UTILIZATION OF PHOSPHATIDYLCHOLINE, A LUNG SURFACTANT COMPONENT, AS A MAJOR NURIENT SOURCE DURING Pseudomonas aeruginosa LUNG INFECTION

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By

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Abstract

*Pseudomonas aeruginosa* can grow to high-cell-density (HCD) during infection of the cystic fibrosis (CF) lung. Phosphatidylcholine (PC), the major component of lung surfactant, has been hypothesized to support HCD growth of *P. aeruginosa in vivo*. Three different pathways, the betaine, glycerol and fatty acid degradation (Fad) pathways, are involved in the degradation of PC components including a phosphorylcholine headgroup, a glycerol molecule, and two long-chain fatty acids (FAs).

The Fad pathway still remains largely uncharacterized in *P. aeruginosa*. During the course of this work, *fadBA1,4,5* operons (3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase) were shown to be the most important operons involved in fatty acid degradation through mutational analysis. Various *fad* mutants and the triple pathway mutant were analyzed extensively by *in vitro* growth analysis, virulence characterization, and competition study. Defect of growth on PC as sole carbon source was most significant on the triple pathway mutants, as expected. This growth defect translated to *in vivo* competition disadvantage in BALB/c mice, suggesting the importance of PC as nutrient source *in vivo*. 
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My greatest appreciation goes to my friends who were always outstanding supporters through my struggles and frustrations in my new life and studies in this country.

I would like to thank my family, especially my parents, for always believing in me, for their love and support in my decisions, and without whom I could not have made it here.
Dedication

This work is dedicated to
my parents
Guoming Sun and Peishuang Wu
# Table of Contents

Abstract ........................................................................................................................................... i
Acknowledgements ......................................................................................................................... ii
Dedication .......................................................................................................................................... iii
List of Figures ................................................................................................................................... v
List of Tables ..................................................................................................................................... vi
List of Abbreviations ....................................................................................................................... vii

Chapter 1. Introduction .................................................................................................................... 1
1.1 *Pseudomonas aeruginosa* ........................................................................................................ 1
1.2 *P. aeruginosa* virulence factors ............................................................................................. 2
1.3 *P. aeruginosa* infections and antimicrobial resistance .......................................................... 7
1.4 *P. aeruginosa* and Cystic Fibrosis ......................................................................................... 10
1.5 Nutrient acquisition during Cystic Fibrosis lung infection ..................................................... 11
1.6 Specific aims ............................................................................................................................. 14

Chapter 2: Materials and Methods .................................................................................................. 15
2.1 Bacterial strains and growth conditions ................................................................................... 15
2.2 General molecular methods ..................................................................................................... 15
2.3 Genetic techniques .................................................................................................................. 18
2.4 Growth characterization of mutants and complementation strains ....................................... 19
2.5 Virulence factors detection ...................................................................................................... 20
2.6 *In vitro* and *in vivo* competition studies ........................................................................... 21

Chapter 3. Characterization of *fadBA*-operons Homologues in *P. aeruginosa* Fatty Acid Degradation Pathway ..................................................................................................................... 24
3.1 Introduction .............................................................................................................................. 24
3.2 Results ....................................................................................................................................... 25

Chapter 4. Characterization of PC Degradation in *P. aeruginosa* ............................................... 30
4.1 Introduction .............................................................................................................................. 30
4.2 The triple pathway mutant experiences dramatic inability to utilize PC *in vitro* .................... 31
4.3 Virulence factors production ................................................................................................... 31
4.4 *P. aeruginosa* triple pathway mutant exhibit significantly decreased fitness *in vitro* .......... 32
4.5 *P. aeruginosa* triple pathway mutant exhibit significantly decreased fitness *in vivo* .......... 33

Chapter 5. Discussion ...................................................................................................................... 35
Tables ................................................................................................................................................. 41
Figures ............................................................................................................................................... 45
Reference Cited ................................................................................................................................. 60
List of Figures

Figure 1. Phosphatidycholine (PC) degradation pathways in P. aeruginosa (A), and the proposed FA degradation pathway in P. aeruginosa based on E. coli β-oxidation (B)…………………………………………………………………….46

Figure 2. Five potential fadBA-operon homologues of P. aeruginosa………………49

Figure 3. Comparison of P. aeruginosa FadBAs with E. coli FadBA……………….50

Figure 4. PCR confirmation of fadBA mutant strains……………………………….51

Figure 5. PCR verification of the mutant strains at the end of growth analysis………52

Figure 6. Growth analysis of different fadBA mutants on medium and long chain-length fatty acid (C_{12:0}, C_{14:0}, C_{16:0} and C_{18:1}^{\Delta9})…………………………………….53

Figure 7. Growth analysis of ΔfadBA1,4,5 mutant on PC as a sole carbon source……54

Figure 8. Growth analysis of various pathway mutants on PC……………………….55

Figure 9. Analyses of protease, hemolysin, lipase, and rhamnolipid production by P. aeruginosa various pathway mutants………………………………………………..56

Figure 10. Detection of virulence factors produced by P. aeruginosa……………..58

Figure 11. In vitro competition studies of the various mutants and their complemented strains in different growth media…………………………………………………59

Figure 12. In vivo competition study of the various mutants and their complemented strains………………………………………………………………………………60
List of Tables

Table 1. Bacterial strains used in this study……………………………………..42
Table 2. Plasmids used in this study…………………………………………………44
Table 3. Primers used in this study…………………………………………………..45
List of Abbreviations

aa  amino acid/amino acids
ABC  ATP-bing cassette
ADPRT  adenosine diphosphate ribosyltransferase
AHL  N-acylated homoserine lactone
AMP  adenosine monophosphate
Ap  ampicillin
AP  apical surface
Ap'  ampicillin resistant/resistance
ATP  adenosine triphosphate
BL  basolateral
BLAST  basic local alignment search tool
bp  base pair
BSA  bovine serum albumin
CAA  casamino acids
Cb  carbenicillin
CIP  calf intestinal alkaline phosphatase
CF  cystic fibrosis
CFTR  cystic fibrosis transmembrane conductance regulator gene
CFU  colony forming unit
CI  competitive index
CoASH  coenzyme A
COX  cyclooxygenase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>DAG</td>
<td>diacyl-glycerol</td>
</tr>
<tr>
<td>DAP</td>
<td>diaminopimelic acid</td>
</tr>
<tr>
<td>DDW</td>
<td>double deionized water</td>
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<tr>
<td>DMG</td>
<td>dimethylglycine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Ec</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substance (EPS)</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FACS</td>
<td>fatty acyl-CoA synthetase</td>
</tr>
<tr>
<td>Fad</td>
<td>fatty acid degradation</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH(_2)</td>
<td>reduced flavin adenine dinucleotide</td>
</tr>
<tr>
<td>flp</td>
<td>gene encoding <em>Saccharomyces cerevisiae</em> recombinase</td>
</tr>
<tr>
<td>FRT</td>
<td>Flp recognition target</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravitational force</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating</td>
</tr>
<tr>
<td>Gm</td>
<td>gentamicin</td>
</tr>
<tr>
<td>Gm(^r)</td>
<td>gentamicin resistant/resistance</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
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</table>
HCD  high cell density
HS   heparan sulfate
HSPGs heparan sulfate proteoglycans
IPTG isopropyl β-D-1-thiogalactopyranoside
k    kilo
kb   kilobase
$k_{\text{cat}}$ enzyme catalytic efficiency
kDa  kilodalton
Km   Michaelis constant
Km   kanamycin
Km'  kanamycin resistant/resistance
l    liter
LB   Luria-Bertani
LCFA long chain fatty acid
LPS  lipopolysaccharide
M    molar
Mex  multidrug efflux
Km   kanamycin
m    milli
Mb   mega base pair (s)
MCFA medium chain fatty acid
m    milli
mg   milligram (s)
min minute
ml milliliter(s)
mM millimolar
MW molecular weight
NAD+ nicotinamide adenine dinucleotide
n nano
NADH reduced nicotinamide adenine dinucleotide
NB nutrient broth
nmol nanomole
OD optical density
ONPG 2-nitrophenyl-β-D-galactopyranoside
ORF open reading frame
oriT origin of transfer for conjugation
PC phosphatidylcholine
PCR polymerase chain reaction
PIA Pseudomonas isolation agar
PIB Pseudomonas isolation broth
PLC Phospholipase C
Pa Pseudomoans aeruginosa
PP_i pyrophosphate
QS quorum sensing
s second
sacB Bacillus subtilis gene encoding levansucrase
<table>
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<th>Definition</th>
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<tr>
<td>SCFA</td>
<td>short chain fatty acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Sp</td>
<td>streptomycin</td>
</tr>
<tr>
<td>Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>streptomycin resistant/resistance</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base- acetate EDTA buffer</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>Tet</td>
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<td>Tet&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
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<td>µ</td>
<td>micro</td>
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<td>µg</td>
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<td>microliter (s)</td>
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<td>µM</td>
<td>micromolar</td>
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<td>U</td>
<td>unit</td>
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<td>UV</td>
<td>ultraviolet</td>
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<td>v</td>
<td>volume</td>
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<td>w</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
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Chapter 1. Introduction

1.1 Pseudomonas aeruginosa

*Pseudomonas aeruginosa* is a gram-negative, rod-shaped, and monoflagellated gamma proteobacteria that has an incredible nutritional versatility. It is a rod ranging from 1-3 µm in length and 0.5-1.0 µm in width. *P. aeruginosa* produces two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. Pyocyanin is produced abundantly in low-iron content media and has an important function in iron metabolism for this bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus" [1,2], which is a characteristic of infections caused by *Pseudomonas aeruginosa*.

