosa is an important organism to study in order to develop treatments that are more effective. Many P. aeruginosa isolates have been investigated using many different Omic and direct virulence factor measures in an attempt to describe shared virulence and metabolic changes during chronic infection of the CF lung. In this study, we used ¹³C Metabolic Flux Analysis and Flux Balance Analysis on one reference strain and six strain isolates from the lungs of CF patients to quantify their metabolic phenotypes. The strains were grown under aerobic and anoxic conditions in a media formulated to mimic the CF lung. Our results highlight the importance of putrescine (a polyamine) metabolism and the use of the glyoxylate cycle of P. aeruginosa during growth in the CF lung while demonstrating low utilization of the Entner-Doudoroff and Oxidative Pentose Phosphate Pathways. We also report four metabolic phenotypes under growth in aerobic and anoxic conditions and the divergence of metabolism of genetically related isolates that have emerged during chronic CF infection.
INTRODUCTION

Cystic fibrosis of the pancreas (CF), also known as mucoviscidosis or more commonly simply as cystic fibrosis, is an autosomal recessive disease caused by genetic defects in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) [1–5]. The CFTR, expressed in many epithelial tissues of the body, is especially important for the correct secretion of chloride and bicarbonate ions in the pancreas, lungs, and duodenum [6–14]. Defective CFTR expression leads to thickened mucus secretions in the body of CF patients, causing symptoms including: pancreatic duct obstruction; pancreatitis and fibrosis of the pancreas; meconium ileus; gastroesophageal reflux disease and peptic ulcers; and Bronchiectasis, which poses the greatest risk of morbidity and mortality in CF [6–9,15–24].

Bronchiectasis, an obstructive lung disease, is caused by widening of the lower bronchioles due to chronic inflammation caused by repeated cycles of infection and immune clearance, damaging lung parenchyma [25–27]. In CF, secreted mucus has abnormally low viscosity, causing pooling of mucus that allows growth of inhaled bacteria in the lung [28–30]. Final stage treatment for decreased lung function caused by bronchiectasis is lung transplantation, though the lack of sufficient organs for transplant makes this option unavailable to most [31,32]. Common infectious species seen in CF include Gram positive bacteria (such as *Staphylococcus aureus, Streptoccocus* sp.), Gram negative bacteria (*Pseudomonas aeruginosa, Haemophilus influenzae*) and non-bacterial species (*Aspergillus* sp.) [33]. Of greatest concern related to the possible lung pathogens in CF is *P. aeruginosa*.

Pseudomonas aeruginosa is a gram negative opportunistic pathogen with a wide range of suitable environments and hosts [34–39]. Current prevalence rates of *P. aeruginosa* infection among CF patients have been reported to be as high as 74% of all CF patients 26 years and older

[40,41]. Due to its potential ubiquity in suitable human habitats, the high rate of infection of CF patients is currently thought to be due to random environmental exposure and, rarely, clinical exposure [42]. Additionally, *P. aeruginosa* is resistant to many current therapies, due to its many intrinsic defenses against current antibacterials (e.g. biofilm, cephalosporinase, and efflux pump expression), as well as its rapid adaption due to the commonly seen hypermutable phenotype (caused by acquired defects in mismatch repair enzymes) [43–47]. These intrinsic defenses and rapid adaptations against antibacterial treatment, along with the pathogen's environmental ubiquity, lead to repeated infection cycles and clinical failure of treatment, thus making *P. aeruginosa* of major clinical interest in the treatment of CF.

Many studies of *P. aeruginosa* CF isolates have been focused on identifying the changes in the organism during chronic infection. Studies have used various Omic tools [48–64], measurements of known virulence factor expression, and measures of physiological changes [42,65–76] to establish a wide range of known adaptations. Evidence in these and other studies generally demonstrate an organism growing in a microaerobic to anaerobic environment within the lung (with nitrate available for respiration), with its primary carbon sources being amino acids, glucose, and lactic acid present in the lung sputum [77,78]. Additionally, studies of *P. aeruginosa* indicated that overproduction of the biofilm component alginate is associated with increased morbidity and mortality in the CF patient population [79–82]. Chronic infection isolates of *P. aeruginosa* also demonstrate decreased production of other known virulence factors [71].

However, these identified changes have not led to a clear understanding of the metabolic adaptations that the organism undergoes for two distinct reasons. First, chronic infection strains of *P. aeruginosa* are often of the hypermutable phenotype, causing significant genomic

instability and diversity, introducing significant uncertainty in direct genomic measurements [62,43,83,84]. Second, different Omic datasets do not correlate well within data sets or in comparisons to other Omic measures of the same samples, rendering uncertain the true meaning of the differences recorded with these techniques [85–87]. To identify which metabolic changes are actually significant for pathogenesis and to measure and predict functional changes in metabolism, a toolbox of network based computational and experimental methods is available. The work in this paper focuses on two steady-state based metabolic modeling techniques, Metabolic Flux Analysis and Flux Balance Analysis.

Metabolic Flux Analysis (MFA) centers on the use of isotopically labeled substrates (usually ¹³C) in experiments allowed to reach a stable pattern of isotopic distribution (end point labeling) [88]. The labeling patterns in metabolic products or intermediates are used to deduce internal metabolic state and a pattern of carbon fluxes through the cellular metabolic system: a flux map [89]. To produce such a map, a standard list of reactions with carbon positional transformations in the organism and stoichiometries must be known, and measurements must be taken of extracellular input and output fluxes such as biomass production, gas exchange, and carbon inputs [89–91]. Metabolism of carbon-containing compounds by enzymatic processes occurs at defined carbon positions within the molecule due to the biochemical specificity of enzymatic catalysis; this fundamental property of enzymatic reactions allows for characteristic labeling patterns to develop for the carbon in the molecule [92]. To describe the re-shuffling of the label for a reaction, an atom mapping matrix is constructed for each reaction in the network [93]. With mapping matrices and isotopomer matrix representations, matrix algebra can be used to predict the labeling pattern at steady state in all metabolites by assuming reaction rates (the values of the metabolic fluxes) [89]. The computed results are then compared to the measured

¹³C labeled measurements and the assumed flux values are iteratively changed to optimize the fit of the model to the measured labeling patterns [89]. This results in MFA quantifying carbon fluxes through metabolism.

The second modeling technique used, Constraints-based Flux Balance Analysis (FBA), is a biochemically-based mathematical modeling approach to the steady state analysis of metabolism that incorporates carbon and non-carbon elements in a large-scale mass balance analysis; energy and redox co-factors are also balanced and no labeling experimental data are needed[94–96]. FBA can extend the metabolic scope beyond carbon and beyond central metabolism for complete genomic scale coverage of metabolism [95,97]. The biochemical equations that make up the individual steps of metabolism are converted into a matrix equation in which the stoichiometry of each reaction is analyzed using software packages such as The COBRA (Constraints Based Reconstruction Analysis) toolbox in MATLAB [98] in order to optimize the model reactions. Additionally, optimization algorithms can be used to maximize or minimize selected individual fluxes or combinations of fluxes (called objective functions) in order to produce the most likely carbon flux of the metabolic network being tested [95].

To date, most MFA studies of microbial systems have focused on questions related to biotechnology and metabolic engineering, microbial physiology, and gene function [99–103]. Several recent studies have analyzed the metabolic interactions of pathologically relevant bacteria within their host environment [104,105], or the metabolic differences between mutant strains [106]. A recent study of pathogenesis-related metabolism in *P. aeruginosa* employed MFA to compare 17 uropathogenic strains [107]. FBA investigations of pathogenic organisms have been used to search for novel drug targets and have identified potential metabolic targets not affected by current therapeutics, such as amino acid production or fatty acid metabolism [108–115]. Recent FBA research on *P. aeruginosa* includes a genome-based metabolic and transport model by Oberhardt and colleagues [116], and uses FBA with transcript data from two CF clinical strains isolated 44 months apart in order to investigate *P. aeruginosa*'s metabolic capabilities and potential metabolic changes during prolonged infection [117]. FBA has also been used to identify potential metabolic drug targets during biofilm growth of *P. aeruginosa* [118].

The combination of both FBA and MFA allows for investigation of suboptimal growth and testing of alternative objective functions that may be optimized besides maximal biomass production. However, there have been surprisingly few studies in which these complementary network flux analysis approaches have been combined [119,85,99,120-125], let alone in direct application to understand bacterial pathogenicity. Here, we performed FBA and ¹³C MFA of reference strain PA01 and 6 selected CF pathogenic isolates to quantify redox production and consumption, metabolic inefficiencies, and the metabolic phenotypes in media that mimics the CF lung. In this study, we highlight the importance of amino acid catabolism by putrescine (polyamine) metabolism, with significant carbon fluxes not described in the scientific literature of any MFA study. We also demonstrate the differences in utilization of the glyoxylate cycle under aerobic and anoxic growth. Among the clinical strains we report four general metabolic phenotypes, two in aerobic growth and two in anoxic growth, which appear during chronic infection of the CF lung. We also demonstrate divergence in metabolic phenotype during chronic infection of two genetically related strains. Finally, we report strain similarities and differences using ¹³C labeling data of proteogenic amino acids and with obtained ¹³C MFA results. While discrimination of strains was obtained in both approaches in aerobic growth results, failure of

discrimination during anoxic growth using ¹³C labeling data alone was inconsistent with ¹³C MFA results.

METHODS AND MATERIAL

Bacterial strains and culture

Pseudomonas aeruginosa strains are described in Table 3.1. Strains AA2, AA43, AA44, and LES 400 were obtained from the Belgian Co-ordinated Collections of Micro-organisms (Ghent, Belgium; LMG numbers:27630, 27631, 27632, and 27623 respectively). *Pseudomonas aeruginosa* strain AMT 0023-30 and AMT 0023-34 was obtained from Cystic Fibrosis Isolate Core (Seattle, Washington). *Pseudomonas aeruginosa* strain PAO1 was obtained from Dr. M. Mulks at Michigan State University. Synthetic Cystic Fibrosis sputum Media (SCFM), a defined minimal media designed to simulate both the carbon sources used by *P. aeruginosa* and the environment of the cystic fibrosis lung, was one of the culture medias used in this experiment [77].

Strain	Origin	Related Isolates	Relevant Characteristics	References			
PAO1	Australia Wound Isolate (1955)	N/A	Commonly Used Reference Strain.	[65,73,74]			
AMT 0023- 30	USA CF - Pediatric Clinical Isolate (~1998)	CF - Pediatric ical Isolate AMT 0023-34 Persister Cells Present (~1998)					
AMT 0023- 34	USA CF - Pediatric Clinical Isolate (~2006)	AMT 0023-30	Persister Cells Present				
LES 400	U.K. CF - Adult Clinical Isolate (2003)	Liverpool Epidemic Strains (LES)	Increased Acute Morbidity Risk Decreased Life Expectancy with Infection Pyrocyanin Overproduction Alginate Overproduction CF Transmissible	[53,67–69,126]			
AA2		AA43, AA44 Parental strain of AA43, AA44					
AA43	German CF – Adult Clinical Sequential Isolates	AA2, AA44	7.5 years total lung colonization time Increased Acute Morbidity risk	[52,70,71]			
AA44	(~1998 & ~2003)	AA2, AA43	Motility defect Protease reduction LPS and PGN changes				

 Table 3.1. List of Pseudomonas aeruginosa strains used in this experiment. LPS – Lipopolysaccharide; PGN – polygalacturan

For the purpose of ¹³C MFA labeling experiments, a reduced carbon source media based on SCFM was prepared (Simplified-SCFM), with the following changes to the carbon sources: L-glutamate 9.2 mM, L-alanine 11 mM, L-ornithine 4.3 mM, L-arginine 1.8 mM, and Laspartate 4.9 mM and retaining all other described components (e.g. salts) [77]. These concentrations and substrates were determined by the major carbon compounds utilized by all experimental strains of P. aeruginosa during logarithmic growth of experimental cultures, the availability and relatively low cost of ¹³C used in this study, and the need to maintain the osmolaric concentration of SCFM. All cultures were pre-cultured from -80°C frozen stock samples onto the media of subsequent experimental culturing (SCFM or Simplified-SCFM) that was solidified with 1.5% agarose. Cultures were grown for 24 hours at 37° C to produce isolated colonies. For aerobic cultures, single colonies were transferred into 30 mL of SCFM or simplified-SCFM liquid media in 250 mL Bellco triple baffled shake flasks, incubated at 37°C and shaken at 120 RPM until stationary phase (12 to 14 hours) before spectrophotometric measurement of cell density as absorbance at 600nm (OD₆₀₀) and inoculation of experimental cultures to initial densities of 0.01 OD₆₀₀. For all anoxic cultures, glass pressure tubes were used and media concentrations of KNO3 were increased to 50mM. All pressure tubes were purged of oxygen content through degassing by bubbling the media with pure N₂ gas for 15 minutes uncovered and then for 5 additional minutes covered by rubber stopper. All tubes were then fully closed by rubber stoppers, sealed in place by a metal crimp, and autoclaved. All culturing techniques involving puncturing the rubber stopper were executed by purging hypodermic needles with N₂ gas before the removal or addition of liquid culture media. For anoxic culture inoculation, single colonies were transferred onto the side of pressure tubes filled with 10 mL of SCFM or simplified-SCFM liquid media in 20 mL pressure tubes, vigorously shaken for 10

minutes, and then incubated at 37° C until stationary phase (24 to 48 hours) before spectrophotometric measurement of cell density as absorbance at 600nm (OD_{600}). Inoculation of experimental cultures occurred at initial densities of 0.01 OD_{600} . For ¹³C labeling experiments, cells were cultured using either 75% (mol/mol) [3-¹³C] L-alanine or 99% (mol/mol) [U-¹³C] Lglutamate.

Cell and media sample harvest

Cell pellets were collected by centrifugation of cultures at mid-log phase ($OD_{600} \approx 0.5$ for aerobic, $OD_{600} \approx 0.2$ for anoxic). 1 mL of culture was centrifuged at 15000 g for 5 minutes. The supernatant was removed and 100 µL of 6N HCl was added to the pellet which was stored at -20° C. Cell pellet sample preparation for amino acid analysis was based on previously reported methods [125,127].

Determination of substrate uptake rates and product secretion

Amino acid uptake rates were determined using at least three biological replicate samples collected during log phase time points. Samples were filtered using Millipore 20 μ m pore size filters with filtered media collected and placed in -20° C for storage. Samples were quantified using a Waters Quattro micro API LC/MS/MS as described by Gu et al., 2012 [128]. Sample preparation used 100 μ L of samples diluted in 900 μ L of milipore filtered H₂O with 100 μ M of phenylalanine-d8 as internal standard.

Glucose and lactate uptake rates and acetate secretion rates for each strain were determined from 1 mL culture supernatants using at least three biological replicate samples for each of three log phase time points taken with 10 mM methylphosphamide added as internal standard. After lyophilization, samples were resuspended in 600 μ L of 99% D₂O, and then lyophilized and resuspended in 600 μ L of 99% D₂O. 1H-NMR was performed on an Agilent DirectDrive2 500MHz instrument using an Alginate OneProbe with Protune for auto-tuning. Spectra were obtained at 500 MHz with a pulse angle of $<45^{\circ}$, acquisition time of 2.05 s, and recycle delay of 2 s. No other secreted products were detected at significant levels in the media after culture growth for any of the strains. Calculation of uptake and secretion rates during exponential growth used the following equation:

$$v = \frac{\mu}{X_i} \frac{C - C_0}{e^{-\mu t} - 1}$$

Where μ is intrinsic growth rate (hr⁻¹); *C* is the concentration of glucose (mM) at time *t*; *X_i* is the initial density (gDW/L) of cells; *t* is time (hours); and *v* is uptake flux (mmol/g·DW-hr).

Determination of CO₂ efflux rates

As described above, aerobic cultures were sealed with gas-tight caps and incubated for two hours until reaching early log phase. Due to the high sensitivity and low maximum range of CO_2 detection of the LICOR CO_2 measurement, *P. aeruginosa* growth was limited to a low range of optical density for this measurement. The cell density of the culture was measured and growth was stopped by adding 1 mL 6N HCl to the culture, which also converted dissolved bicarbonate to CO_2 . Total CO_2 was measured using a LICOR LI-6400 with dry CO_2 free air as input into the culture flask at a rate of 500 µL/s. Readings of total CO_2 were recorded over 5 minutes (1 s time resolution) to allow for removal of CO_2 from the flask. Total CO_2 evolved was measured in at least three replicate cultures with CO_2 levels integrated for total CO_2 efflux. CO_2 efflux was calculated as described in determination of substrate uptake rates and product secretion.

Determination of NO₃ uptake rates

Nitrate uptake rates were determined using at least three biological replicate samples collected during log phase time points of anoxic cultures. Samples were filtered using Millipore 20 µm pore size filters with filtered media collected and placed in -20° C for storage. Sample

preparation used 100 μ L of samples diluted in 900 μ L of milipore filtered H₂0 with 10 μ M of ¹⁴NO₃ as internal standard. A 3200 QTRAP® LC/MS/MS coupled to a Shimadzu (Columbia, MD) LC-20ADvp HPLC system and SIL-HTC autosampler was used for LCMS analysis. A ZIC®-pHILIC column (50 × 2.1 mm, 5 μ m particle size) was used with column oven temperature at 40° C. The injection volume was 1 L, and the flow rate was 0.2 mL/min using water/acetonitrile (A/B) gradient at ambient temperature. The initial gradient (A/B)=10/90, was held until 2 minutes, followed by a linear gradient to 30/70 at 3 minutes, held at 30/70 until 4 minutes, followed by a ramp to 10/90 at 4.01. Mass spectra were acquired using turbo VTM ion source and multiple ion monitoring scan type in negative ion mode. Masses of 62 (unlabeled nitrate) and 63 (¹⁵N labeled nitrate) were measured at retention time of 1.3 min. To determine the concentration of ¹⁵N labeled and unlabeled nitrate, a standard curve was run for authentic ¹⁵N labeled nitrate standard with concentration range from 0 to 100 M. Data were processed for calibration and for quantification of the analytes with QTRAP analyst software.

Analysis of Amino Acid labeling

Cell pellets were suspended in 1mL of 6N HCl, incubated at 100° C for 24 hours, and dried at 60° C under a stream of N₂. Amino acids were derivatized using N-Methyl-N-[tert-butyldimethyl-silyl] trifluoroacetimide (MTBSTFA,SIGMA-ALDRICH) in a 25 μ L pyrimidine and 25 μ L 1% MTBSTFA solution incubated at 40° C for 1 hour as previously described [125,127,129].

GC-MS analyses of derivatized amino acids were performed on an Agilent 5973GC/quadrupole MS. GC-MS signals were corrected for natural isotope abundance [127]. Amino acid fragments used in ¹³C MFA were based on reported reliability [129]. Unlabeled amino acid samples collected were used to confirm the accuracy of natural abundance correction.

GC-MS amino acid data was corrected for natural abundance isotopic contents with average values and standard deviation calculated for biological replicates used as model inputs.

Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA)

GC-MS measured mass fragment mass abundance data for amino acids of strains harvested after growth in 75% $3 \cdot C^{13}$ L-alanine or 100% uniformly labeled (1,2,3,4,5- C^{13}) Lglutamate was collected. Amino acid fragments that were measureable in three biological replicates were then processed in MATLAB (2010a) using the PCA function to produce a principal component analysis [130]. HCA was performed on this data set as well as on fluxes determined by MFA that were normalized to each strain's glucose uptake rate. HCA was implemented in the programming language R using complete linkage criteria which use Euclidian distance [131].

¹³C Metabolic Flux Analysis

A carbon isotopic network of *Pseudomonas aeruginosa* central metabolism was constructed by an approach similar to that previously used for *E coli* and previous *P. aeruginosa* work [125,132].The iM01056 FBA model [116] reaction network was simplified by only including carbon atoms of reactants and products, by introducing both net and exchange fluxes for reversible fluxes, by combining linear reaction sequences that do not alter carbon positions, and by condensing secondary metabolic pathways into a growth equation that consumes central metabolic intermediates as precursors to produce cellular biomass as previously defined [116,133]. Additionally, the preservation of the architecture of the FBA model allowed the results of FBA and ¹³C MFA to be directly compared and the ¹³C MFA results to be used to constrain the FBA [125]. Measured rates of glucose uptake, CO_2 efflux, and ¹³C labeling patterns in amino acids, together with growth rates, were used to estimate internal fluxes of central carbon metabolism [133] by fitting flux values to the experimental data using the ¹³C-FLUX software as previously described [92,125,134]. For labeling data, variation among biological replicates was very small (see Supplemental 3.1); since experimental standard deviations (SDs) are not a reliable estimate of true population SDs when 5 or fewer replicates are analyzed, the experimentally observed SD's were increased to 2% of mean values for MFA modeling as previously described [125,132]. Measurements with a replicate standard deviation greater than 2% were adjusted to this value to account for the measurement uncertainty. Measurements with only one replicate had their deviation adjusted to 5% of the mean value. This also avoids excessively constraining modeling results to labeling data at the expense of direct flux measurements and reduces the potential for distortions due to precise but inaccurate mass isomer quantification [125,132]. To allow for uncertainties in *P. aeruginosa* biomass compositions, production rates were constrained to be within 50% of the *E. coli* biomass values [125].

To minimize the risk that solutions represented local rather than global optimization minima, multiple randomly generated initial fluxes constrained by sampling the feasible solution space were used. First, at least 100 randomly generated feasible starting points that produced optimized fits by the ¹³C-FLUX program were found. Next, the 10 starting points that yielded the lowest final residuum values were used in the second stage to generate 1000 more starting points by randomly perturbing the 10 starting points to yield 100 new points each. The final, lowest residuum optimized flux values that resulted in the best fit to the data were used. Confidence intervals for flux values were estimated using a Monte Carlo approach to randomly generate values of: biomass, glucose uptake rate, CO_2 efflux rate, and amino acid labeling data for each

strain [135] based on the experimentally determined standard deviations. At least 20 such datasets were then fitted, using the 10 best fit (lowest SS_{res}) starting and ending flux values determined for each strain as described above to yield best-fit flux maps for each strain. These flux value sets were then used to calculate 90% confidence intervals for each flux modeled for each strain.

All MFA computations were performed at the High Performance Computer Center, Michigan State University, using a parallel 1536 core cluster of 192 nodes (two four-core Intel Xeon E5620s at 2.4 GHz with 24 GB of RAM and 250 GB local disk space per node). Global SS_{res} for each strain is listed in Supplemental 3.2.

Flux Balance Analysis

The genome-derived stoichiometric *Pseudomonas aeruginosa* model iM01056 developed by Oberhardt et al. with previously described updates were used for FBA [116,132]. The model was modified to more fully account for alanine catabolism (see Supplemental 3.3), resulting in a total of 1016 reactions and 876 metabolites. The COBRA Toolbox 2.0 in MATLAB (2014a) with the implementation of the Gurobi 6 optimizer as optimization solver was used for FBA, using the objective function of maximal biomass production [97]. FBA simulations used measured amino acid uptake rates, NO₃ uptake rates, and acetic acid and CO₂ efflux rates by strain. To determine the range of fluxes that allow 99% of the maximal growth rates, the Flux Variability Analysis (FVA) function of the COBRA Toolbox was used [98].

RESULTS

To determine the preferred carbon sources in the *P. aeruginosa* isolates in the cystic fibrosis lung and to reduce the complexity and cost in ¹³C substrate labeling experiments, strains were grown under aerobic and anoxic conditions in the defined complex Synthetic Cystic

Fibrosis sputum Media (SCFM) [77]. Filtered media samples of *P. aeruginosa* strains grown in SCFM under aerobic conditions were quantified using HPLC with the results shown in Figure 3.1. The average concentrations and uptake rates of *P. aeruginosa* grown in SCFM under aerobic conditions measured at early-log phase growth demonstrate average strains preference for Llactate, ornithine, proline, alanine, glycine, leucine, lysine and aspartate under uptake rates, carbon uptake rates, and lowest % remaining compared to original media concentrations. Individual strain results (see Supplemental Table S3.1) demonstrate strain AA43 having little to no uptake of leucine and lysine and strain AMT0023-30 having little uptake of glycine under aerobic SCFM growth. Consideration of similar catabolic pathway utilization allows for the selection of L-lactate or alanine as well as glutamate or proline, with little reduction in determination of metabolic pathways. Anoxic growth in SCFM results also demonstrate similarities in uptake preference in aerobic SCFM, with a notable increase in leucine consumption and decrease in L-Lactate (see Supplemental Table S3.2). These combined results led to a proposed Simplified-SCFM (S-SCFM) for ¹³C labeling experiments containing the amino acids of glutamate, alanine, ornithine, arginine (included for proposed anoxic growth utilization in P. aeruginosa [136]), and aspartate in P. aeruginosa strains. Similar amino acid uptake preferences were also demonstrated by Palmer et al., with the exception of ornithine [77].

Comparisons of P. aeruginosa intrinsic growth rates in Simplified-SCFM and SCFM under aerobic and anoxic conditions for each strain are shown in Figures 3.2 and 3.3. Figure 3.2 demonstrates similar growth rates (within 20%) between S-SCFM and SCFM of all strains under aerobic and anoxic growth. Figure 3.3 demonstrates the similarity of growth rate order between strains under aerobic and anoxic conditions, with high R² values demonstrating similarity of growth to SCFM (see Supplemental Figure S3.1).



Figure 3.1. Analysis of amino acids, glucose, and lactate consumption in aerobic Synthetic Cystic Fibrosis sputum Media (SCFM) growth of seven strains of *Pseudomonas aeruginosa*. Media harvest occurred at early logarithmic growth (~3-5 hrs post inoculation, with strain AMT 0023-34 at ~8 hrs post inoculation). A - Averaged values of compound uptake rates, units are in mMol/(g·DW-hr). B - Averaged values of the carbon uptake, Units are in C-mMol/(g·DW-hr). C - Averaged values of the % of the compound remaining in SCFM media. D - % of total carbon uptake from the media by compound. All measures are n=21 (3 measures per strain); standard deviation ranges are represented as error bars.



Figure 3.2. Analysis of amino acids, glucose, and lactate consumption in anoxic Synthetic Cystic Fibrosis sputum Media (SCFM) growth of seven strains of *Pseudomonas aeruginosa*. Media harvest occurred at early logarithmic growth (~ 3-5 hrs post inoculation, with strain AMT 0023-34 at ~8 hrs post inoculation). A - Averaged values of compound uptake rates, units are in mMol/(g·DW-hr). B - Averaged values of the carbon uptake, Units are in C-mMol/(g·DW-hr). C - Averaged values of the % of the compound remaining in SCFM media. D - % of total carbon uptake from the media by compound. All measures are n=21 (3 measures per strain); standard deviation ranges are represented as error bars.