*P. aeruginosa* has an incredible metabolic versatility and can metabolize over seventy organic substances as nutrient and energy sources, e.g., citrate, ribose, choline, glycerol, and fatty acids [3,4]. It has the ability to thrive in most natural and man-made environments, such as soil, water, plants and animals, and infect multiple hosts [5,6]. It comes as no surprise that it occupies a wide range of environmental niches, considering its genome (6.3Mb) [7] is larger than that of *Haemophilus influenzae* (1.8 Mb) [8], *Bacillus subtilis* (4.2 Mb) [9], *Mycobacterium tuberculosis* (4.4 Mb) [10] and *E. coli* (4.6Mb) [11], with hundreds of genes encoding for transport and metabolism of various nutrients [7]. The species has adapted to a broad range of hosts, including humans [12], other animals (e.g., *Caenorhabditis elegans* [13], fruit flies [14], zebrafish [15], mink and chinchillas [16-19]), plants (e.g., lettuce and thale cress [19,20]) and microbes (e.g., amoeba [21]) in which it cause diseases. The role of *P. aeruginosa* as an opportunistic
human pathogen is of particular concern, especially because it is a frequent cause of
nosocomial infections such as pneumonia, urinary tract infections, and bacteremia
[2,22,23]. *P. aeruginosa* infection in the respiratory tract of cystic fibrosis (CF) patients
causes a rapid deterioration in lung function and thus patient survival [24,25]. The
pathogenicity of *P. aeruginosa* infection in CF patients has been extensively studied in
terms of biofilm production [26-29] and quorum sensing (QS) [30-32]. However, little
effort has focused on understanding the contribution of *P. aeruginosa* nutrient acquisition
in its ability to replicate to high cell density during lung infection.

### 1.2 *P. aeruginosa* virulence factors

The virulence of a microorganism is defined by its ability to associate itself with a
prospective host, to invade and multiply within that host, to produce local and/or
systemic disease, and ultimately to overwhelm and even kill the host [33]. Virulence
factors refer to bacterial products or strategies that contribute to the capability of the
bacterium to cause infection. *P. aeruginosa* produces a large variety of virulence factors
which make it a successful opportunistic pathogen in a range of host tissues and organs,
and *P. aeruginosa* infections are difficult to treat [2]. The most notable virulence factors
include pili, flagella, exotoxins, biofilm, lipopolysaccharide (LPS), protease and
exoenzymes secreted by the Type III secretion system [34]. The N-acylated homoserine
lactone (AHL) quorum sensing (QS) regulatory system is one of the key mechanisms
used by *P. aeruginosa* to control the expression of many virulence factors (e.g. elastases,
proteases, pyocyanin, exotoxin A, and biofilm development). To ensure that host immune
response will not be able to destroy the whole bacterial population, virulence genes are
expressed under the control of QS when *P. aeruginosa* grows to high-cell density [2,35,36].

Cystic fibrosis (CF) patients frequently suffer from chronic *P. aeruginosa* pulmonary infections [37,38]. These pulmonary infections are thought to be initiated by attachment to and subsequent colonization of the mucosal epithelium of the upper respiratory tract by *P. aeruginosa*. At least three separate adhesins (pili, exo-S and alginate), are used by this bacterium to bind to a patient’s respiratory epithelial cells [39]. Pili and exo-S have the highest apparent affinity constants for respiratory epithelial cells, appearing to be responsible for initiating *P. aeruginosa* colonization [39]. Alginate is the lowest affinity adhesin. When there are significantly larger populations of *P. aeruginosa* in the airway, alginate likely contributes significantly to colonization of the lower airway [39]. Iwona et al.[40] demonstrated that Type IV pili (Tfp) are necessary and sufficient for maximal binding to host N-glycans [41] at the apical (AP) surface of polarized epithelium, while flagella are necessary and sufficient for maximal binding to heparan sulfate (HS) chains of heparan sulfate proteoglycans (HSPGs) [41] at the basolateral (BL) surface.

*P. aeruginosa* injects one set of toxins, known as effector proteins, directly into host cells through a macromolecular syringe called a Type III secretion system (T3SS). T3SS has been shown to be a major reason for the cytotoxicity of *P. aeruginosa* for a wide range of host cells [42]. This system consists of components of a secretory apparatus and a translocon, and factors that regulate secretion [42,43]. Effectors thought to alter normal host cell processes are exported from across the bacterial cell envelope by the secretion apparatus, and injected into the host cell cytoplasm. Four effector proteins have been identified in *P. aeruginosa*: ExoS, ExoT, ExoU and ExoY; the first three have been
closely linked to virulence [42,44,45]. ExoS and ExoT are closely related bifunctional toxins sharing 76% amino acid identity with an N-terminal GTPase-activating (GAP) domain and C-terminal adenosine diphosphate ribosyltransferase (ADPRT) domain [44,45]. Targeted host cells result in actin cytoskeleton disruption, apoptotic-like cell death and inhibition of DNA synthesis, endocytosis and vesicular trafficking [44]. The exoS gene is found in approximately 70% of clinical isolates, while the exoT gene is present in all P.aeruginosa strains [45]. ExoU is the most virulent effector when comparing the relative contributions of the effectors to mortality, persistence of the bacteria in the lung, and bacterial dissemination in the animal model infection [42,44,45]. ExoU is a potent cytotoxin that encodes phospholipase A2 activity, which leads to rapid cell death by directly damaging the plasma membrane. Its phospholipase activity may also lead to excessive inflammation, increased tissue damage, and bacterial dissemination by producing large amounts of arachidonic acid, which serves as a substrate for the cyclooxygenase (COX) and lipoxygenase pathways [42,46]. The role of ExoY remains unclear. But this adenylate cyclase was shown to be toxic in yeast cells [47] and induced cell rounding in eukaryotic immune cells [48]. Hritonenko et al. [49] showed that the adenylate cyclase domain of ExoY could mediate “bleb-niche” formation in epithelial cells resembling “bleb-niched” formation by ExoS. ExsA, a transcriptional activator that binds to an ExsA consensus element in the promoter of T3SS genes and to its own promoter, and thus regulates transcription of the T3SS genes [44,45]. Three proteins, ExsC, ExsD, ExsE, are involved in the complex upstream regulatory network to ensure that ExsA is only available when secretion activity is ongoing [40,45].
Proteases synthesized and secreted by *P. aeruginosa* result in tissue damage with extensive degradation of basement membrane constituents, and modifications of the cellular and humoral immune response [39]. *Pseudomonas* elastase (PE) and an alkaline protease (PAP) are major potent proteases that have been isolated, characterized and extensively studied [50-52]. Both PAP and PE are synthesized and secreted at the same point in the *P. aeruginosa* growth cycle, i.e., late exponential or early stationary phase, but the extracellular release mechanism of these two proteases is independent [39]. It has been shown that proteases are released extracellularly *in vivo*. Two particular observations prove this. First, the proteases have been detected in the sputum of many cystic fibrosis (CF) patients who suffer from chronic *P. aeruginosa* infections of the respiratory tract [50]. Second, antibodies to the proteases in *P. aeruginosa* were detected in infected patients [53]. Protease degradations of many extracellular matrix component constituents of connective tissues, including elastin, collagen types III and IV, and laminin are cytotoxic [54-56].

*P. aeruginosa* produces two hemolysins, a heat stable glycolipid, and a heat-labile lecithinase (Phospholipase C or PLC) [39]. PLCs are phosphodiesterases that hydrolyze the phosphodiester bond between glycerol and phosphate in glycerophospholipids, yielding diacyl-glycerol (DAG) and a phosphorylbase, e.g., phosphorycholine, or phosphorylinositol [57]. Two studies indicate that PLC is produced during *P. aeruginosa* infection. Hamood *et al.* (1996) showed that a high level of PLC is produced by most *P. aeruginosa* isolates from infection sites in the trachea, urinary tract, and wounds [58]. Phosphorylcholine or its derivatives, along with oxygen, are the two factors required for PLC production [59]. In human lungs, both factors are found in relative abundance. It is
well known that *P. aeruginosa* is the major pathogen in CF lungs, where thick, dehydrated mucus inhibits clearance of bacteria. CF patients are colonized by *P. aeruginosa* at an early age, and periodically experience acute pulmonary infections by this species [59]. A previous serum antibodies study in CF patients chronically colonized with *P. aeruginosa* showed that all had elevated antibody titers to PLC [60].