Figure 3.3. Specific growth rates of *Pseudomonas aeruginosa* **strains under the growth conditions.** Aerobic SCFM (black; AA2 n=4, LES400 n=4, others n=5), Aerobic Simplified-SCFM (dark grey; n=3), Anoxic SCFM (light grey; PA01 n=3, all others n=5), and Anoxic Simplified-SCFM (white; n=3). Error bars represent standard deviation of measurements.

Uptake and efflux rates determined under aerobic and anoxic growth in S-SCFM are shown in Tables 3.2 and 3.3 and in Figures 3.4 and 3.5. Strains growing under aerobic S-SCFM uptake rates in Table 3.2 demonstrate a nearly threefold difference in alanine (12.15 mmol gDW 1 hr⁻¹ vs 5.85 mmol gDW⁻¹ hr⁻¹), arginine (0.94 mmol gDW⁻¹ hr⁻¹ vs 3.00 mmol gDW⁻¹ hr⁻¹), and ornithine (0.52 mmol gDW⁻¹ hr⁻¹ vs 1.54 mmol gDW⁻¹ hr⁻¹) uptake rates, a nearly sixfold difference in the uptake rate of aspartate (0.59 mmol $gDW^{-1} hr^{-1} vs 3.52 mmol <math>gDW^{-1} hr^{-1}$), and a twofold difference in glutamate uptake rate (2.51 mmol gDW⁻¹ hr⁻¹ vs 5.69 mmol gDW⁻¹ hr⁻¹). Total percentage uptake of carbon by compound demonstrates a range of amino acid by uptake as well, shown in Figure 3.4. The two compounds that make up a majority of the carbon uptake in each strain are alanine and glutamate. The cross-strain ranges of the percentage of total carbon uptake made up by these two compounds demonstrates large variation in the percentage of alanine in total carbon uptake (23% in PA01 to 50% in AA44) and narrower variation in the percentage of glutamate in total carbon uptake (22% in AA44 to 37% in PA01). Measured uptake rates of amino acids by P. aeruginosa during growth in anoxic S-SCFM also demonstrate large ranges over substrate uptakes. As shown in Table 3.3, alanine uptake rates demonstrate a fourfold difference (15.09 mmol gDW⁻¹ hr⁻¹ vs 1.43 mmol gDW⁻¹ hr⁻¹); arginine, a sixfold difference (1.43 mmol gDW⁻¹ hr⁻¹ vs 0.22 mmol gDW⁻¹ hr⁻¹); aspartate, a fivefold difference (6.88 mmol gDW⁻¹ hr⁻¹ vs 1.24 mmol gDW⁻¹ hr⁻¹); and glutamate, a fivefold difference (6.88 mmol gDW⁻¹ hr⁻¹ vs 1.24 mmol gDW⁻¹ hr⁻¹). Additionally, ornithine uptake rates demonstrate a range of 19% excretion of total carbon uptake to 16% of total carbon uptake (3.79 mmol gDW⁻¹ hr^{-1} vs -0.97 mmol gDW⁻¹ hr^{-1}). Figure 3.5 also demonstrate the ranges of total carbon uptake by amino acid. These rates also demonstrate ranges of 35% (PA01) to 69% (AA2) for alanine and 12% (AA2 and AMT 0023-34) to 29% (PA01 and AA43) for glutamate, the two compounds

which collectively form the majority of carbon uptake. Based on the consistency of carbon flow in both anoxic and aerobic growth, a parallel ¹³C labeling experimental scheme of 75% ¹³C-3 alanine and 100% U-¹³C glutamate was used for ¹³C labeling for the MFA studies.

Proteogenic ¹³C labeled amino acids obtained from parallel ¹³C labeling experiments were used to construct a Principal Component Analysis plot of the aerobic and anoxic strain in this experiment to determine strain relationships under aerobic and anoxic growth. Figure 3.6A shows clear differences under aerobic growth of strains, while Figure 3.6B indicates that strains AA2, AA44, and LES400 demonstrate labeling overlap in anoxic growth. Full proteogenic amino acid labeling data can be found in electronic Supplemental 3.1.

The ¹³C MFA flux map for reference strain PA01 during aerobic growth of S-SCFM is shown in Figure 3.7, with the list of fluxes shown in the flux map provided in Table 3.4 (the complete flux listing can be found in electronic Supplemental 3.2). The MFA model shows the funneling of carbon through ornithine from glutamate towards succinate with only a third of the incoming glutamate flux towards α -ketoglutarate directly. The flux model also demonstrates cycling of carbon from the TCA (tricarboxcylic acid) cycle in the form of malate to pyruvate and then back as citrate. The model also demonstrates flux through the glyoxylate cycle with all glyoxylate being converted to phosphoenolpyruvate. The Entner-Doudoroff and Oxidative Pentose Phosphate Pathways have little relative carbon flux through them, and demonstrate minimal flux allowing for necessary biosynthesis of bases and amino acids for biomass production. Most fluxes in this model demonstrate 90% CI values of 20% or less of the total flux, with the exception of the urea cycle components in this model demonstrating potential reversibility and utilization of this pathway.

Strain	Alanine	Arginine	Aspartate	Glutamate	Ornithine	Total Carbon Uptake	CO ₂ Efflux
PA01	5.97±1.38	1.49±0.26	3.52±0.89	5.69±1.51	1.54 ± 0.47	77.1±19.1	31.25±6.41
AA2	6.60±1.22	0.94±0.29	1.79±0.11	3.80±0.63	0.92±0.29	56.2±10.4	31.4±1.68
AA43	5.85±1.26	1.29±0.29	1.32±0.08	2.51±0.63	0.52±0.26	45.8±10.3	43.1±5.78
AA44	10.17 ± 2.09	1.32±0.36	1.52±0.44	2.75 ± 1.44	0.59 ± 0.32	61.2±18.96	31.2±1.48
LES400	$10.88{\pm}1.85$	1.12±0.21	2.10±0.97	4.62 ± 1.49	1.18 ± 0.78	76.7±22.0	27.9±3.28
AMT 0023-30	12.15±2.53	3.00±0.51	2.35±1.51	4.97±2.38	1.24±0.35	94.9±30.3	73.0±3.08
AMT 0023-34	7.29±1.79	1.22±0.40	0.59±0.31	3.25±0.24	0.66±0.19	51.1±11.2	44.7±3.47

Table 3.2. Uptake and CO₂ efflux rate of *Pseudomonas aeruginosa* strains under aerobic growth conditions in Simplified-SCFM. All units are in mMol/(g-DW-hr) except total carbon uptake, which is in C-mMol/(g-DW-hr) For amino acid uptake rates: PA01 n=6; AA2 n=5; AA44, LES400, AMT30 n=4; AA43 and AMT34 n=3. For CO₂ efflux rates n=6 for PA01, AA43, AA44, and AMT 0023-34; n=3 for AA2, LES400, AMT 0023-30.

Strain	Alanine	Arginine	Aspartate	Glutamate	Ornithine	Total Carbon Uptake	Nitrate Uptake	Acetate Secretion
Pa01	6.82±0.37	0.29±0.09	2.59±0.35	3.33±0.36	1.80±0.19	58.2±5.82	17.3±3.68	0.68±0.16
AA2	11.1↔16.6	1.05↔1.17	2.91↔4.41	0.16↔2.28	-0.25↔-0.45	49.8↔83.6	103±37.3	3.00±0.65
AA43	3.57±0.08	0.26 ± 0.05	1.24 ± 0.01	1.68 ± 0.18	0.63±0.33	28.8±3.13	13.3±1.78	0.28 ± 0.09
AA44	6.21±0.72	0.22±0.12	1.44 ± 0.94	1.95 ± 0.68	0.37±0.77	37.3±13.9	68.8±0.96	0.74 ± 0.14
LES400	15.1±2.81	1.43±0.16	6.88±1.24	3.24↔3.35	3.79±2.49	107±27.6	28.5 ± 8.09	1.36±0.49
AMT 0023-30	10.9↔15.1	0.91↔1.34	1.41↔1.60	0.86↔1.83	-0.69↔0.39	40.4↔70.8	38.2±9.91	ND
AMT 0023-34	4.76±1.07	0.51±0.23	1.55 ± 1.53	0.65 ± 0.80	-0.97 ± 1.42	21.9±21.8	27.6±3.91	ND

Table 3.3. Amino acid and nitrate uptake rates and acetate efflux rates of *Pseudomonas aeruginosa* strains under anoxic growth conditions in Simplified-SCFM. All units are in $mMol/(g \cdot DW-hr)$ except Total carbon uptake, which is in C- $mMol/(g \cdot DW-hr)$. Measurements with only two measurements are given as the range of measurement. Amino acid uptake rates for strains AA2 and AMT 0023-30, n=2; LES400 Glutamate measurement n=2; all others n=3. Nitrate uptake rates n=3. Acetate secretion rates for strains for PA01 and AA44 n=4; all others n=3; ND= not detected.



Figure 3.4. Percent of total carbon uptake from Simplified Synthetic Cystic Fibrosis Media (S-SCFM) during aerobic growth by *Pseudomonas aeruginosa* strains by compound. Alanine (black) Arginine (white), Aspartate (dark grey), Glutamate (medium grey), and Ornithine (light grey). PA01 n=6; AA2 n=5; AA44, LES400, AMT30 n=4; AA43 and AMT34 n=3. Error bars represent standard deviation of measurements.



Figure 3.5. Percent of total carbon uptake from Simplified Synthetic Cystic Fibrosis Media (S-SCFM) during anoxic growth by *Pseudomonas aeruginosa* strains by compound. Alanine (black) Arginine (white), Aspartate (dark grey), Glutamate (medium grey), and Ornithine (light grey). Uptake rates for strains AA2 and AMT 0023-30, n=2; LES400 Glutamate measurement n=2; all others n=3. Error bars represent standard deviation of measurements or as ranges where measurements are n=2.

Flux Balance Analysis of *P. aeruginosa* reference strain PA01 was performed using a modified genome-derived model together with the amino acid uptake rates measured for PA01 under aerobic conditions. A predicted flux map based on maximum growth efficiency (maximal biomass production for amino acid uptake rates) as the objective function is shown in Figure 3.8, with the listing of flux ranges for 99% of maximal biomass determined by Flux Variability Analysis shown. The FBA results demonstrate striking differences from ¹³C MFA flux, showing that optimal growth in *P. aeruginosa* strain PA01 would involve carbon flux into L-glutamate and then into the TCA cycle via α -ketoglutarate. Most carbon flux from the TCA cycle would be through the conversion of oxaloacetate to phosphoenolpyruvate under optimized growth.

The normalized to total carbon uptake ¹³C MFA flux results for all strains grown aerobically are shown in Figure 3.9 with values listed as a percentage of total carbon uptake through each reaction pathway for each strain. A complete listing of absolute fluxes in the figure can be found in Table 3.4 and a full result listing in electronic Supplemental 3.2. These results demonstrate a carbon flux similar to that for PA01, with movement of glutamate to ornithine and then to the TCA cycle making up a range of 30%-77% of the total uptake carbon for all strains. Strains AA44 and AMT0023-30 utilize glutamate dehydrogenase to move carbon from the TCA cycle to glutamate. The α -ketoglutarate to succinate flux shows great variability in the strains, with a range of no flux to more than half of the carbon flux through this pathway across all of the strains. The MFA demonstrates cycling of carbon from the TCA cycle in the form of malate to pyruvate and then back as citrate in all of the strain models. The fluxes (Table 3.4) also demonstrate little flux through the EDP and OPPP pathways for each strain.



Figure 3.6. Determination of Strain Diversity by ¹³**C-Labeling fingerprinting. 3.6A:** Principal Component Analysis of the isotopomers of proteinogenic amino acids and their fragments during **aerobic** growth. **Figure 3.6B**: Principal Component Analysis of the isotopomers of proteinogenic amino acids and their fragments during **anoxic** growth. Steady state labeling was measured in three biological replicates from 100% $3 \cdot C^{13}$ Alanine and 100% $U_5 \cdot C^{13}$ Glutamate Aerobic experiments (see materials and methods). Strains: AMT0023-30 (filled circle), AMT0023-34 (empty circle), AA2 (triangle), AA43 (square), AA44 (diamond), LES 400 (pentagram), and PA01 (hexagram).



Figure 3.7. Flux map from ¹³C MFA analysis for strain PA01 grown Simplified-SCFM under aerobic conditions. Strain PA01 was grown in defined S-SCFM with either 100% $1-C^{13}$ or 20% U_6-C^{13} Glucose (n=3 replicates for each substrate) to steady state labeling during exponential growth. Labeling and external fluxes were measured and modeled as described in materials and methods. Arrow thicknesses are proportional to net fluxes. Numbers represent net carbon fluxes in units of c-mmol flux/(gDW•hr); values are given +/- 90% confidence intervals (CI). Non-standard or potentially ambiguous abbreviations: 6PGC, 6-phospho-gluconate; G3P,Glyceraldehyde 3-phosphate; H6P, hexose-6-phosphate; P5P, Pentose-5-Phosphate; G5SH, glutamate-5-semialdehyde; CITR, citrulline.

Strain P		401	1 AA2		AA43		AA44		LES 400		AMT 0023-30		AMT 0023-34	
Flux	NetFlux	Xch Flux	NetFlux	Xch Flux	NetFlux	Xch Flux	Net Flux	Xch Flux	NetFlux	Xch Flux	NetFlux	Xch Flux	NetFlux	Xch Flux
Alanine Uptake	7.35±0.24	NA	6.93±0.36	NA	6.99±0.54	NA	10.67±0.20	NA	9.03±0.27	NA	9.62±0.19	NA	5.50±0.15	NA
Arginine Uptake	1.23±0.09	NA	1.14±0.12	NA	1.41±0.05	NA	1.09±0.10	NA	1.08±0.07	NA	3.51±0.15	NA	1.62±0.13	NA
Aspartate Uptake	2.63±0.24	NA	1.81±0.03	NA	1.42±0.06	NA	1.28±0.11	NA	2.38±0.08	NA	3.86±0.32	NA	0.90±0.08	NA
Glutamate Uptake	7.07±0.24	NA	4.42±0.13	NA	3.78±0.28	NA	4.16±0.09	NA	4.28±0.10	NA	7.02±0.11	NA	3.49±0.06	NA
Ornithine Uptake	1.07±0.12	NA	1.09±0.11	NA	0.44±0.15	NA	0.27±0.07	NA	1.20±0.13	NA	1.58±0.12	NA	0.85±0.07	NA
$ARG \rightarrow ORN + CO2$	0.64±0.78	NA	2.30±0.45	NA	1.18±0.46	NA	0.64±0.15	NA	0.69±0.09	NA	3.35±3.09	NA	1.55±0.25	NA
$ORN \rightarrow SUCC + CO2$	10.09±1.49	NA	9.61±1.10	NA	3.41±0.87	NA	5.58±0.89	NA	6.28±0.48	NA	9.38±0.79	NA	9.55±1.04	NA
$ORN + CO2 \rightarrow CITR$	0.00±0.83	NA	1.46±0.49	NA	0.00±0.46	NA	0.00±0.15	NA	0.00±0.04	NA	0.00±3.19	NA	0.00±0.16	NA
ORN→G5SH	-8.38±1.48	0.95±0.14	-7.66±1.08	0.95±0.12	-1.81±0.92	0.69±0.12	-4.67±0.89	0.78±0.03	-4.40±0.39	0.95±0.03	-4.45±0.63	0.95±0.01	-7.16±0.97	0.95±0.02
Glu→G5SH	8.43±1.48	0.95±0.01	7.71±1.08	0.95±0.11	1.84±0.92	0.95±0.10	4.71±0.89	0.95±0.00	4.44±0.39	0.95±0.01	4.48±0.63	0.95±0.00	7.17±0.97	0.95±0.00
H6P→6PGC	0.00±0.01	NA	1.37±0.64	NA	1.60±0.50	NA	0.00±0.10	NA	0.00±0.00	NA	0.00±0.14	NA	0.02±0.06	NA
6PGC→G3P+PYR	0.00±0.19	NA	1.37±0.27	NA	0.00±0.31	NA	0.00±0.00	NA	0.00±0.00	NA	0.00±0.12	NA	0.02±0.05	NA
G3P→PEP	-4.44±0.37	0.95±0.12	-5.57±0.54	0.00±0.16	-4.17±0.72	0.00±0.15	-2.95±0.22	0.00±0.11	-3.14±0.10	0.00±0.03	-7.30±0.35	0.00±0.02	-4.38±0.43	0.00±0.12
PEP→PYR	-2.01±0.28	0.00±0.09	-0.46±0.63	0.74±0.12	-2.49±0.86	0.11±0.09	-3.68±0.35	0.00±0.14	-2.55±0.08	0.00±0.04	-1.68±0.31	0.00±0.05	-2.26±0.15	0.00±0.03
2 G3P→H6P	1.46±0.22	NA	2.32±0.30	NA	1.13±0.33	NA	0.89±0.10	NA	1.00±0.03	NA	0.99±0.15	NA	0.39±0.09	NA
6PGC→2R5P	0.00±0.19	NA	0.00±0.67	NA	1.60±0.42	NA	0.00±0.10	NA	0.00±0.00	NA	0.00±0.10	NA	0.00±0.04	NA
2 R5P→S7P + G3P	-0.19±0.09	0.58±0.09	-0.12±0.24	0.81±0.10	0.44±0.14	0.95±0.10	-0.01±0.03	0.50±0.05	-0.08±0.01	0.50±0.08	-0.11±0.02	0.44±0.06	-0.04±0.02	0.40±.08
$S7P + G3P \rightarrow R5P + E4P$	-0.19±0.09	0.58±0.09	-0.12±0.24	0.81±0.10	0.44±0.14	0.95±0.11	-0.01±0.03	0.50±0.10	-0.08±0.01	0.50±0.03	-0.11±0.02	0.44 ± 0.06	-0.04 ± 0.02	0.40±0.02
$R5P + E4P \rightarrow H6P + G3P$	-0.73±0.11	0.95±0.00	-0.46±0.24	0.00±0.11	0.23±0.15	0.66±0.13	-0.48±0.03	0.95±0.00	-0.45±0.03	0.95±0.07	-0.52±0.03	0.00±0.00	-0.16±0.04	0.00±0.15
PYR→CO ₂ +AcCoA	6.85±0.33	NA	5.75±0.89	NA	6.28±0.82	NA	7.67±0.27	NA	7.12±0.19	NA	11.43±0.33	NA	6.08±0.18	NA
OAA + AcCoA → CIT	6.03±0.30	NA	4.46±0.36	NA	5.83±0.33	NA	6.86±0.14	NA	5.97±0.15	NA	10.57±0.29	NA	4.83±0.22	NA
CIT→ICIT	6.03±0.30	NA	4.46±0.36	NA	5.83±0.33	NA	6.86±0.14	NA	5.97±0.15	NA	10.57±0.29	NA	4.83±0.22	NA
$ICIT \rightarrow \alpha KG + CO_2$	4.42±0.54	0.95±0.01	3.86±0.95	0.00±0.14	4.78±0.92	0.95±0.08	5.83±0.23	0.95±0.17	4.45±0.14	0.95±0.00	9.32±0.50	0.90±0.09	3.86±0.43	0.00±0.11
$\alpha KG \rightarrow SUCC + CO_2$	2 34+1 67	NA	0 10+0 93	NA	641+071	NA	4 66+1 11	NA	373+039	NA	11 32+1 00	NA	0 00+1 33	NA
$SUCC \rightarrow FUM$	14 04+0 49	0 01+0 00	10 30+0 38	0 01+0 00	10 88+0 46	0 01+0 00	11 27+0 22	0 01+0 01	11 53+0 29	0 01+0 00	21 95+0 28	0 01+0 00	10 53+0 28	0 01+0 00
$FUM \rightarrow MAI$	14 59+1 00	0.95+0.16	12 19+0 44	078+012	11 11+0 64	0.00+0.16	11 67+0 28	0.04+0.16	11 96+0 32	0.95+0.06	22 36+3 26	0.92+0.13	10 72+0 18	0.00+0.00
	9.37+0.89	0.00+0.10	11 81+0 59	0.03+0.12	7 53+0 94	0.00+0.03	7 65+0 49	0.00+0.17	7 01+0 24	0.00+0.00	14 97+2 90	0.00+0.04	7 05+0 37	0.00+0.00
$ICIT \rightarrow GIX + SUCC$	1 61+0 33	NA	0.59+0.94	NA	1 05+0 90	NA	1 03+0 16	NA	1.52+0.06	NA	1 25+0 22	NA	0.98+0.25	NA
$GIX + AcCoA \rightarrow MAI$	0.00+0.02	NA	0 59+0 65	NA	0 01+0 74	NA	0.00+0.26	NA	0 17+0 01	NA	0 00+0 24	NA	0 84+0 25	NA
$MAI \rightarrow PYR + CO_2$	5 22+0 39	0.61+0.10	0.97+0.72	0.93+0.12	3 60+0 64	0.00+0.09	4 02+0 47	0.09+0.04	5 12+0 14	0.00+0.04	7 39+0 36	0.93+0.01	4 51+0 11	0.65+0.03
$PEP + CO_2 \rightarrow OAA$	-3.51+0.27	0 70+0 05	-5 79+0 48	0.86+0.13	-2 09+0 76	071+012	-0.20+0.36	0.85+0.02	-1 34+0 14	0 78+0 01	-6 44+0 40	0.88+0.06	-2 36+0 32	0 78+0 04
$G3P \rightarrow GLYC$	0 23+0 04	NA	0 17+0 03	NA	0 11+0 02	NA	0 17+0 03	NA	0 20+0 03	NA	0 16+0 02	NA	0.07+0.01	NA
$2 \text{ GIX} \rightarrow \text{PG3} + \text{CO}_2$	0.80+0.17	NA	0.00+0.34	NA	0 52+0 47	NA	0.51+0.06	NA	0 67+0 03	NA	0.62+0.10	NA	0 07+0 05	NA
$\alpha_{\rm KC} \rightarrow \rm CIII$	2 07+1 41	0.95+0.06	3 76+1 04	0.01+0.12	-1 63+0 72	0 94+0 07	1 17+0 93	0.95+0.09	0 72+0 37	0.95+0.00	2 00+0 64	0.95+0.00	3 86+0 95	0.95+0.02
$CITR + ASP \rightarrow APG + FUM$	0.00+0.83	NA	1 46+0 49	NA NA	0.00+0.46	NA	0.00+0.15	NA	0.00+0.04	NA	0.00+3.19	NA	0.00+0.16	NA
CO- Efflux	36 21+1 85	NA	30 44+0 77	NA	36 66+1 00	NA	31 36+0 53	NA	7 94+0 22	NA	73 77+1 28	NA	38 02+1 58	NA
Wall Efflux	0.10+0.03	NA	0 14+0.03	NΔ	0.08+0.02	NΔ	0.15+0.03	NΔ	0 10+0.02	NΔ	0.16+0.02	ΝΔ	0.07+0.01	NA
LPS Efflux	0.23+0.04	NA	0.11+0.01	NA	0.09+0.02	NA	0.17+0.02	NA	0.20+0.02	NA	0.15+0.02	NA	0.07+0.01	NA
End End Efflux	0.23+0.04	NA	0.17+0.03	NA	0 17+0 03	NA	0.18+0.03	NA	0.22+0.03	NA	0.18+0.02	NA	0.08+0.01	NA
Glycogen Efflux	0 22+0 03	NA	0 16+0 03	NA	0.09+0.02	NA	0 16+0 03	NA	0 19+0 02	NA	0 15+0 02	NA	0.07+0.01	NA
Final Residuum	119	.936	23.3	3704	222	.005	248	908	130	.518	177	.613	166	.846

Table 3.4. Net and exchange (Xch) fluxes determined by ¹³**C MFA of aerobic SCFM with 90% confidence intervals.** (See materials and methods for description of CI calculation) Residuum is the minimal sum-of-squares optimized value found using C13-Flux. Abbreviations as for Figure 3.7, NA - flux description is not applicable.



Figure 3.8. Flux map from FBA analysis for strain PA01 grown in Simplified-SCFM under aerobic conditions. Uptake of amino acids, production of CO₂, and growth rate were used to constrain a stoichiometric genome-derived metabolic model (see materials and methods) with the objective function being maximal growth (corresponding to maximal growth yield on S-SCFM defined amino acids). Numbers are net fluxes as in Figure 3.4. Ranges are from flux variability analysis (range of each flux consistent with 99-100% of maximal growth).

The ¹³C MFA flux map for reference strain PA01 during anoxic growth in S-SCFM is shown in Figure 3.10, with the complete list of fluxes listed in Table 3.5. As seen in the aerobic ¹³C MFA results, carbon flux from glutamate is moved through ornithine from glutamate towards succinate with additional carbon converted from α -ketoglutarate to glutamate. The glyoxylate cycle is utilized heavily in anoxic conditions, as is an incomplete TCA cycle. Most fluxes in this model demonstrate 90% CI values of 20% or less of the total flux, with the exception of the urea cycle components in this model demonstrating potential reversibility and utilization of this pathway.

The FBA results for optimized biomass growth under anoxic conditions with the FVA determined ranges of 99% optimized biomass are shown in Figure 3.11. The FBA results demonstrate movement of amino acid carbon from ornithine to glutamate to be taken into the TCA cycle as α -ketoglutarate. FBA also demonstrates full utilization of the TCA cycle and no flux through the glyoxylate cycle. These results stand in stark contrast with the ¹³C MFA results for PA01. The FBA results are in agreement with the MFA results of little carbon flux through the EDP and OPPP.



Figure 3.9. Averaged carbon-uptake normalized flux map from ¹³C MFA analysis of *P. aeruginosa* cystic fibrosis isolates grown in aerobic Simplified-SCFM conditions. MFA determined c-mol fluxes of individual experimental strains normalized to total carbon uptake averaged with standard deviation determined. Arrow thicknesses are proportional to averaged % of total carbon uptake. Arrows are colorized to demonstrate variability of total uptake carbon through experimental strains.



Figure 3.10. Flux map from ¹³C MFA analysis for strain PA01 grown in Simplified-SCFM under anoxic conditions. Strain PA01 was grown in defined S-SCFM with either 100% $1-C^{13}$ or 20% U_6-C^{13} Glucose (n=3 replicates for each substrate) to steady state labeling during exponential growth. Labeling and external fluxes were measured and modeled as described in materials and methods. Arrow thicknesses are proportional to net fluxes. Numbers represent net carbon fluxes in units of c-mmol flux/(gDW•hr); values are given +/- 90% confidence intervals (CI).