Diacylglycerol is a product of PLC hydrolysis of lung surfactant, and plays an important role as a second messenger in eukaryotic cell regulation through protein kinase C dependent and independent pathways [61]. Arachidonic acid is released when diacylglycerol is cleaved by endogenous or bacterial lipases to act as the precursor of a variety of inflammatory mediators [62].

Rhamnolipids, another virulence factor, are produced and secreted by *P. aeruginosa*; they are rhamnose-containing glycolipid biosurfactants [63,64]. The study from Zulianello *et al.* (2006) showed that rhamnolipids promote early infiltration of primary human airway epithelia by *P. aeruginosa* via the mechanism that alters the tight-junction by incorporation of secreted bacterial rhamnolipids within the host cell membrane [64]. The biosynthesis of rhamnolipid in *P. aeruginosa* is regulated by QS systems in a cell-density-dependent manner [31]. Two autoinducer (N-acylated homoserine lactones, HSL) mediated transcriptional regulatory circuits, Las and Rhl, have been characterized [64]. Once a sufficient amount of autoinducer molecules have accumulated, they bind to their cognate transcriptional activators LasR and RhlR. N- (3-oxododecanoyl)-HSL is synthesized by the product of the *lasI* gene, and N-butyryl-HSL is produced by RhII. LasR bound with N- (3-oxododecanoyl)-HSL regulates the transcription of several
virulence genes, including *lasA*, *lasB*, and *toxA*. RhlR bound with N-butyryl-HSL enhances transcription of *lasB* and the rhamnolipid synthesis genes *rhlAB* [31,32,64].

Biofilms are known as “bacterial cities”, highly organized, microbial communities embedded in a matrix and attached to a surface [42]. The microbial cells account for less than 10% of the dry mass, whereas the matrix can account for over 90% in most biofilms [65]. The matrix is extracellular material known as extracellular polymeric substances (EPS). EPS mainly contains extracellular polysaccharides, but also proteins, nucleic acids, lipids and other biopolymers such as humic substances [65]. It has been reported that the matrix protects organisms against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations, ultraviolet radiation, many (but not all) protozoan grazers and host immune defenses [65]. Biofilms become a serious medical problem when attached to a surgical implant, endotracheal tube, catheter, or the airways of individuals with CF. *P. aeruginosa* causes a chronic infection in the Cystic Fibrosis (CF) lungs by establishing an alginate-containing biofilm [66]. A stable mucoid CF sputum isolate with hyper-production of alginate due to a deletion in *mucA* was used to establish a chronic lung infection in BALB/c mice, providing an improved method for evaluating the interaction between bacteria, host and antibacterial therapy [66]. MucA encodes an anti-sigma factor of AlgU required for expression of the alginate biosynthetic operon, leading to a mucoid phenotype [66]. *P. aeruginosa* biofilms have become an obvious target for efforts aimed at therapeutic interventions in terms of the importance in pathogenesis [42].

1.3 *P. aeruginosa* infections and antimicrobial resistance
P. aeruginosa rarely causes infection in healthy people, who often carry these bacteria without problems. Serious infections commonly occur in hospital patients, and immune-compromised individuals. P. aeruginosa is the leading problem for nosocomial infections, accounting for ~12% of the reported infections. Specifically, it has been reported that this microbe causes ~10% of urinary tract infections in patients with indwelling catheters, ~9% of surgical wound infections, ~17% of lower respiratory tract infections with a tube placed in their airway to support breathing (ventilator patients), and ~11% of bacteremia in surgical patients [12]. In hospitals, the bacteria can easily spread through medical equipment that has not been properly cleaned, on the hands of healthcare workers and even in food. P. aeruginosa is well known for its ability to infect burns, and it is the main cause of death in burn patients who survive the initial burn trauma. These patients may also acquire lung infections due to inhaling fire-heated air [67]. This microbe is also a concern for people who wear contact lenses, because it can infect small scratches on the cornea, caused by improper use of contact lenses. The resulting infections can become serious if the patients are not treated promptly and effectively [67].

P. aeruginosa is a key problem in CF lung infections, with infection of the respiratory tract causing a rapid deterioration in lung function and patient survival [24,25].

What makes these infections particularly troublesome is that P. aeruginosa exhibits innate resistance to multiple antimicrobial agents. The synergy between a low-permeability of the outer membrane (1/100 of the permeability of E. coli outer membrane[68]) and expression of a number of broadly specific multidrug efflux (Mex) systems (MexAB-OprM and MexXY-OprM participate simultaneously in nature; MexCD-OprJ, MexEF-OprN and MexJK-OprM act only in acquired resistance [69,70]).
causes this intrinsic multidrug resistance [71]. Furthermore, the chromosomally encoded AmpC cephalosporinase provides impressive resistance to β-lactams [72].

β-lactams, quinolones, aminoglycosides, and polymixins are antibiotics that can be used against *P. aeruginosa*. β-lactams prevent cross-linking of peptidoglycan to stop cell-wall synthesis, thus inhibiting bacterial growth [67]. Several β-lactams are available, such as carboxypenicillins (carbenicillin, ticarcillin), ureidopenicillins (azlocillin, piperacillin), some third generation cephalosporins (ceftazidime, cefsulodine, cefoperazone), all the fourth generation cephalosporins, the monobactam aztreonam, and the carbapenems imipenem and meropenem [73]. Aminoglycosides inhibit protein synthesis by binding irreversibly to the 30S ribosome subunit, thus preventing bacterial growth. The effective aminoglycosides include gentamycin, tobramycin, isepamicin, and amikacin [74]. Quinolones, ciprofloxacin and norfloxacin, interfere with DNA replication and transcription by inhibiting bacterial DNA gyrase that is responsible for the unwinding of DNA. [75]. Colistin (polymyxin E) and polymyxin B are effective antipseudomonal agents used against *P. aeruginosa* that are highly resistant to other drugs. These drugs disrupt the bacterial cell membrane[36]. Although many antibiotics could be used against *P. aeruginosa*, it is still a very challenging fight against the multi-drug resistant and highly resistant strains. Those strains undergo mechanisms of developing antimicrobial resistance by diminishing this outer membrane permeability; overexpressing multidrug efflux systems with wide substrate profiles; de-repressing chromosomal AmpC cephalosporinase; producing plasmid or integrin-mediated β-lactamases; producing aminoglycoside-modifying enzymes, and structural alteration of gyrases. These mechanisms usually happen simultaneously [73].
1.4 *P. aeruginosa* and Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive genetic disease that is most commonly lethal among Caucasians, with an incidence of about 1 in 3500 newborns in the United States and Canada [76-79]. CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), which is an epithelial cell chloride channel regulating chloride channel in the apical plasma membrane. CFTR protein contains two ATP-hydrolysis domains and 12 membrane-spanning alpha helices, belonging to the ATP-binging cassette (ABC) transporter family [76,80]. Although more than 1000 other mutations have been documented in the CF gene, the ΔF508 mutation with the deletion of phenylalanine at position 508 of the polypeptides is present in 70% of CF chromosomes [76]. The deficiency of CFTR function results in abnormal regulation of electrolyte and fluid balance in specific tissues. CF affects many organ systems, including the reproductive tract and sweat glands, but the morbidity and mortality are primarily related to disease of the gastrointestinal and respiratory tracts [76]. The median predicted survival is approximately 37 years, despite impressive advances in life expectancy in CF during the last 3 decades [81]. Some individuals with CF still die from lung infection during their teenage years, and the severity of lung disease become the major determinant for those who can survive into adulthood.

An electrolyte imbalance causes dehydration in the lungs. Compounded with the production of a viscous mucus, mucociliary clearance is significantly impaired, and allows persistent bacterial colonization to occur [82]. *P. aeruginosa* is the key problem in CF lung infections, and more than 80% of CF patients over the age of 26 are colonized by
this species, although CF patients can become infected with other microorganisms such as *Staphylococcus aureus*, *Burkholderia cepacia*, and *Haemophilus influenzae* [76]. *S. aureus* is the most common initial pathogen in the first few years of life, but it has been demonstrated that the opportunistic pathogen *P. aeruginosa* is the most important pathogen in progressive, severe CF lung disease [80]. $10^9$ colony forming units (CFU) of *P. aeruginosa* can be found in one milliliter of older patients’ sputa [76]. Permanent eradication of *P. aeruginosa* from the airways of CF patients seems to be impossible, but specific therapy directed at *P. aeruginosa* infection does lead to clinical improvement. According to information from the Cystic Fibrosis Foundation, inhaled tobramycin administered by a nebulizer is used to treat people with CF who are chronically infected with *P. aeruginosa*.