Strain	PA01		AA2		AA43		AA44		AMT0023-30		AMT0023-34		LES 400	
Flux	NetFlux	Xch Flux	NetFlux	Xch Flux	Net Flux	Xch Flux	NetFlux	Xch Flux	Net Flux	Xch Flux	NetFlux	Xch Flux	NetFlux	Xch Flux
Alanine Uptake	6.46±0.10	NA	9.90±0.26	NA	3.49±0.02	NA	5.50±0.21	NA	10.05±0.40	NA	3.69±0.24	NA	6.46±0.11	NA
Arginine Uptake	0.27±0.04	NA	1.03±0.02	NA	0.21±0.02	NA	0.21±0.04	NA	1.04±0.10	NA	0.28±0.04	NA	0.27±0.04	NA
Aspartate Uptake	2.32±0.09	NA	2.90±0.13	NA	1.23±0.00	NA	1.74±0.12	NA	3.72±0.20	NA	0.67±0.10	NA	2.31±0.05	NA
Glutamate Uptake	3.69±0.07	NA	2.72±0.16	NA	1.87±0.04	NA	2.63±0.17	NA	4.97±0.15	NA	1.45±0.13	NA	3.69±0.03	NA
Ornithine Uptake	1.73±0.06	NA	-0.00±0.04	NA	0.30±0.04	NA	0.38±0.07	NA	0.12±0.12	NA	-0.00±0.01	NA	1.73±0.00	NA
$ARG \rightarrow ORN + CO2$	0.16±2.02	NA	0.84±0.34	NA	0.12±0.05	NA	0.08±0.10	NA	0.89±0.10	NA	0.15±0.10	NA	0.16±0.04	NA
ORN → SUCC + CO2	6.58±0.48	NA	0.73±0.67	NA	0.52±0.41	NA	2.71±0.48	NA	4.81±0.79	NA	0.00±0.51	NA	6.57±0.39	NA
$ORN + CO2 \rightarrow CITR$	0.00±2.04	NA	0.00±0.34	NA	0.00±0.04	NA	0.00±0.10	NA	0.00±0.07	NA	0.00±0.08	NA	0.00±0.00	NA
ORN→G5SH	-4.69±0.47	0.47±0.94	0.12±0.65	0.00±0.02	-0.10±0.38	0.41±0.02	-2.25±0.47	0.95±0.03	-3.80±0.71	0.95±0.06	0.15±0.50	0.95±0.05	-4.68±0.38	0.94±0.01
Glu→G5SH	4.71±0.47	0.47±0.95	-0.11±0.65	0.88±0.01	0.10±0.38	0.89±0.01	2.26±0.47	0.95±0.00	3.81±0.71	0.95±0.06	-0.14±0.50	0.80±0.06	4.69±0.38	0.95±0.00
Glu→PRO	0.01±0.00	NA	0.01±0.00	NA	0.01±0.00	NA	0.01±0.00	NA	0.01±0.00	NA	0.01±0.00	NA	0.01±0.00	NA
H6P→6PGC	0.01±0.07	NA	0.00±0.01	NA	0.00±0.30	NA	0.00±0.42	NA	0.00±0.06	NA	0.00±0.09	NA	0.00±0.01	NA
6PGC→G3P+PYR	0.01±0.07	NA	0.0±0.01	NA	0.00±0.01	NA	0.00±0.02	NA	0.00±0.05	NA	0.00±0.02	NA	0.00±0.01	NA
G3P→PEP	-3.92±0.32	0.95±0.15	-3.52±0.19	0.95±0.14	-2.66±0.19	0.95±0.07	-2.27±0.23	0.95±0.15	-6.66±0.45	0.95±0.09	-0.84±0.28	0.95±0.16	-3.93±0.12	0.95±0.17
PEP→PYR	-1.38±0.28	0.71±0.00	0.00±0.08	0.00±0.01	-1.20±0.21	0.00±0.08	-1.33±0.17	0.00±0.05	-2.92±0.10	0.00±0.05	-1.21±0.07	0.30±0.06	-1.49±0.06	0.70±0.00
2 G3P→H6P	0.34±0.08	NA	0.38±0.03	NA	0.22±0.10	NA	0.48±0.16	NA	0.45±0.08	NA	0.31±0.07	NA	0.32±0.04	NA
6PGC→2 R5P	0.00±0.00	NA	0.00±0.00	NA	0.00±0.30	NA	0.00±0.42	NA	0.00±0.00	NA	0.00±0.08	NA	0.00±0.00	NA
2 R5P→S7P + G3P	0.01±0.01	0.01±0.02	0.01±0.01	0.12±0.01	-0.03±0.10	0.95±0.10	-0.07±0.14	0.95±0.08	0.02±0.01	0.00±0.06	0.01±0.03	0.23±0.07	0.01±0.01	0.03±0.06
$S7P + G3P \rightarrow R5P + E4P$	0.01±0.01	0.00±0.01	0.01±0.01	0.12±0.07	-0.03±0.10	0.95±0.09	-0.07±0.14	0.95±0.08	0.02±0.01	0.00±0.06	0.01±0.03	0.23±0.09	0.01±0.01	0.00±0.01
$R5P + E4P \rightarrow H6P + G3P$	-0.19±0.02	0.02±0.08	-0.22±0.02	0.95±0.14	-0.11±0.10	0.00±0.16	-0.23±0.14	0.00±0.14	-0.27±0.03	0.00±0.13	-0.18±0.03	0.95±0.11	-0.19±0.02	0.00±0.13
PYR→CO ₂ +AcCoA	6.96±0.26	NA	11.28±0.34	NA	3.53±0.12	NA	5.15±0.26	NA	10.88±0.51	NA	3.23±0.29	NA	6.94±0.08	NA
PYR→Acetate	0.84±0.07	NA	3.66±0.26	NA	0.37±0.03	NA	0.88±0.06	NA	0	NA	0	NA	0.84±0.19	NA
$OAA + AcCoA \rightarrow CIT$	6.61±0.24	NA	5.39±0.35	NA	2.28±0.11	NA	3.73±0.30	NA	8.63±0.23	NA	2.25±0.22	NA	6.59±0.08	NA
CIT→ICIT	6.61±0.24	NA	5.39±0.35	NA	2.28±0.11	NA	3.73±0.30	NA	8.63±0.23	NA	2.25±0.22	NA	6.59±0.08	NA
ICIT $\rightarrow \alpha KG + CO_2$	1.13±0.47	0.95±0.17	-2.69±0.35	0.95±0.15	-1.70±0.31	0.95±0.00	-0.24±0.31	0.95±0.15	-0.99±0.35	0.95±0.04	-1.02±0.38	0.95±0.12	1.12±0.17	0.95±0.14
$\alpha KG \rightarrow SUCC + CO_2$	0.00±0.66	NA	0.00±0.72	NA	0.00±0.36	NA	0.00±0.44	NA	0.00±0.62	NA	0.47±0.68	NA	0.00±0.42	NA
$SUCC \rightarrow FUM$	12.06±0.32	0.01±0.00	8.80±0.50	0.14±0.00	4.50±0.18	0.01±0.00	6.68±0.48	0.01±0.00	14.43±0.44	0.01±0.00	3.73±0.36	0.01±0.00	12.05±0.10	0.01±0.00
$FUM \rightarrow MAL$	12.21±1.99	0.95±0.11	8.89±0.60	0.95±0.13	4.56±0.19	0.95±0.00	6.84±0.43	0.95±0.10	14.62±0.47	0.95±0.08	3.80±0.42	0.95±0.12	12.20±0.10	0.95±0.10
$MAL \rightarrow OAA$	7.94±1.84	0.00±0.02	7.41±0.45	0.00±0.00	3.11±0.21	0.00±0.006	4.09±0.29	0.00±0.04	10.29±0.57	0.00±0.00	2.13±0.38	0.00±0.02	7.83±0.11	0.00±0.00
ICIT→ GLX + SUCC	5.48±0.39	NA	8.07±0.22	NA	3.98±0.26	NA	3.97±0.27	NA	9.62±0.42	NA	3.26±0.41	NA	5.48±0.14	NA
$GLX + AcCoA \rightarrow MAL$	0.00±0.00	NA	5.46±0.05	NA	1.05±0.09	NA	1.00±0.07	NA	1.72±0.47	NA	0.61±0.18	NA	0.00±0.06	NA
$MAL \rightarrow PYR + CO_2$	4.27±0.19	0.83±0.12	6.94±0.24	0.95±0.04	2.51±0.16	0.74±0.03	3.74±0.22	0.63±0.00	6.05±0.16	0.87±0.00	2.29±0.15	0.88±0.06	4.37±0.10	0.83±0.00
$PEP + CO_2 \rightarrow OAA$	-2.94±0.22	0.54±0.02	-3.97±0.17	0.00±0.03	-1.62±0.18	0.00±0.06	-1.27±0.17	0.21±0.05	-4.30±0.48	0.00±0.05	0.00±0.26	0.00±0.07	-2.83±0.11	0.55±0.03
$G3P \rightarrow GLYC$	0.06±0.01	NA	0.07±0.01	NA	0.03±0.01	NA	0.07±0.01	NA	0.09±0.01	NA	0.06±0.01	NA	0.06±0.01	NA
$2 \text{ GLX} \rightarrow \text{PG3} + \text{CO}_2$	2.74±0.19	NA	1.31±0.11	NA	1.46±0.14	NA	1.49±0.14	NA	3.95±0.06	NA	1.33±0.12	NA	2.74±0.07	NA
$\alpha KG \rightarrow GLU$	1.13±0.47	0.95±0.09	-2.69±0.62	0.95±0.08	-1.70±0.37	0.95±0.07	-0.24±0.40	0.95±0.08	-0.99±0.64	0.95±0.07	-1.48±0.50	0.95±0.01	1.12±0.38	0.95±0.08
CITR + ASP \rightarrow ARG + EUM	0.00±2.04	NA	0.00±0.34	NA	0.00±0.04	NA	0.00±0.10	NA	0.00±0.07	NA	0.00±0.08	NA	0.00±0.00	NA
CO ₂ Efflux	32.06±0.92	NA	37.61±1.55	NA	12.41±0.42	NA	18.41±1.37	NA	40.32±1.43	NA	8.58±1.07	NA	32.01±0.35	NA
Wall Efflux	0.07+0.01	NA	0.09+0.01	NA	0.04+0.01	NA	0.09+0.01	NA	0.11+0.03	NA	0.07+0.01	NA	0.07+0.01	NA
LPSEfflux	0.06±0.01	NA	0.07±0.01	NA	0.03±0.01	NA	0.07±0.01	NA	0.08±0.01	NA	0.06±0.01	NA	0.06±0.01	NA
Fatty Acid Efflux	0.07±0.01	NA	0.11±0.01	NA	0.05±0.01	NA	0.10±0.01	NA	0.13±0.01	NA	0.09±0.01	NA	0.07±0.01	NA
Glycogen Efflux	0.06±0.01	NA	0.07±0.01	NA	0.03±0.01	NA	0.07±0.01	NA	0.08±0.01	NA	0.05±0.01	NA	0.06±0.01	NA
Final Residuum	320.	275	788.	462	669.351		179	149	757.507		493.121		320.	162

Table 3.5. Net and exchange (Xch) fluxes determined by ¹³**C MFA of anoxic SCFM with 90% confidence intervals.** (See materials and methods for description of CI calculation) Residuum is the minimal sum-of-squares optimized value found using C13-Flux. Abbreviations as for Figure 3.7, NA - flux description is not applicable.

Net carbon fluxes normalized to total carbon uptake rates of the ¹³C MFA results for all strains are shown in Figure 3.12, with values listed as a percentage of total carbon uptake through each reaction pathway for each strain. A complete listing of fluxes in the figure can be found in Table 3.5 and a full result listing in Supplemental 3.2. These results demonstrate that all strains but AMT0023-34 have an incomplete TCA cycle, with most strains (besides PA01 and LES400) transferring carbon from α -ketoglutarate to isocitrate into the glyoxylate cycle. The glyoxylate cycle also has a large carbon flux for all strains, having a range of 57%-87% of total input carbon. These results also demonstrate the cycling of carbon from the TCA cycle in the form of malate to pyruvate and then back as citrate. The movement of carbon towards the OPPP and ED pathways represents 11%-29% of total carbon uptake.



Figure 3.11. Flux map from FBA analysis for strain PA01 grown in Simplified-SCFM under anoxic conditions. Uptake of amino acids, production of CO_2 , and growth rate were used to constrain a stoichiometric genome-derived metabolic model (see materials and methods) with the objective function being maximal growth (corresponding to maximal growth yield on S-SCFM defined amino acids). Numbers are net fluxes as in Figure 3.4. Ranges are from flux variability analysis (range of each flux consistent with 99-100% of maximal growth).



Figure 3.12. Flux map from ¹³**C MFA analysis of** *P. aeruginosa* **cystic fibrosis isolates grown in anoxic Simplified-SCFM conditions.** MFA determined c-mol fluxes of individual experimental strains normalized to total carbon uptake averaged with standard deviation determined. Arrow thicknesses are proportional to averaged % of total carbon uptake. Arrows are colorized to demonstrate variability of total uptake carbon through experimental strains.



Figure 3.13. Hierarchical Cluster Analysis of MFA derived flux values. Hierarchical Clustering Analysis using MFA derived flux values was employed to identify the main groupings as discussed in the text. **A**- Results from the Aerobic S-SCFM ¹³C MFA determined fluxes. **B** - Results from the Anoxic S-SCFM ¹³C MFA determined fluxes.
	Aerobic										
Strain	NADPH	NADH	АТР	FADH2	ATP Equivalence	ATP/µ	TCA/GLX	Orn/Glu	PEP/PYR	G6P/R5P	
PA01 FBA	7.51 [6.61]	11.53 [31.87]	7.51 [5.10]	9.39 [9.39]	69.21 [58.94]	53.50	NA	NA	-2.3	-1.28	
AA43	4.51±0.93	16.02±3.89	2.08±0.19	10.88±0.46	69.73±12.92	79.17±0.20	5.53±0.14	-2.09±0.10	-1.72±0.10	3.20±0.68	
AMT 0023-30	4.81±1.11	28.42±4.23	5.16±2.51	21.95±0.28	121.15±11.25	138.57±0.16	8.47±0.03	-4.69±0.07	-1.15±0.02	-1.28±0.16	
PA01	-4.06±1.21	16.20±2.38	-8.10±0.94	14.04±0.49	43.31±8.77	35.22±0.21	3.75±0.04	4.86±0.14	-1.49±0.03	-1.33±0.21	
AA2	-3.89±1.56	17.23±3.61	-9.52±0.01	10.30±0.38	39.29±13.50	31.47±0.34	7.50±0.26	2.55±0.06	-0.17±0.19	-3.38±0.54	
AA44	1.08±1.25	18.20±1.31	-3.73±0.42	11.27±0.22	61.39±7.13	54.62±0.12	6.68±0.03	4.77±0.16	-19.98±0.45	-1.23±0.13	
AMT 0023-34	-3.33±1.35	12.63±1.47	-9.43±0.35	10.53±0.28	29.61±7.82	45.30±0.27	4.95±0.04	2.48±0.05	-1.91±0.03	-1.60±0.32	
LES400	-0.04±0.37	15.44±0.68	-3.27±0.05	11.53±0.29	52.54±3.10	55.61±0.13	3.93±0.01	8.67±0.10	-3.83±0.03	-1.47±0.08	

	Anoxic										
Strain	NADPH	NADH	ATP	FADH2	ATP Equivalence	ATP/µ	TCA/GLX	Orn/Glu	PEP/PYR	G6P/PPP	
PA01 FBA	7.86 [3.89]	16.94 [38.28]	7.77 [7.09]	9.62 [10.87]	84.20 [61.46]	130.00 [61.46]	NA	NA	-1.45	-1.28	
AA2	-2.59±0.69	17.59±2.69	0.11±0.19	8.80±0.50	50.79±9.01	114.10±0.25	0.67±0.01	-0.27±0.19	-0.57±0.13	-1.17±0.12	
AA43	-1.81±0.65	3.69±1.93	-1.31±0.14	4.50±0.18	10.15±6.85	48.47±0.75	0.57±0.01	-0.31±0.16	-0.65±0.03	-1.32±1.02	
AMT 0023-34	-0.88±0.75	3.51±2.44	-0.60±0.16	3.73±0.36	11.56±8.70	32.21±0.76	0.69±0.03	0.00±1041	0.0±4048996	1.15±0.29	
PA01	-4.72±2.22	26.12±3.92	-7.21±0.00	12.06±0.32	64.37±15.83	169.25±0.26	1.21±0.01	5.81±0.08	-0.69±0.02	-1.18±0.27	
LES400	-3.59±0.43	25.93±1.28	-6.18±0.11	12.05±0.10	67.75±4.52	144.20±0.15	1.20±0.00	5.88±0.07	-0.65±0.01	-1.14±0.18	
AA44	-2.51±0.78	13.04±2.48	-3.59±0.03	6.68±0.48	32.75±8.88	74.66±0.30	0.94±0.02	-11.32±0.34	-0.34±0.04	-1.36±0.68	
AMT 0023-30	-4.82±0.55	23.13±3.53	-6.73±0.06	14.43±0.44	60.70±10.79	113.43±0.28	0.90±0.01	-4.85±0.13	-0.71±0.03	-1.15±0.29	

Table 3.6. Flux ratio tables of experimental strains in major points of carbon diversion in central metabolism and estimated energy production of MFA determined strains flux rates. Flux ratios and cofactor production rates for *P. aeruginosa* strains. The ratios of fluxes at major central metabolic branch points, as discussed in the text, with significant flux and strain variability shown in Figure 3.9 and 3.13. Estimated cofactor production rates are from MFA determined flux maps normalized to the glucose uptake rate for each strain (direct summation of FBA results listed in brackets). ATP, substrate level phosphorylation; NADP(H) total cellular NADH + NADPH production. Flux ratios calculated using: GLX/TCA = $V_{(OAA+AC-COA+CTT)}/V_{(ICIT+GLX+SUCC)}$; ORN/GLU = $V_{(ORN+SUCC+CO2)}/V_{(GLU+\alpha KG)}$; PEP/PYR = $V_{(OAA+PP+CO2)}/V_{(MAL+PYR+CO2)}$; G6P/R5P = $V_{(G3P+G3P+GP)}/V_{(G3P+S7P+E4P+R5P)}$.

Anoxic									
Strain	AA2	AA43	AMT 0023-34	PA01	LES400	AA44	AMT 0023-30		
ATP Synthesis	126%	124%	133%	60%	121%	105%	74%		
NADPH Synthesis	411%	414%	133%	241%	296%	123%	76%		
NADH Synthesis	122%	120%	139%	83%	134%	165%	102%		
Biomass	69%	69%	49%	45%	80%	28%	45%		
NH4 Production	119%	117%	125%	137%	114%	145%	137%		
	Aerobic								
Strain	PA01	AA44	AA2	AMT 0023-30	AA43	AMT 0023-34	LES400		
ATP Synthesis	91%	93%	92%	59%	67%	71%	134%		
NADPH Synthesis	79%	184%	107%	265%	193%	190%	86%		
NADH Synthesis	104%	117%	139%	107%	98%	154%	136%		
Biomass	78%	85%	83%	52%	53%	32%	86%		
NH4 Production	88%	126%	131%	249%	257%	335%	-11%		

Table 3.7. MFA constrained FBA to FBA maximized growth results. Results higher than 100% correspond to increased production compared to growth optimized results.

Hierarchical Cluster Analysis of the carbon uptake normalized ¹³C MFA flux results are shown in Figure 3.13.. These results demonstrate two flux patterns in the aerobic and anoxic results. Under aerobic growth (Figure 3.13A), strains LES 400 and AMT 0023-34 show the most similarity in comparison to the other strains. Under anoxic growth (Figure 3.13B), AMT0023-34, AA2 and AA43 show the most similarity in comparison to the other strains. To understand the flux differences that HCA has shown, the ATP, redox cofactors, and flux ratios of major metabolic flux divisions were calculated. As listed in Table 3.6, ratios of TCA cycle flux to glyoxylate cycle flux are higher under aerobic growth compared to similar to higher flux ratios through the glyoxylate cycle flux under anoxic conditions. Movement of carbon from glutamate or ornithine shows the utilization of ornithine to succinate conversion by all strains under aerobic growth, but via glutamate by strains AA2, AA43, and AMT 0023-34 under anoxic conditions. Most strains also demonstrate net consumption of NADPH and ATP during growth (except aerobic growth of AA43 and AMT0023-30). To determine differences in the ATP and redox cofactor production in relation to optimized biomass growth, FBA constrained by ¹³C MFA results was compared to FBA optimized biomass growth. As shown in Table 3.7, MFA constrained FBA shows increased NADPH and NADH production over biomass synthesis reduction.

DISCUSSION

Many studies of *Pseudomonas aeruginosa* have been conducted to better understand the various adaptations that happen during chronic infection of CF patients. A frequent concern related to *P. aeruginosa* metabolism in the CF lung has been understanding its metabolic flexibility under micro-aerobic to anoxic conditions, especially as the organism's growth is likely to be under these conditions in the CF lung [77,78,137]. Additionally, various Omic measures of

serial CF isolates have been conducted to measure the genomic [48,49,51–54,56,57,75,138], transcriptomic [51,58–62], and proteomic [58,63,123] changes in chronic infection adaptations to the CF lung environment. This study provides the first direct quantization and comparison of the metabolic phenotypes of *P. aeruginosa* under CF lung-like conditions and the metabolic changes seen under oxygen and nitrate respiration.

This study was based on the work of Palmer et al. on defining the likely carbon and salt environment seen in the CF lung [77]. While this media lacks a source of lipids, especially those seen in the CF lung, the findings in this study would be compatible with possible uptake of a lipid for catabolism as the TCA cycle is the principal catabolic pathway utilized by these strains [59]. This study also demonstrates an uptake preference of amino acids and lactate similar to that reported by Palmer et al., with the notable exception of ornithine uptake preference in the strains of this study [77]. Due to cost and availability concerns, ¹³C L-lactate was not used in this study. Simulated FBA results of L-lactate uptake under aerobic growth, using MFA flux rates as internal flux constraints with SCFM L-lactate uptake rates, did show ready uptake and increase in biomass synthesis rates in all strains (see Supplemental 3.3). These results showed a significant utilization of the enzyme L-Lactate dehydrogenase ubiquinone, which converted Llactate to pyruvate with reduced ubiquinone available for cellular respiration. Additionally, leucine was a major carbon source in anoxic growth but was not used in these experiments to allow direct comparison to aerobic and anoxic growth. Simulated FBA results of SCFM measured leucine uptake rates with constrained MFA did demonstrate uptake in some strains and allowed for additional reductant production in L-leucine catabolism (see Supplemental 3.3). Finally, aromatic amino acids, which have been shown to increase virulence factor production in CF isolates of P. aeruginosa, were not included in this study [139]. While this may affect central

carbon metabolism due to increased production of virulence factors, the current MFA shows that production fluxes of these aromatic amino acids are minimal and thus likely to have a minimal direct effect on aromatic amino acid anabolism.

The ¹³C MFA and FBA results demonstrate notable phenotypic properties of *P*. *aeruginosa* under CF lung-like growth conditions. With oxidative bursts a common anti-bacterial strategy of many species [140–143], the high rates of reductant production seen in these *P*. *aeruginosa* strains are notable. Comparisons of the ¹³C MFA constrained FBA to optimized growth also demonstrates both significant increases in NAD(P)H production over that in biomass optimized growth and the reduction in biomass production rates. FBA constrained by ¹³C MFA results and nitrate uptake rates intriguingly showed incomplete reduction of nitrate in some strains, being excreted as nitrite (see supplemental 3.3), pointing to the possibility of incomplete respiration of nitrate under anoxic conditions.

The inability to demonstrate similarity of anoxic ¹³C labeled proteogenic amino acids PCA results to ¹³C MFA HCA results is again noted. The efforts to build a high throughput system for analysis of metabolic similarities and differences using ¹³C label patterns of metabolites while avoiding the time intensive work of ¹³C MFA is desirable [101,144]. While previous results showed that PCA of ¹³C labeled proteogenic amino acids may differentiate strain similarity but not true metabolic phenotypes [132], the results shown in this study demonstrate that ¹³C MFA determined fluxes can demonstrate strain phenotypes even in PCA determined strains with amino acid label similarities.

Previous work on CF isolates' metabolism in minimal media demonstrates some similarities to the findings in this work. The HCA of M9 aerobic growth and S-SCFM aerobic

growth reproduces the close flux relationships between strains AA44 and PA01 and between strains AA2 and AA43 [132]. During aerobic growth in S-SCFM, strains PA01 and LES 400 and strains AA44 and AA2 demonstrate greater differences than were shown in aerobic minimal media growth [132]. While not surprising results, this does demonstrate that different metabolic phenotypes can be expressed by each strain than can be predicted under more simplified media conditions. These results do represent important factors in the consideration of the role of metabolism in pathogenicity, as under both media conditions the utilization of the glyoxylate cycle is higher than would be predicted under maximized biomass growth demonstrated in FBA [132]. These studies' results also indicate that metabolic phenotypes of multiple strains may fall in a few generalized categories, such as two phenotypes demonstrated in the previous study and those demonstrated in this study [132]. These fewer phenotypes resolved by ¹³C MFA stand in contrast to the range and number of genotypes seen in genomic based methods, but analysis of more strains would be needed to fully understand the metabolic flexibility of *P. aeruginosa* in chronic CF infection [48,52,53,56,60].

The glyoxylate cycle is important to consider both as a metabolic target for pharmaceutical development and as a potential pathogenic factor in *P. aeruginosa* infection. A study conducted on the reduction of pathogenicity of *P. aeruginosa* infection of alfalfa seeds seen with the removal of the glyoxylate cycle enzyme isocitrate lyase also demonstrated reduction in rates of lung infection [145]. The work in this study as well as that in previous studies that utilized M9 minimal media demonstrates the high utilization of this pathway even in growth without lipids, as the catabolism of lipids found in the lung has been proposed as a possible explanation of glyoxylate cycle utilization in chronic infection isolates of *P. aeruginosa* as well as other pathogenic organisms of the lung [58,132,146]. The ¹³C MFA results also

demonstrate the utilization of glyoxylate to form phosphoglycerate under anoxic conditions by using glyoxylate carboligase, 2-hydroxy-3-oxopropionate reductase NAD(P), and glycerate kinase, but not utilizing the common second enzyme of the glyoxylate cycle, malate synthase.