### 1.5 Nutrient acquisition during Cystic Fibrosis lung infection

*P. aeruginosa* can reach $>10^9$ CFU/ml during high-cell-density (HCD) replication in the lung of CF patients [83-85]. The HCD replication is highly energy demanding, requiring efficient nutrient acquisition. However, unlike the gastrointestinal tract where rich nutrients are readily available, nutrients in the lung environment remain scarce and which nutrients allow HCD *P. aeruginosa* growth and maintenance *in vivo* remains unclear. Lungs are naturally coated by lung surfactant, which is composed of 10% protein and 90% lipids, and about 80% of the lung surfactant lipids are phosphatidylcholine (PC) [86-88]. Lung surfactant is essential for the proper lung function, maintaining alveolar patency to prevent small airway collapse [89], promoting mucociliary clearance [90,91], preventing pulmonary infections by acting as part of the innate immune response and
host defense [92-94], holding anti-inflammatory features [95] and scavenging oxygen-radicals and increasing antioxidant activities inside the cell [96]. The hypothesis is that PC, the most abundant lipid in lung surfactant, may provide a nutrient for HCD bacterial growth in vivo. In accordance with this hypothesis, an initial study suggests that PC is a major nutrient source for P. aeruginosa during lung infection, and that it supports HCD replication [85].

It has been shown that P. aeruginosa produces phospholipase C (heat-labile hemolysin) and lipases in vivo that can cleave exogenous PC into three components, a phosphorylcholine headgroup, glycerol, and two fatty acid (FA) tails, the richest nutrient [85] (Fig. 1A). These three components can be further metabolized by the betaine, glycerol and fatty acid degradation (Fad) pathway (Fig. 1B), respectively. Glycerol and choline metabolism by P. aeruginosa are well characterized [97-101]. However, P. aeruginosa fatty acid degradation and the genes involved in this process are yet to be defined.

The enzymatic activity of phospholipase C on PC releases the phosphorylcholine headgroup and the diacylglycerol (DAG) molecule (Fig. 1A). The phosphorylcholine headgroup is first transported across the cell membrane and dephosphorylated by a phosphatase [97,102,103] to yield choline, which has previously been shown to be sufficient for P. aeruginosa to grow on as a sole carbon, nitrogen, and energy source [104]. P. aeruginosa BetAB (a choline dehydrogenase and a glycine betaine aldehyde dehydrogenase) catalyzes the conversion of choline to glycine betaine [97]. Glycine betaine is successively demethylated to form dimethylglycine (DMG), sarcosine (monomethylglycine), and finally glycine [98,105]. The DAG molecule is cleaved by the
P. aeruginosa lipase, liberating a glycerol molecule and two long-chain fatty acids (LCFAs). Glycerol metabolism has been well characterized in P. aeruginosa. The operon primarily consists of glpD (a sn-glycerol-3-phosphate dehydrogenase [106]), glpF (a membrane-associated glycerol diffusion facilitator [101,107]), glpK (a glycerol kinase [101,107]), glpM (a membrane protein affecting alginate synthesis [100]), and glpR (a regulator of the glp operon [99]).

P. aeruginosa has a large genome (6.3 Mb) compared to E. coli, indicating possible redundancies and a high level of complexity. 6-10 times more genes for Fad were predicted in P. aeruginosa than E. coli [7]. The well-established aerobic Fad pathway in E. coli was used as a model to characterize P. aeruginosa Fad. E. coli possesses only a single copy of each fad gene [108,109], and the cyclic degradation of fatty acids by two carbons per cycle is primarily catalyzed by an acyl-CoA dehydrogenase coded by fadE, and the products of the fadBA-operon, 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, respectively. Two fadBA-operon homologues (fadBA1 and fadBA5), encoding 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, have been investigated so far. The fadBA5-operon (PA1736 and PA1737) was shown to be involved in LCFAs (C_{14:0} – C_{18:1}{^\Delta^9}) degradation, and the fadBA1-operon was strongly induced by medium-chain fatty acids (MCFA C_{10:0} and C_{12:0}), to a lesser extent by LCFA [3,110]. Three fadLs have been investigated, but their exact role in fatty acid transport in P. aeruginosa is still unclear [111]. Two of P. aeruginosa fatty acyl-CoA synthetase genes (fadD1 and fadD2) were identified and determined to have broad specificity for fatty acids of different chain length [112]. It has been found that FadD1 has a preference for long chain fatty acids whereas FadD has higher activities for shorter chain FAs [112]. We have recently
identified four new \textit{fadD} homologues PA1617, PA2893, PA3860 and PA3924 out of 11 potential candidates, and PA1617 (\textit{fadD4}) was found to be the major contributor to Fad [113].

PC metabolism could offer a very rich nutrient source upon which \textit{P. aeruginosa} could multiply in the lung environment. It has been shown that the action of phospholipases significantly affect the pulmonary surfactant phospholipids, which contribute to the severe pathology associated with \textit{P. aeruginosa} related lung infections [114]. As a result, PC degradation not only contributes to HCD growth by liberating nutrient and energy sources, but also directly leads to disease.

\textbf{1.6 Specific aims}

The goal of the research described here is to demonstrate that \textit{P. aeruginosa} can metabolize the lung surfactant component, PC, as a potential nutrient source to support its HCD growth during infection in the CF lung. This was conducted through the following specific aims:

1. Genetic characterization and growth analysis of \textit{fadBA}-operons, 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, involved in fatty acid degradation

2. Characterize individual pathway mutants \textit{in vitro} by growth analysis and monitoring virulence factor production; determine the competitive fitness of individual pathway mutants by \textit{in vitro} and \textit{in vivo} competition studies
Chapter 2: Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *E. coli* EP-Max10B was used as a cloning strain and cultured in Luria-Bertani (LB) medium (Difco). *Pseudomonas* Isolation Agar or Broth (PIA or PIB; Difco) or LB medium were used to culture *P. aeruginosa* strain PAO1 and derivatives.

Antibiotics were added to media for selection or plasmid maintenance as follows: 100 µg/ml ampicillin (Ap), 15 µg/ml gentamicin (Gm), 35 µg/ml kanamycin (Km), 25 µg/ml streptomycin (Sp), and 10 µg/ml tetracycline (Tet) for *E. coli*; 500 µg/ml carbenicillin (Cb), 150 µg/ml Gm, and 100 µg/ml Tet for *P. aeruginosa*. All antibiotics were purchased from Teknova and stock solutions were prepared as recommended by the manufacturer. *E. coli Δasd* (E0464) was grown in a medium supplemented with 100 µg/ml diaminopimelic acid (DAP, Sigma) prepared as described previously [115]. All fatty acids (FAs) stocks were made in house as previously described [112].

Strains for growth analyses were cultured in a minimal medium containing 1X M9 salts [116] + 0.5 mM MgSO$_4$ + 0.02 mM CaCl$_2$ + 0.2% (w/v) Brij-58 (Sigma) + 1% (w/v) casamino acids (CAA) or 0.4% (w/v) of the individual FA, C$_{12:0}$ to C$_{16:0}$, or C$_{18:1}^{Δ9}$ (Sigma; Fig. 6) and 1x M9 minimal medium + 0.2% (w/v) Brij-58 (Sigma) + 0.4% (w/v) phosphatidylcholine (PC, Sigma; Fig. 8), at 37°C with shaking at 200 r.p.m. The *in vitro* competition studies (Fig. 11) were performed as previously described [112].

2.2 General molecular methods
Oligonucleotides were synthesized by Integrated DNA Technology (Table 3). All molecular methods and their components were employed as previously described [117].

2.2.1 Reagents

All restriction enzymes, DNA markers, T4 DNA polymerase, T4 DNA ligase, calf intestinal alkaline phosphatase (CIP), deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs (NEB) and used as recommended by the manufacturer. \textit{Pfu} and \textit{Taq} DNA polymerases were purchased from Stratagene®.

2.2.2 Polymerase chain reaction (PCR)

PCR was generally performed by initial denaturation at 94°C for 3 min and 30 cycles of 15 s at 94°C, 15 s at 50 -70°C (determined by the melting temperature of primers), and 1 min per kb at 72°C, with a final step of 10 min’s extension at 72°C. 15 to 30 pmol of forward and reverse primers, 10 -100ng DNA template, and 5 U of DNA polymerase were used per 50 µl reaction. 2.5 – 10% (v/v) of dimethyl sulfoxide (DMSO) was supplemented to PCR reaction when some of the GC rich chromosomal regions of \textit{P. aeruginosa} were amplified.

2.2.3 Gel electrophoresis and DNA extraction

DNA samples may be separated based on their size difference on a 1-2% agarose gel by running at 110V for 60 min. SYBR® Safe stain, a less hazardous alternative to ethidium bromide, was used for the visualization of the DNA fragments. DNA bands of desired sizes were excised from agarose gel and DNA was extracted in the Zymo Gel Recovery Kit, following the manufacturer’s protocol.
2.2.4 Isolation of bacterial chromosomal DNA and plasmid DNA

*E. coli* and *P. aeruginosa* chromosomal DNA was isolated from 3 ml of overnight culture utilizing phenol-chloroform extraction [116].

The Zyppy plasmid miniprep I kit (Zymo Research Corporation) was used to isolate plasmid DNA from *E. coli* overnight cultures following the manufacturer’s instruction. Various volumes of overnight culture were used according to requirements, e.g., different origins of replication.

DNA concentration was measured in a Thermo Scientific NanoDrop™ 1000 Spectrophotometer according to the manufacturer’s protocol.

2.2.5 Restriction enzyme digestion and ligation

All restriction enzymes were purchased from NEB and supplemented with buffers. Restriction enzyme digests were usually incubated at 37°C for at least 2h. To dephosphorylate vectors, CIP was added directly to the digestion mixture followed by 30 min incubation at 37°C. 1 µl of 2 mM dNTPs and 3U of T4 DNA polymerase were added directly to the mixture and incubated at 37°C for 30 min, if blunt-end DNA fragments were needed. T4 DNA polymerase was deactivated by incubation at 75°C for 20 min to allow subsequent enzymatic digestion. As an alternative, gel electrophoresis can be used to purify the restriction enzyme digestion mixture to allow subsequent enzyme digest.

A 10:1 molar ratio of insert DNA fragments to vector DNA was used for ligation. Generally, a final volume of 10 µl was used for a ligation reaction with 1 U T4 DNA ligase, purchased from NBE, in 1X ligation buffer. The ligation reaction was incubated at 16°C for at least 4 h. Ligation mixtures were routinely transformed into different *E. coli* strains according to the requirement.
Electro-competent *E. coli* cells were prepared as described previously [115], and chemically competent *E. coli* cells were prepared through the MgCl$_2$/CaCl$_2$ method [116].

### 2.3 Genetic techniques

#### 2.3.1 Bacterial conjugation

*E. coli* mobilizable strain E0464 was routinely used as donor strain to introduce vectors containing oriT into *P. aeruginosa* recipient strains. Helper plasmid pRK2013 was used as an alternative to mobilize plasmids into *E. coli* and *P. aeruginosa*. Briefly, the donor and recipient strains were grown to mid-log phase in LB broth. 0.5 ml of each culture was gently mixed in a sterile 1.5 ml microfuge tube and spun down at 7,000x g. The cell pellet was resuspended in 50 µl LB broth and then spotted onto a pre-warmed LB agar plate. After incubation overnight at 37°C, the cells were scraped from the medium and twice washed in 1 ml 1X M9 salt buffer, and dilutions were plated on selective media.

#### 2.3.2 Construction of mutants and complementation strains

All mutants were constructed as described previously [118]. Briefly, the *fadAB* (*fadAB1, fadAB2, fadAB3, fadAB4, fadAB5*) operons, *betBA* operons, and *glpD* gene were amplified by PCR using respective upstream and downstream primer pairs (Table 3). PCR products were purified from the gel, digested with restriction enzymes, and cloned into the gene replacement vector pEX18T, digested with the same restriction enzymes, to yield each of the pEX18T-target gene construct. After deletions were made in each of the *fadBA*-operons, the *glpD* gene, and the *betBA*-operon through restriction digestion
(fadAB1: PstI, BamHI; fadAB2: StuI, BamHI; fadAB3: NorI, SmaI; fadAB4: EcoRV; fadAB5: SphI, PstI) and blunt-ended (except for glpD), the 1.1 kb FRT-GmR-FRT cassette obtained from pPS856 digested with SmaI was inserted into each gene. The newly constructed pEX18T vectors were transformed into E.coli SM10 or ER2566-mob, and conjugated into PAO1 to engineer the unmarked mutations as previously described [118].

The single copy integration vector, miniCTX2, was used to engineer the complemented strains for each triple-pathway mutant as previously described [119]. Briefly, fadAB5 and betBA were PCR amplified with primers 888/889 and 522/895, respectively. The miniCTX2-fadBA5 was yielded by inserting fadBA5 fragment into miniCTX2, both digested with XhoI and BamHI. The betBA gene was sub-cloned in using SacI and SpeI, yielding miniCTX2-fadAB5-betBA. glpD was first cloned into pUC19 by digesting the PCR product with HindIII and SacI, which was then amplified using primers 896/519. P lac-glplD fragment was amplified using primers 519/927 from pUC19-glplD and cloned into miniCTX2-fadAB5 and miniCTX2-fadAB5-betBA to yield miniCTX2-fadAB5-glplD and miniCTX2-fadAB5-betBA-glplD, respectively.

The newly engineered mutant strains ΔfadAB145, ΔfadAB145 ΔglpD, and ΔfadAB145 ΔbetBA ΔglpD, were complemented using the relevant gene(s) on the miniCTX2 single copy integration vector as previously described [119]. The resulting strains ΔfadAB145/ complement, ΔfadAB145 ΔglpD/ complement, ΔfadAB145 ΔbetBA/ complement, ΔfadAB145 ΔbetBA ΔglpD/ complement were used in the growth curve experiment (Fig. 8).

2.4 Growth characterization of mutants and complementation strains
Growth curve analyses have been described previously [112]. Briefly, all strains utilized were initially grown overnight in *Pseudomonas* Isolation Broth (PIB). Overnight cultures were then centrifuged and the cell pellets were washed twice in 1X M9 minimal medium. A 1:50 dilution was made into 25 ml of the respective media (described above) for different growth curves. To clarify any insoluble fatty acid, individual cultures were diluted 4-fold in 4% Brij-58, pre-incubated at 42°C, prior to taking OD$_{540}$ measurement at each time point.

### 2.5 Virulence factors detection

Protease, phospholipase, lipase, and rhamnolipid were detected as previously described[112]. Various mutant strains, ΔfadAB145, ΔfadAB145 ΔglpD, ΔfadAB145 ΔbetBA, ΔfadAB145 ΔbetBA ΔglpD and the complement strains for each mutant were used in the assays (Table 1). Strains were grown in LB medium and the cell concentrations were measured at OD$_{540}$ at various time points. Clarified supernatant was used for protease, phospholipase, and lipase assays. Briefly, at the 24 h time point, 1 ml culture was centrifuged (16,000x g) for 2 min at 4°C and filtered though 0.2 µm hydrophilic PVDF filters (Fisher Scientific). 4-mm diameter holes were punched into 2% skim milk NB agar protease plates, blood agar phospholipase plates (PML microbiological) and rhodamine B agar lipase plate. Both skim milk and blood agar plates were pipetted with 50 µl of each cell-free supernatant into the holes, respectively. After 8 hours, another 50 µl cell-free supernatant was added. Both were incubated at 37°C for 18 h before analyzing. For the rhodamine B agar plate, 50 µl of the same cell-free supernatants were applied five times into each hole [120], and the plates were
imaged using a UV transilluminator after incubation at 37°C for 3 days to visualize lipase activity. Assays were conducted in triplicate and the diameters of clear zone on skim milk and blood agar plates, or diameters of the fluorescent halo on the rhodamine B plates were measured and compared by percentage conversion relative to the wildtype PAO1 value, and presented as a mean ± s.e. [112].

The methylene blue complexation assay [121] was used to assess rhamnolipid production. As described above, all strains were grown for 24 h in LB medium and 1.5 ml of clarified supernatant for rhamnolipid extraction was obtained from each culture by centrifugation at room temperature at 16,000x g. This assay was performed in triplicates and the average absorbance was compared by percentage conversion relative to the wildtype PAO1 value, and expressed as a mean ± s.e. [112].

2.6 In vitro and in vivo competition studies

In vitro and in vivo competition studies were performed as previously described [112]. Various alginate-overproducing strains, ΔfadAB145-mucA⁻, ΔfadAB145ΔglpD-mucA⁻, ΔfadAB145ΔbetBA-mucA⁻, ΔfadAB145ΔbetBAΔglpD-mucA⁻ and the complement strains for each mutant utilized in this study are listed in Table 1.

P. aeruginosa usually undergoes phenotypic change in the lung environment, and the mucoid strain is the most commonly isolated P. aeruginosa strain from the CF lungs. A mucA insertional mutation was introduced into all strains to overproduce alginate, since we used a mouse model to allow these mucoid strains to survive and replicate in the lung as previously described [66]. Briefly, oligos #973 and # 974 were used in PCR to amplify a 450-bp internal region of the mucA gene, which was cloned into the PvuI site of
pUC18. The newly constructed vector pUC18-‘*mucA’ was electroporated into the various pathway mutants/complemented strains and the mucoid transformants were selected on PIA (*Pseudomonas* Isolation Agar) plates supplemented with 500µg/ml carbenicillin (Cb500). A single colony of each mutant/complemented strain was picked and inoculated into 3 ml PIB (*Pseudomonas* Isolation Broth) + Cb500. After 24h incubation with shaking at 37°C, all cultures were diluted 100X into 5 ml fresh PIB + Cb500. After overnight growth, 3 ml of each culture was spun down (20,000g) for 10 min at 4°C and the clarified supernatant was collected. Standard 10-fold serial dilutions were plated on LB plates to determine the cell density. Each culture was then adjusted to 2x10⁸ CFU/ml in its own clarified supernatant that was collected earlier. Each mutant strain and its complemented pair were mixed at a CFU ratio of 1:1, and the resulting mixtures were used for *in vitro* competition or *in vivo* competition studies.