This study also helps to shed light on published results of the various Omic techniques used to understand chronic infection adaptation of *P. aeruginosa*. The ¹³C MFA results contextualize the transcriptomic work on chronic infection isolates from CF lungs by Hoboth et al., with both showing the utilization of glutamate and ornithine metabolism [58]. Importantly, ¹³C MFA demonstrated that the carbon flow was the reverse of that theorized by Hoboth et al. [58]. Other published transcriptomic studies show results similar to this study's ¹³C MFA, with upregulation of ornithine and arginine catabolic pathways and downregulation of glutamate dehyrdogenase and EDP related enzymes [147]. The Entner-Doudoroff and Oxidative Pentose Phosphate Pathways may not be particularly important in planktonic growth in the CF lung, as MFA results demonstrate little carbon flux through these pathways, especially after biosynthesis of biomass components are taken into account. These results may be different in the CF lung, due to the presence of carbohydrates and direct oxidative stress, as well as measured transcriptomic changes of *P. aeruginosa* CF isolate strains demonstrating increased EDP and OPPP gene expression [51,148].

Putrescine and other polyamines are common molecules found in many organisms with multiple described functions (e.g. antioxidant, nucleic acid stabilization, acid resistance, sidephores, etc.) [149]. Pathogenesis in several bacterial species (e.g., *Staphylococcus* sp., *Yersinia pestis, Bacillus anthracis*, and *Vibrio cholerae*) has been linked to the expression of various polyamines [150]. The importance of polyamine production in biofilm formation of several bacteria species (e.g., *Vibrio cholerae, Yersinia pestis, Bacillus subtilis*, and *Staphylococcus aureus*) has been more recently demonstrated, with defects in enzymes necessary for polyamine metabolism, or exposure to certain polyamines, reducing biofilm formation but not affecting growth [151–157]. The metabolism of *P. aeruginosa* heavily utilizing putrescine metabolism during catabolism of amino acids, as well as multiple biofilm forming species failing to form biofilms without intact polyamine metabolic pathways, may demonstrate a shared metabolism in biofilm forming pathogenic bacteria and a possible shared pharmaceutical target.

Direct production and utilization of polyamines by *P. aeruginosa* has also been described. Polyamine localization to the outer membrane of *P. aeruginosa* have been described and shown to be protective against extracellular DNA, oxidative damage, and antibiotic action [158]. A high amount of putrescine production has been reported in *P. aeruginosa* strain PA01 grown under rich media conditions and showing decreased production when supplemented with addition of glucose or fructose to the media [159]. Reports of samples of CF sputum during exacerbation have shown an increase in putrescine concentration and may be explained by the metabolism of *P. aeruginosa* demonstrated in this work [160]. These possible utilizations of putrescine rely on the accumulation of the metabolite, and kinetic data for these enzymes would be beneficial in determining the possibility of this accumulation.

Putrescine or agmatine being utilized as a form of signaling in *P. aeruginosa* may be another possible explanation of the ¹³C MFA results described in this work. Genetic and transcriptomic work by Chou et al. during growth of *P. aeruginosa* mutants to characterize metabolism of agmatine and putrescine demonstrated induction of enzymes responsible for catabolism when exposed to agmatine and acetyl-putrescine [161]. Chou et al. also report the induction of the *oprH-phoP-phoQ* operon in *P. aeruginosa* exposed to exogenous agmatine and putrescine, allowing for the expression of known antibacterial resistance genes [161]. A gene that functions to enhance biofilm growth and whose promoter binds to agmatine, *agu2ABCA'*, has been described and found in some *P. aeruginosa* CF isolates [162]. Other findings of various polyamine exposures at millimolar concentrations causing an increased susceptibility to penicillin-class antibiotics and polymyxin B but decreasing susceptibility towards other classes (aminoglycosides, quinolones, etc.) in *P. aeruginosa* add to evidence of the role of polyamines as a possible regulatory agent in this species, especially in the reactivation of persister cell growth [163–165]. Other organisms have also demonstrated some of these adaptations, with work in *Escherichia coli* showing the role of putrescine metabolism in persister cell development [166]. A study of modulation of biofilm formation though exogenous exposure of various polyamines in pathogenic bacteria also demonstrate a possible role of polyamines in biofilm formation signaling or as a metabolic factor, but this relationship remains to be fully elucidated [156,157].

ACKNOWLEDGEMENTS

We wish to thank Matthew Juergens for performing the Hierarchical Clustering Analysis. We also wish to thank Seattle Children's Hospital Cystic Fibrosis Isolate Core (NIH P30 DK089507) for their donation of strain AMT0023-30 and the RTSF Mass Spectrometry and Metabolomics Core for at Michigan State University for their analytical support. Dr. Gemma Reguera graciously provided anoxic culturing technique training and equipment. Michael Opperman was supported in part by funds from the Cellular and Molecular Biology Graduate Program and College of Human Medicine at Michigan State University and by a MD-PhD Fellowship grant by Spectrum Health. APPENDICES

APPENDIX A - Electronic Supplemental Descriptions

Descriptions of the supplemental material uploaded as an electronic supplement of this dissertation.

Supplemental 3.1: ¹³C labeling data of amino acids used in Principal Component Analysis and Metabolic Flux Analysis models of this study.

Supplemental 3.2: Complete list of reactions in Metabolic Flux Analysis model with complete flux table results and list of biomass values set for each strain.

Supplemental 3.3: Complete flux results for Flux Balance Analysis of biomass optimized growth of strains PA01, AA2, AA43, AA44, LES 400, AMT 0023-30, and AMT 0023-34 in aerobic and anoxic growth conditions. Flux Variability Analysis results for 99% and maximally optimized biomass flux rate are also shown. Additional modeling (e.g. lactate and leucine uptake) can also be found here. Alterations to the biomass equation and additional reactions added to Opperman et al. FBA model are also described.

Supplemental 3.4: Current version of formatted Flux Balance Analysis model used in this study (.xls format).



APPENDIX B - Additional Tables and Figures

Figure S3.1. Correlation of *Pseudomonas aeruginosa* **strain intrinsic growth rates under media and oxygen conditions.** Intrinsic growth rates of *P. aeruginosa* strains under aerobic (squares) and anoxic (triangles) conditions plotted SCFM intrinsic growth rates against Simplified-SCFM intrinsic growth rates. Correlation coefficients for both conditions show strong correlations, demonstrating the similar biomass growth rates and thus metabolic conditions in both media conditions.



Figure S3.2. Carbon conversation efficiency (CCE) calculation of *Pseudomonas aeruginosa* strains under aerobic growth conditions in Simplified-SCFM with 90% CI. Empty bars show values calculated from amino acid uptake and biomass production; Filled bars show values calculated from CO₂ efflux and biomass production. 2-sided student's t-test of CCE demonstrate no significant difference (P >0.05) for each strain. CCE% calculation based on amino acid uptake for PA01 and AA43 n=6; AA2 n=5; AA44, LES400, and AMT 0023-30 n=3; AMT 0023-34 n=3. CCE% calculation based on CO₂ efflux for PA01, AA44, AA43, and AMT 0023-34 n=6; all rest are n=3.



Figure S3.3. Nitrogen conversation (NC) percentage calculation of *Pseudomonas aeruginosa* strains under aerobic and anoxic growth conditions. Aerobic (filled) and anoxic growth (empty) conditions in Simplified-SCFM with standard deviation as error bars. NC% calculation based on aerobic growth for PA01 and AA43 n=6; AA2 n=5; AA44, LES400, and AMT 0023-30 n=3; AMT 0023-34 n=3. N=3 for all NCE% anoxic calculations. NCE% greater than 100% imply uptake of nitrogen from sources other than amino acids. NCE% less than 100% imply secretion of nitrogenous waste due to amino acid catabolism.

		Ala	Arg	Asp	Cys	Glu+Gln	Gly	His	lle	Leu	Lys	Met	Phe	Pro	Ser	Trp	Tyr	Val	Thr	Orn	Glucose	Lactate
	Average	46%	29%	-1%	103%	7%	42%	90%	59%	87%	86%	89%	89%	47%	54%	71%	85%	81%	68%	-1%	76%	24%
Pa01	Stdev	11%	4%	0%	24%	1%	19%	4%	2%	3%	2%	2%	4%	5%	11%	3%	3%	4%	10%	0%	65%	17%
	Average	19%	18%	-1%	90%	6%	46%	80%	50%	75%	76%	80%	57%	50%	45%	30%	66%	73%	55%	-1%	0%	11%
AA2	Stdev	5%	1%	0%	6%	0%	7%	1%	2%	6%	3%	3%	9%	5%	4%	7%	3%	3%	12%	0%	0%	2%
	Average	70%	36%	34%	102%	39%	62%	92%	64%	99%	91%	92%	99%	75%	63%	88%	87%	88%	65%	3%	51%	17%
AA43	Stdev	8%	5%	5%	71%	5%	13%	4%	1%	5%	3%	4%	2%	2%	10%	7%	4%	2%	15%	1%	26%	4%
	Average	17%	19%	10%	101%	20%	60%	88%	58%	88%	82%	89%	87%	34%	51%	69%	73%	81%	51%	1%	41%	12%
AA44	Stdev	12%	0%	10%	73%	12%	5%	4%	3%	7%	4%	5%	8%	23%	9%	5%	2%	3%	10%	1%	34%	3%
	Average	9%	18%	-1%	87%	6%	31%	78%	49%	70%	72%	78%	57%	21%	42%	51%	63%	67%	46%	-1%	17%	11%
LES400	Stdev	3%	1%	0%	46%	0%	8%	2%	2%	2%	3%	0%	5%	7%	1%	3%	1%	2%	1%	0%	15%	2%
	Average	21%	21%	-1%	50%	7%	92%	80%	85%	73%	74%	66%	76%	39%	103%	20%	61%	67%	81%	-1%	43%	12%
AMT30	Stdev	14%	6%	0%	13%	2%	68%	7%	54%	14%	9%	29%	7%	11%	77%	8%	20%	14%	46%	0%	23%	2%
	Average	55%	17%	-1%	59%	8%	-5%	9%	4%	-11%	8%	9%	2%	128%	255%	351%	12%	14%	10%	0%	0%	0%
AMT34	Stdev	11%	0%	0%	34%	0%	1%	0%	0%	0%	0%	0%	0%	11%	17%	9%	0%	1%	1%	0%	NA	NA

Table S3.1. Average and standard deviation of remaining carbon compounds during growth in Synthetic Cystic FibrosisMedia (SCFM) under aerobic conditions.Amino Acids measured by LCMS and remaining Glucose and Lactate measured by NMR.

-																						
		Ala	Arg	Asp	Cys	Glu+Gln	Gly	His	lle	Leu	Lys	Met	Phe	Pro	Ser	Trp	Tyr	Val	Thr	Orn	Glucose	Lactate
	Average	40%	44%	14%	98%	19%	ND	92%	91%	11%	79%	77%	13%	32%	92%	ND	ND	70%	8%	8%	123%	59%
Pa01	Stdev	4%	5%	0%	8%	0%	ND	6%	7%	3%	4%	3%	0%	8%	8%			25%	0%	0%	101%	22%
	Average	75%	58%	35%	78%	56%	ND	81%	83%	3%	85%	78%	54%	60%	77%	ND	ND	54%	40%	40%	58%	264%
AA2	Stdev	6%	2%	11%	24%	7%	ND	2%	3%	2%	3%	3%	6%	16%	1%			42%	11%	11%	33%	127%
	Average	80%	55%	60%	90%	69%	ND	87%	86%	2%	88%	85%	76%	64%	87%	ND	ND	81%	68%	68%	61%	156%
AA43	Stdev	7%	1%	1%	5%	5%		7%	6%	2%	5%	7%	7%	8%	7%			10%	7%	7%	29%	33%
	Average	72%	60%	57%	78%	66%	ND	85%	84%	5%	87%	80%	53%	70%	79%	ND	ND	62%	57%	57%	93%	158%
AA44	Stdev	7%	2%	7%	38%	5%		5%	5%	2%	5%	8%	4%	7%	8%			36%	12%	12%	77%	28%
	Average	77%	50%	13%	81%	24%	ND	85%	85%	5%	87%	79%	19%	61%	79%	ND	ND	112%	8%	8%	73%	137%
LES400	Stdev	18%	2%	10%	14%	15%	ND	9%	9%	3%	10%	6%	10%	13%	6%			40%	7%	7%	74%	24%
	Average	70%	65%	45%	94%	59%	ND	81%	81%	4%	83%	76%	52%	51%	77%	ND	ND	76%	47%	47%	115%	163%
AMT30	Stdev	3%	2%	4%	6%	3%		4%	5%	2%	5%	2%	3%	29%	5%			30%	2%	2%	99%	31%
	Average	54%	41%	27%	93%	31%	ND	94%	92%	12%	79%	80%	13%	56%	96%	ND		55%	29%	29%	56%	74%
AMT34	Stdev	22%	4%	6%	1%	11%	UND	8%	8%	4%	11%	8%	0%	12%	9%			48%	8%	8%	26%	21%

Table S3.2. Average and standard deviation of remaining carbon	compounds during growth in Synthetic Cystic Fibrosis
Media (SCFM) under Anoxic conditions. Amino Acids measured b	y LCMS and remaining Glucose and Lactate measured by NMR.

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CHAPTER 4 –

Conclusions and Future Directions

The work described in this thesis was concerned with the metabolic changes during adaptation to the cystic fibrosis lung environment and pathogenesis by the bacterium *Pseudomonas aeruginosa* in chronic infection. The research used Metabolic Flux Analysis and Flux Balance Analysis to quantify central carbon metabolism in CF isolates of *P. aeruginosa* to add to the understanding of the bacterium's metabolism and the changes induced by exposure to and growth in the cystic fibrosis lung environment. Due to the significance of *P. aeruginosa* as a cause of morbidity and mortality in cystic fibrosis (CF) and its intrinsic and acquired resistance against antibiotic therapy, the additional goal of this study was to propose possible metabolic targets for potential future pharmaceutical development.