### 2.6.1 *In vitro* competition study

Growth media with LB, glucose, CAA, PC, C_{18:1}^{Δ9}, glycerol, or choline as sole carbon source were used in this study. Each mutant/complement equal cell density mixture was diluted 100X into those media and grown at 37°C with shaking for 1-2 days, until the total cell densities reached ~1x10⁹ CFU/ml. Then the total number of bacterial cells was quantified by plating dilutions onto LB plates; the number of complemented bacteria was determined by plating dilutions onto LB plates with tetracycline (to inhibit the growth of mutant strains). These numbers were used to determine the *in vitro* CI (Competitive Index), which was defined by the ratio of mutant CFU to CFU of the corresponding complemented strain [122]. If CI is smaller than 1, the mutant is less competitive than its complement strain. The smaller the number, the less competitive of
the mutant strain. Experiments were performed in triplicate, and statistical analyses were performed in Graphpad Prism 5.0.

2.6.2 In vivo competition study

6-8 week old male BALB/c mice used in this study were purchased from Charles River Laboratories [66]. Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10mg/kg xylazine, and then 30 µl of the mutant/complement strain mixture (3x10⁶ CFU of each) was inoculated intratracheally using the BioLITE Intubation System (Braintree Scientific). Mice were humanely euthanized and their lungs were harvested and homogenized at 24h and 48h postinfection. Bacteria were quantified by plating dilutions onto growth media with and without tetracycline, as described above in the in vitro competition study. The in vivo CI (CFU\text{mutant}/CFU\text{complement} when grown in mouse lungs) was calculated [122]. Statistical analyses were performed in Graphpad Prism 5.0.
Chapter 3. Characterization of \textit{fadBA}-operons Homologues in \textit{P. aeruginosa} Fatty Acid Degradation Pathway

3.1 Introduction

HCD replication requires much energy, and efficient nutrient acquisition and transformation. However, unlike in the gastrointestinal tract where nutrients are readily available, nutrients in the lung environment remain scarce, and which nutrients allow (HCD) \textit{P. aeruginosa} growth and maintenance \textit{in vivo} remains unclear. Lungs are naturally coated by a lung surfactant composed of 10% protein and 90% lipids, and about 80% of the lung surfactant lipids are phosphatidylcholine (PC) [86-88]. Thus, PC, may provide a nutrient for HCD bacterial growth \textit{in vivo}. In accordance with this hypothesis, an initial study suggests that PC is a major nutrient source for \textit{P. aeruginosa} during lung infection, and that it supports HCD replication [85].

\textit{P. aeruginosa} produces phospholipases C (heat-labile hemolysin) and lipases that can cleave exogenous PC into three components: a phosphorycholine headgroup, glycerol and two fatty acids (FAs) tails, the richest nutrient [85] (Fig. 1A). These three components can be further metabolized by the betaine, glycerol and fatty acid degradation pathway (Fig. 1B), respectively. Glycerol and choline metabolism by \textit{P. aeruginosa} are well characterized [97-101]. However, fatty acid degradation by \textit{P. aeruginosa} and the genes involved in this process are yet to be elucidated.

\textit{P. aeruginosa} has a larger genome (6.3 Mb) than \textit{E. coli}, indicating possible redundancies and a high level of genome complexity. It was predicted that there are 6-10 times more genes for Fad in \textit{P. aeruginosa} than in \textit{E. coli} [7]. Two \textit{fadBA}-operon homologues (\textit{fadBA1} and \textit{fadBA5}), encoding 3-hydroxyacyl-CoA dehydrogenase and
acyl-CoA thiolase, have been investigated so far. The \textit{fadBA5}-operon (PA1736 and PA1737) was shown to be involved in long-chain fatty acids (LCFA, C\textsubscript{14:0} – C\textsubscript{18:1\text{\^9}}) degradation, and the \textit{fadBA1}-operon was strongly induced by medium-chain fatty acids (MCFA C\textsubscript{10:0} and C\textsubscript{12:0}), to a lesser extent of LCFA [3,110]. Three \textit{fadLs} have been investigated, but their exact role in fatty acid transport in \textit{P. aeruginosa} is still unclear [111]. Two \textit{P. aeruginosa} fatty acyl-CoA synthetase genes (\textit{fadD1} and \textit{fadD2}) were identified in a previous study, and were determined to have broad specificity for fatty acids of different chain lengths. It has been found that FadD1 has a preference for long chain fatty acids whereas FadD has higher activities for shorter chain FAs [112]. We have recently identified four new \textit{fadD} homologues PA1617, PA2893, PA3860 and PA3924 out of 11 potential candidates (Fig. 1), and PA1617 (\textit{fadD4}) was found to be the major contributor to Fad [113].

The purpose of this study is to further characterize \textit{fadBA}-operons involved in \textit{P. aeruginosa} Fad. Identification and characterization of this Fad would provide valuable information about pathogenesis of \textit{P. aeruginosa} in CF lung infection.

### 3.2 Results

#### 3.2.1 Identification of five \textit{fadBA}-operons potentially involved in \textit{P. aeruginosa} fatty acid degradation

The well-established aerobic fatty acid degradation (Fad) pathway in \textit{E. coli} was used as a model to characterize \textit{P. aeruginosa} Fad. \textit{E. coli} possesses only a single copy of each \textit{fad} gene [108,109], and the cyclic degradation of fatty acids by two carbons per cycle is primarily catalyzed by an acyl-CoA dehydrogenase coded by \textit{fadE}, and the products of
the *fadBA*-operon, 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, respectively. Up to five potential *fadBA*-operons were identified in *P. aeruginosa* by BLAST analysis of the *E.coli* fadBA sequence against the *P. aeruginosa* genome, including *fadBA1* (PA1737, PA1736), *fadBA2* (PA3590, PA3589), *fadBA3* (PA2554, PA2553), *fadBA4* (PA4786, PA4785), and *fadBA5* (PA3014, PA3013) (Fig. 2 and Fig. 3). Of these five FadBAs, FadBA5 showed the greatest homology to the *E.coli* FadBA with FadB5 having 72% similar (54% identical) and FadA5 having 76% similar (61% identical) to the *E.coli* FadBA, respectively [3,123].

Considering the larger size of the *P. aeruginosa* genome (6.29Mb) [7] compared to that of *E. coli*, and wide range of environmental niches it occupies, it is not surprising to identify up to five *fadBA*-operon homologues. However, one would question whether this redundancy serves a purpose, and if every operon is equally involved in the metabolism of fatty acids.

3.2.2 Significant involvement of *fadBA1,4,5*-operons in fatty acid and PC degradation

Readily available long-chain fatty acids (LCFAs) are released by enzymatic cleavage of PC by *P. aeruginosa* lipases [124,125]. Since most fatty acids hydrolyzed from PC (50 to 60%) are C16:0, with ~10 to 20 % of each of C14:0, C16:1, C18:1 Δ9, and C18:2 constituting the rest [94], growth analyses were performed in 1X M9 minimal medium supplied with each of C12:0 (medium-chain fatty acid), C14:0, C16:0, and C18:1 Δ9 (LCFAs) as sole carbon sources (Fig. 6). Accordingly, the PC we used in this experiment contains mostly LCFAs, approximately 33% C16:0, 13% C18:0, 31% C18:1 Δ9, and 15% C18:2.
Our previous work showed that \textit{fadBA5}-operon is involved in LCFA utilization, but the \textit{ΔfadBA5} mutant still grew on LCFAs as a carbon source, indicating the existence of other potential \textit{fadBA}-operons in fatty acid degradation [3]. The \textit{fadBA1}-operon was shown to be induced by medium-chain fatty acids (MCFAs) and to a lesser extent by LCFAs [110] suggesting that \textit{fadBA1}-operon might have overlapping functions with the \textit{fadBA5}-operon in the metabolism of different chain length fatty acids. However, the involvement of other \textit{fadBA}-operons in Fad has not been characterized.