Comparison of Metabolism

A central question of this thesis is whether metabolic differences can be determined in the role of adaptation towards chronic infection of lung, with the comparison of *P. aeruginosa* CF lung isolates to a wound isolate as well as to other published studies. The results show the extensive metabolic range of *P. aeruginosa*, both in the range of flux patterns though carbon metabolism under different conditions as well as in the carbon metabolic phenotypes present in genetically similar and diverse strains. Both of the studies in this dissertation also help to contextualize the metabolism of *P. aeruginosa* in comparison to other ¹³C MFA organisms under aerobic conditions with glucose as the carbon source and under conditions mimicking the CF lung.

The minimal media ¹³C MFA study describes a standardized growth condition that can be used to identify and quantify differences in the growth physiology and metabolism between bacterial species. The results in this study also contribute to a comparison of metabolism based on metabolism of glucose through the Embden-Meyerhof-Parnas Pathway (EMP) versus the Entner-Doudoroff Pathway (EDP). While theories of reduced protein production cost and energy yield as explanations of the loss of EMP with conservation and utilization of EDP in some organisms have been demonstrated mathematically, direct demonstration in organisms have not been shown [1]. The measured utilization rates of the EDP in several of the *P. aeruginosa* strains (especially AA44) and *E. coli* utilization of EMP during aerobic growth in minimal media demonstrate similar rates of flux ratios through these pathways with increased growth rates in *P. aeruginosa* [2]. The study of aerobic metabolism during growth on glucose also allowed the direct comparison of a related species *P. fluorescens* to *P. aeruginosa*. The findings demonstrated not only the shared metabolic phenotypes in these species, but also provide evidence that one of the minimal media growth phenotypes in this study may be demonstrating a proto-biofilm synthesis metabolism [3].

The S-SCFM ¹³C MFA study allowed a detailed elucidation of the carbon fluxes through central metabolism in *P. aeruginosa* under more CF lung-like conditions. The metabolic findings of the study can be compared and contrasted to the proteomic work by Hoboth et al. (2009). That group's findings of increased levels of Tricarboxylic acid (TCA) cycle enzymes and of proteins responsible for the interconversion of ornithine, arginine, and glutamate and TCA cycle intermediates in late CF isolates in comparison to early CF isolates are consistent with the large carbon fluxes in the S-SCFM ¹³C MFA flux maps obtained here [4]. But, ¹³C MFA quantification of carbon fluxes here demonstrated that the utilization of amino acids by these strains under both aerobic and anoxic conditions drives carbon flux from glutamate to ornithine to succinate, which is opposite to the conclusions of the prior Omic study [4]. The additional analysis of the anoxic growth condition to better understand differences in aerobic and anoxic growth in the presence of substrates present in the CF lung also provided insight into the

utilization of amino acids and the role of the glyoxylate cycle enzyme isocitrate lyase under anoxic growth. The analyses in lung-like media under both aerobic and anoxic conditions highlight the value of studying metabolic phenotypes under experimental conditions that mimic relevant environmental circumstances. Indeed there is evidence that the CF lung includes a range of microenvironments from aerobic to anoxic [5–7].

Metabolic Fingerprinting and Metabolic Flux Analysis

Metabolic Fingerprinting using stable isotopic labeling is a method that has been proposed to increase the speed and throughput of metabolic phenotyping by identifying metabolic fluxes without full flux analysis [8,9]. Metabolic flux fingerprinting involves using LC-MS, GC-MS, and/or NMR to produce a set of labeling data on the system of interest and applying statistical methods (Analysis of Variance, ANOVA; Principal Component Analysis, PCA; or Independent Component Analysis, ICA) to identify differences and/or degrees of difference between treatments or genotypes. Features revealed may either describe the system's metabolic features or identify specific measures that could be used for identification of a specific organism [10]. A proposed refinement of this method is the use of stable isotopic labeling to produce labeled metabolites that capture the underlying metabolic network that produced the measured metabolites [11]. This process is meant to identify metabolic similarities and differences more efficiently (days of analysis) than using ¹³C MFA (months of modeling).

We investigated the power of this approach to discriminate between metabolic flux phenotypes exhibited by different strains and whether degrees of similarity among strains identified by fingerprinting agreed with the results of full flux analysis. The results of these studies cast doubt on the ability of ¹³C metabolic fingerprinting to differentiate reliably between strains of the same species. Thus we found that HCA using fluxes obtained by ¹³C MFA was able

to clearly distinguish among *P. aeruginosa* strains grown anoxically in S-SCFM; whereas the ¹³C labeling data used in the MFA was insufficient to distinguish the strains using PCA. Under aerobic growth (either in minimal or S-SCFM media) ¹³C metabolic fingerprinting was able to identify that each strain was different from the others, but the overall degree of difference between one strain and another, did not accord with the degrees of difference/relatedness shown by HCA of ¹³C MFA flux analyses.

Because of the time-consuming nature of rigorous ¹³C MFA and the value of increasing throughput, further investigation should be conducted to determine which if any statistical tools can be used to determine metabolic differences among strains of the same species. It may be that specific labeling information (e.g. increased measurements of labeling in particular metabolites), and/or different statistical methods (e.g. Independent Component Analysis) may increase the predictability of these analyses. It seems likely that progress in this direction will be easier on a case-by-case basis for specific metabolic processes – that is using a particular labeled substrate to discriminate particular fluxes, and that developing profiling methods for system-wide untargeted differences will be harder, especially where multiple substrates are utilized simultaneously.

Virulence and Metabolism

A central question motivating the work of this thesis was "Can metabolism and its changes be meaningfully related to an organism's adaptation towards greater virulence?" By quantifying metabolic changes in strains that have adapted to a pathogenic environment, these changes could be general to organism adaptation in certain body environments. Such differences could provide a basis for the production of tests to determine the course of chronic infections, and investigated as potential targets to reduce or eliminate infections. Presented below is a summary of the metabolic findings of this thesis, which could be potential targets for pharmaceutical intervention.

The glyoxylate cycle has been shown to be an important flux in both minimal media and CF lung-like growth of *P. aeruginosa*. With the use of FBA in these studies, it has been shown that optimized biomass production (growth) in P. aeruginosa does not explain these fluxes. The observation of a correlation between growth rate and glyoxylate cycle flux observed here, is consistent with the results of a previous study that demonstrated reduced virulence in both alfalfa seeds and mouse lung by *P. aeruginosa* in a strain with defective isocitrate lyase [12]. The finding of a functional glyoxylate cycle as being an important metabolic pathway for persistence in the body in pathogenic organisms (especially Mycobacterium tuberculosis) has been noted in the scientific literature and in the context of gluconeogenesis during lipid metabolism [13]. This utilization of the glyoxylate cycle by P. aeruginosa during non-lipid metabolism and especially anoxic amino acid metabolism is intriguing, as the generally accepted role of this cycle is for anabolism from two carbon acetyl-CoA units derived from lipid catabolism. The utilization of this cycle by *P. aeruginosa* strains under the conditions used here could potentially be an inherent signaling response that may be expressed at higher levels under anoxic conditions, but more direct measures (such as transcriptomics and metabolic analyses of mutant and transgenic strains) would need to be conducted to further elucidate the interplay between environment and glyoxylate cycle expression. The results of this study also demonstrate that isocitrate lyase and 2hydroxy-3-oxopropionate reductase, operating without a complete glyoxylate cycle, is heavily utilized during anoxic S-SCFM growth in *P. aeruginosa* and could possibly be pharmaceutical targets to reduce or eliminate this organism from the CF lung environment.
The catabolism of the amino acids glutamate, ornithine, and arginine via putrescine into the TCA cycle and not via α-ketoglutarate is a novel finding. As shown by FBA growth optimization, putrescine production is less efficient for growth, likely due to the increased ATP consumption by this pathway. A possible explanation of this pathway, though kinetic data for these enzymes would be beneficial in determining the possibility of accumulation, in is the utilization of polyamines, such as putrescine, in *P. aeruginosa* as a general cellular and plasma membrane anti-oxidant [14]. The expression of polyamines, especially putrescine, in the cell membrane surface of *P. aeruginosa* has been demonstrated in the context of antimicrobial stress (e.g. extracellular DNA) [15]. A polyamine metabolite derived from arginine decarboxylation, agmatine, has also been demonstrated as a signaling molecule in biofilm formation that develops in some *P. aeruginosa* isolates, providing additional evidence of an adaptation of metabolism towards signaling of immune stress in this organism [16]. The discovery in this study of substantial fluxes via putrescine also provide a possible explanation for the observed correlation between CF exacerbations and increases in putrescine concentration from sputum samples [17].

These studies also provide evidence of metabolism of *P. aeruginosa* towards production of reductant at the expense of biomass synthesis. ¹³C MFA of strains growing in Minimal media demonstrated a metabolic phenotype that differed from the one predicted by FBA for optimized biomass production. A key difference is towards the overproduction of reductant that is seen in the increased OPPP flux through relative to fluxes through the ED pathway and TCA cycle. This overproduction of reductant in comparison to optimized biomass production was further accentuated in S-SCFM growth as seen in the calculation of reductant produced in ¹³C MFA constrained fluxes of FBA optimized growth compared to FBA optimized growth. A possible

explanation for this finding is that *P. aeruginosa* uses high rates of NADPH production to survive in the oxidative environment of the CF lung or in other hostile environments.

Future Directions

To better understand the metabolic adaptation of *P. aeruginosa* to the CF lung environment, a more life-like replication of the CF lung environment would be useful. ¹³C-label experiments would ideally be conducted in an animal model infected with P. aeruginosa, providing the most accurate in vivo study of metabolism and infection. Unfortunately, such an experiment would be both expensive (in ¹³C substrates), and difficult to interpret, since measuring uptake rates and accounting for metabolic transformations outside the P. aeruginosa cells would be invasive and extremely challenging. Additionally, while the genetic cause of CF has been well established, the underlying pathology has not been well replicated in organ systems of any model animal used [18]. Under these circumstances, it is more reasonable to propose the use of biofilm cultivation techniques under lung-like conditions as the next step in experimental techniques [19]. Using such a system to produce biofilm under more lung-like conditions would complicate the ability to perform steady state MFA due to the timescales necessary for complete isotopic labeling of analytic substrate as well as the heterogeneous environment of the biofilm [20]. While isotopically non-stationary MFA techniques have been established that would allow for such a biofilm study to be feasible [21], technical obstacles remain. These include designing and verifying a suitable biofilm growth chamber; methodological development of consistent growth of *P. aeruginosa* biofilm cultures; identification, harvesting and isolation of biofilm layer(s) of phenotypic difference; and measurement of uptake and secretion rates of metabolites. However, such an investigation should be feasible and represents a logical next step in this research.

Besides discussion of the natural progression of experiments conducted in this thesis, other experiments could be conducted to verify and explore the conclusions reported. Growth of the strains in these experiments to measure and quantify biofilm differences in the media used in these experiments could be completed. These experiments could be used to confirm whether probiofilm growth phenotypes are expressed under planktonic growth. Measurement of biofilm production rates and type of biofilm for each strain could then be compared to the identified biofilm and non-biofilm metabolic phenotypes demonstrated under M9 growth to identify if MFA determined phenotypes under planktonic growth do correlate with alginate biofilm producers. Biofilm production difference by strains grown in S-SCFM may provide additional insight into the metabolic differences seen as well. Additionally, all the metabolic results found could be further augmented with measurement of known virulence factors, quorum signaling molecules, and transcriptomic measurements. These measurements may provide finer quantification of pathogenicity or virulence under these growth conditions that may provide a contrastable difference of virulence that may explain the underlying metabolic phenotypes reveled in these studies.

Additional ¹³C MFA experiments could also be conducted using a lipid source instead of amino acid sources of carbon. These result could help to shed light on the differences in lipid and amino acid metabolism of these CF isolates and may help in determining possible metabolic utilization differences that may occur is some strains. Measurement of secondary metabolites (known virulence factors, quorum signaling molecules, etc.) could also be used to further constrain metabolic measurements in FBA constrained MFA that may lead to additional discoveries about *P. aeruginosa* metabolism.

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Additional strains of *P. aeruginosa* could be analyzed using ¹³C MFA to further validate and identify additional metabolic phenotypes. As demonstrated in this work, the reference strain PA01 did not show significant difference from the CF isolates used in this study. The use of environmental strains, as opposed to pathogenic strains like PA01, as the metabolic reference may help in the understanding of pathogenic adaptation seen in this organism. Other CF isolates could also be included to further encompass identified phenotypes beyond the genetically sequential isolates chosen for this study.

Finally, identification and utilization of inhibitors of key enzymatic pathways, or of *P*. *aeruginosa* mutants defective in these pathways, could be used to validate the results and proposed mechanisms of this thesis. Inhibition of the enzyme of the Entner-Doudoroff pathway would validate the ability of *P. aeruginosa* to continue to grow under minimal and complex media. Use of an inhibitor or known mutants in polyamine metabolism would be of additional interest, and would help in the illumination of polyamine metabolism and biofilm formation [22]. These experiments would also need to be paired with biofilm growth experiments to fully validate the relationship between polyamines and biofilm formation. REFERENCES

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