In this study, triple mutants (\textit{ΔfadBA125}, \textit{ΔfadBA135}, \textit{ΔfadBA145}, \textit{ΔfadBA235}, \textit{ΔfadBA245}, \textit{ΔfadBA345}) and a quintuple mutant \textit{ΔfadBA1-5} (Table 1) were generated for growth analysis on MCFA and LCFAs as sole carbon sources to further characterize the \textit{fadBA}-operons. PCR confirmation of genotypes of all the \textit{fadBA} mutants is shown in Fig. 4. Two colonies of individual mutants were picked for PCR verification. The PCR product of the wildtype \textit{fadBA1} locus is 3.4 kb, and mutated it is \textasciitilde 1.3 kb with primers #1039 and #1040. The PCR product for the mutated \textit{fadBA2}-operon is 1.4 kb, whereas for the wildtype it is 3.1 kb with primers #218 and #219. For \textit{fadBA3}-operon, the wildtype PCR product is 2.6 kb and the mutated is 1.2 kb with primers #220 and #221. The wildtype PCR product of \textit{fadBA4}-operon is 3 kb, \textasciitilde 1.4 kb for the mutated product with primers #1496 and #1496. The mutated \textit{fadBA5}-operon is 2.8 kb with the Gm resistance marker, the wildtype PCR product is \textasciitilde 3.4 kb with primers #272 and #273. Later, a pair of primers, #1881 and #1882, was designed to optimize PCR, and used to confirm the strains at the end of growth curve (Fig. 5). After the last time point of the growth curve, all the strains were streaked for single colony isolation. Two individual colonies were used for colony PCR verification.
Growth defects were indicated by slower growth rate and lower overall final cell densities compared to the wild type, which suggested a reduced rate in metabolizing these FAs and the possible accumulation of a growth inhibiting intermediates. Interestingly, the ΔfadBA145 triple mutant showed the same growth defect as ΔfadBA1-5 quintuple mutant on all fatty acids tested (Fig. 6), indicating a less important role of fadBA2 and fadBA3-operons in metabolizing medium and long chain fatty acids. This was further confirmed by the fact that the ΔfadBA235 triple mutant showed less growth defects than both the ΔfadBA245 and ΔfadBA345 mutants. In addition, with the absence of fadBA5 and fadBA1-operons, the different level of growth defects shown by ΔfadBA125, ΔfadBA135 and ΔfadBA145 mutants unveiled the various roles that fadBA2, fadBA3 and fadBA4-operons played in Fad. On C18:1Δ9, ΔfadBA125 and ΔfadBA135 mutants had minimum growth rate reaching the final cell density of OD540 of ~0.7 after 20 h, while ΔfadBA145 mutant shown the same growth pattern as ΔfadBA1-5 quintuple mutant picking up slightly after 24 h and grew to an even lower final cell density of OD540 of ~0.4. The same growth pattern was detected on all the other fatty acids tested. Slight growth of the ΔfadBA145 triple mutant and ΔfadBA1-5 quintuple mutant after 24 h suggested there might be more fadBA homologues, considering redundancy in the P. aeruginosa genome.

In the growth analysis on PC (Fig. 7), the ΔfadBA145 triple mutant displayed reduced growth with delayed log-phase and lower final cell density compared to wild type PAO1. The cleavage rate of exogenous PC into its useable components should not affect the growth of the ΔfadBA145 mutant, because the ΔfadBA145 mutant had the same lipase and phospholipase expression compared to the wild type and the complement strain (Fig. 9). Since the mutant strain can degrade phosphorylcholine and glycerol as well as the
complement strain (data not shown), I concluded that the reduced growth of the mutant strain on PC is due to a reduced ability to degrade LCFAs. All the evidence we have here strongly suggests the involvement of \( \textit{fadBA}1,4,5\)-operons in Fad and PC degradation.

It was also found that these three \( \textit{fadBA} \)-operons are not equally involved in the metabolism of FAs, and have different substrate preferences. The \( \textit{fadBA}5\)-operon plays the most significant role in LCFA degradation, while the \( \textit{fadBA}1\)-operon is more important for MCFA (C\(_{12:0}\)). The \( \textit{fadBA}4\)-operon displays less involvement in metabolizing the FAs tested compared to both \( \textit{fadBA}1 \) and \( \textit{fadBA}5\)-operons. I complemented \( \Delta \textit{fadBA}145 \) mutant strain by integrating miniCTX2-\( \textit{fadBA}5 \) as a single copy into the \( \Delta \textit{fadBA}145 \) mutant background (Table 1). The complemented strain was fully restored to wild type growth on PC, further supporting the role of the \( \textit{fadBA}5\)-operon in metabolizing LCFAs.
Chapter 4. Characterization of PC Degradation in *P. aeruginosa*

4.1 Introduction

Enzymatic activity of phospholipase C on PC releases the phosphorylcholine headgroup and the diacylglycerol (DAG) molecule (Fig. 1A). The phosphorylcholine headgroup is first transported across the cell membrane and dephosphorylated by a phosphatase [97,102,103] to yield choline, which has previously been shown to be sufficient for *P. aeruginosa* to grow on as a sole carbon, nitrogen, and energy source [104]. *P. aeruginosa* BetAB (a choline dehydrogenase and a glycine betaine aldehyde dehydrogenase) catalyzes the conversion of choline to glycine betaine [97]. Glycine betaine is successively demethylated to form dimethylglycine (DMG), sarcosine (monomethylglcine), and finally glycine [98,105]. The DAG molecule is cleaved by the *P. aeruginosa* lipase, liberating a glycerol molecule and two LCFAs. Glycerol metabolism has been well characterized in *P. aeruginosa*. The operon primarily consists of *glpD* (a sn-glycerol-3-phosphate dehydrogenase [106]), *glpF* (a membrane-associated glycerol diffusion facilitator [101,107]), *glpK* (a glycerol kinase [101,107]), *glpM* (a membrane protein affecting alginate synthesis [100]), and *glpR* (a regulator of the *glp* operon [99]).

Since the previous data showed that *betAB* and *glpD* were expressed *in vivo* [85], they could potentially be involved in PC degradation during lung infection. Double pathway mutants ΔfadAB145ΔbetBA and ΔfadAB145ΔglpD, and a triple pathway mutant ΔfadAB145ΔbetBAΔglpD (Table 1) were engineered to further determine whether these mutants are deficient in growth on PC. Through growth analysis, I was able to
demonstrate that the inability of the triple pathway mutants to utilize individual nutrient led to the dramatic growth defect on PC.

**4.2 The triple pathway mutant experiences dramatic inability to utilize PC in vitro**

The Fad pathway mutant $\Delta fadAB14$, the double pathway mutants $\Delta fadAB145\Delta betBA$ and $\Delta fadAB145\Delta glpD$, and a triple pathway mutant $\Delta fadAB145\Delta betBA\Delta glpD$ were used in this experiment. Growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wild type PAO1 strain. As expected, all these mutants experienced various growth defects with decreased maximum cell density and delayed log phase on PC as a sole carbon source (Fig. 8). The triple pathway mutant $\Delta fadAB145\Delta betBA\Delta glpD$ exhibited a dramatic inability to utilize PC, as no growth was observed within the first 30 h. Even after 64 h, it only reached a final cell density of $OD_{540}$~0.7, while the complemented strains grew to wild type levels (Fig. 8). All mutants showed the same growth pattern when their growth in LB medium was determined (Fig. 9 E).

**4.3. Virulence factors production**

*P. aeruginosa* produces a large variety of virulence factors which contribute to its success as an opportunistic pathogen in a range of host tissues and organs, and infections by *P. aeruginosa* are difficult to treat [2]. It is important to confirm my hypothesis by investigating if pathway mutants experience a differential virulence expression pattern due to genome manipulation. The production of protease, phospholipase, lipase (Fig 10),
and rhamnolipid (Fig. 9) were monitored when all strains were grown under the same conditions in vitro, particularly different pathway mutants, their complement pairs and wild type strain. However, no difference in virulence factor production was observed in mutants and complemented strains, supporting my conclusion that the growth defect shown by the mutants was due to a reduced ability to metabolize the three components of PC (LCFAs, glycerol, and phosphorylcholine).

4.4 *P. aeruginosa* triple pathway mutant exhibit significantly decreased fitness in vitro

An in vitro competition study was performed between different pathway mutants (ΔfadAB145, ΔfadAB145ΔbetBA, ΔfadAB145ΔglpD, ΔfadAB145ΔbetBAΔglpD) and their complements to examine whether the mutation reduced their ability to metabolize various carbon sources [112]. Seven growth media with different carbon sources, including Luria-Bertani (LB) medium, casamino acids (CAA), glucose, PC, C₁₈:₁^Δ⁹, choline, and glycerol, were used. CFU were determined after being inoculated into each medium for 24-48 h. The competitive index (CI) was calculated as the CFU ratio of mutant/wild type recovered at each time point divided by the CFU ratio of mutant/wild type in the input inoculum (Fig. 11A) [122]. The smaller the CI value, the more significant was the reduced fitness of the mutant. As expected, all the pathway mutants were less competitive than their respective complements in media containing PC, and also other sole carbon sources involved in the related pathways (Fig. 11B). The triple pathway mutant was almost totally outcompeted by its complement strain with the CI reduced to 0.1 when growing in media containing PC, C₁₈:₁^Δ⁹, choline, or glycerol (Fig. 11B).
4.5 *P. aeruginosa* triple pathway mutant exhibit significantly decreased fitness 

*in vivo*

Since PC is the major component of lung surfactant in mammals, including mice [87], a mouse lung infection model [66] was utilized for our *in vivo* competition study to evaluate the fitness of different pathway mutants in the lung environment. As previously described [66,112], the BALB/c mice were inoculated via intratracheal intubation with equal ratios of each mutant and its complement pair (6 x 10^6 CFU/ animal) (Fig. 12 A). At 24 h post-infection, bacterial CFU recovered from the lungs were counted, followed by CI calculation. For all the strains, the average total CFU/mouse recovered was greater than the initial inoculum (Fig. 12 B and C), suggesting that all strains maintained the ability to replicate within the mouse lung. The mean CI value for the triple pathway mutant group (ΔfadAB145ΔbetBAΔglpD and complement pair) indicated that the triple pathway mutant had a significantly reduced ability to survive and multiply in mice compared to the complement pair. With similar levels of virulence factors (i.e., proteases, hemolysins, lipases) secretion observed between these two strains (Fig. 9), the low CI is most likely due to its inability to utilize PC as a nutrient source. The CIs of the remaining three pathway mutants (ΔfadAB145, ΔfadAB145ΔbetBA, ΔfadAB145ΔglpD) were higher than that of the triple pathway mutant group (Fig. 11 B), with ΔfadAB145ΔbetBA showing the greatest decrease in competitive fitness *in vivo* compared to its complement and the ΔfadAB145 single pathway mutant exhibiting the highest fitness. Clearly, the altered ability for the pathway mutants to metabolize PC as a nutrient source was mirrored by their competitive fitness within the lung. The infections were allowed to
persist for 48 h, with the exception of the tripe pathway mutant group. Mutant
(ΔfadAB145, ΔfadAB145ΔbetBA, ΔfadAB145ΔglpD) groups exhibited the same trend of
reduced fitness in the lung environment (Fig. 12 B and C). Surprisingly, none of the mice
inoculated with the triple pathway mutant group (ΔfadAB145ΔbetBAΔglpD and
complement pair) survived for 48 h. It was noticed that at 24 h post-infection, the triple
pathway mutant group reached 3.6 x 10^8 CFU/mouse, which was the highest average total
CFU/mouse among all the mutants and complement pairs. Correspondingly, more severe
lung damage was observed in the mice inoculated with the triple pathway mutant group
(data not shown). This unexpected and interesting phenotype needs to be explored in
future research. The in vivo study provided direct evidence to support the role of PC as an
important nutrient source during P. aeruginosa lung infection.
Chapter 5. Discussion

_P. aeruginosa_ causes the most morbidity and mortality in CF patients. It possesses an impressive repertoire of virulence factors, and their expression only occurs during the HCD replication, and is regulated by QS [126]. _P. aeruginosa_ requires large amounts of readily available energy to reach and maintain HCD in order to produce high-energy dependent virulence factors like biofilm. However, unlike the gastrointestinal tract where nutrients are readily available, energy and carbon sources in the lung environment are scarce. Nutrients that allow HCD _P. aeruginosa_ growth and maintenance in the lung environment have still not been determined. Exploration of the nutrient sources supporting this energy intensive process is of great importance, especially for the CF patients with _P. aeruginosa_ lung infections. In addition, identification of the genes and pathways for _P. aeruginosa_ HCD replication in CF lungs provides fundamental knowledge for the pathogenesis, and aids in the development of potential therapeutic strategies to directly inhibit the metabolism of lung surfactant PC. Ultimately, this could lead to effective treatment for CF patients infected by _P. aeruginosa_.

Lungs are naturally coated by lung surfactant, which is composed of 10% protein and 90% lipids, and about 80% of the lung surfactant lipids are phosphatidylcholine (PC) [86-88]. Palmer et al. (2007) suggested that amino acids are one of the nutrient sources in the lung for _P. aeruginosa_. A previous _in vivo_ study showed that _P. aeruginosa_ produces phospholipases C (heat-labile hemolysin) and lipases that can cleave exogenous PC into three components: a phosphorycholine headgroup, glycerol and two fatty acids (FAs) tails [85] (Fig. 1A). These three components can be further metabolized by the betaine, glycerol and Fad pathways (Fig. 1B), respectively. It was also shown that genes for
individual PC component degradation, \textit{fadD2, beta, glpK, and glpD}, were expressed by \textit{P. aeruginosa} in sputum collected from CF patients. All these studies led to the hypothesis for this study, that PC, the most abundant lipid in lung surfactant, is one of the major nutrient sources during \textit{P. aeruginosa} CF lung infection[127].

The two highly reduced long-chain fatty acids (LCFA) are the major carbon sources in the PC molecule. Glycerol and choline metabolism by \textit{P. aeruginosa} are well characterized. However, the Fad by \textit{P. aeruginosa} and the genes involved in this process remain to be elucidated. Many genes involved in Fad were shown to be expressed by \textit{P. aeruginosa}, e.g. \textit{fadD1, fadD2, fadA5} and \textit{fadB5} [127]. It was also reported that mutants defective in Fad had decreased fitness during mouse lung infection, and mutations in \textit{fadD} genes influenced two modes of motility and virulence factor expression (proteases, phospholipases, rhamnolipids, and lipases) [112]. With a larger genome, \textit{P. aeruginosa} could potentially have more FA degradation genes than \textit{E. coli}, indicating possible redundancies and higher levels of complexity in this pathway [128]. Up to six \textit{fadD} homologues in \textit{P. aeruginosa} have been investigated [128]. Two \textit{fadBA}-operons homologues, \textit{fadBA1} and \textit{fadBA5}, have been studied so far. The \textit{fadAB1} (PA1736 and PA1737) operon was shown to be strongly induced by medium-chain FAs, and to a lesser extent, LCFA [110]. The \textit{fadBA5} (PA3014 and PA3013) operon was determined to be involved in LCFA degradation and to be induced by LCFA [3]. Three additional \textit{fadBA}-operons (\textit{fadBA2, fadBA3, fadBA4}) in \textit{P. aeruginosa} together with \textit{fadBA1} and \textit{fadBA5} are investigated here. Triple mutants (\textit{ΔfadBA125, ΔfadBA135, ΔfadBA145, ΔfadBA235, ΔfadBA245, ΔfadBA345}) and a quintuple mutant \textit{ΔfadBA1-5} (Table 1) were used for growth analysis on MCFA and LCFAs as sole carbon sources, to further characterize the
fadBA-operons. Interestingly, the ΔfadBA145 triple mutant showed the same growth defect as the ΔfadBA1-5 quintuple mutant on all fatty acids tested (Fig. 6), indicating a less important role of fadBA2 and fadBA3-operons in metabolizing medium and long chain fatty acids. It is possible that fadBA2 and fadBA3-operons are responsible for short chain fatty acid (SCFA) degradation. Additionally, these three fadBA-operons are not equally involved in the metabolism of fatty acids. Rather, they have different substrate preferences. The fadBA5-operon plays the most significant role in LCFA degradation, while the fadBA1-operon is more important for MCFA (C12:0). The fadBA4-operon displays less involvement in metabolizing fatty acids tested compared to both fadBA1 and fadBA5-operons. However, the lack of growth for ΔfadBA145 on MCFAs and LCFAs strongly indicates that these three operons are significantly involved in MCFAs and LCFAs metabolism. Furthermore, the ΔfadBA145 triple mutant displayed reduced growth with delayed log-phase and lower final cell density compared to wild type PAO1 in the growth analysis on PC as a sole nutrient source (Fig. 7), indicating that fatty acids are important nutrient source for supporting bacterial growth.

Additionally, along with genes encoding proteins involved in Fad, betAB and glpD were shown to be expressed in vivo [85], indicating that they were potentially involved in PC degradation during lung infection. Double pathway mutants ΔfadAB145ΔbetBA and ΔfadAB145ΔglpD, and a triple pathway mutant ΔfadAB145ΔbetBAΔglpD (Table 1) were engineered to further determine whether these mutants were deficient in growth on PC. As expected, ΔfadAB145ΔbetBA and ΔfadAB145ΔglpD exhibited reduced growth capability compared to ΔfadAB145. The triple pathway mutant, ΔfadAB145ΔbetBAΔglpD, exhibits dramatic growth defects, which strongly supports that
*P. aeruginosa* utilizes the betaine, glycerol and fatty acid degradation pathways to degrade individual components of the PC molecule *in vitro*.

Additional experiments were performed to test my hypothesis. *P. aeruginosa* produces a large variety of virulence factors to make itself a successful opportunistic pathogen in a range of host tissues and organs [2]. It is important to further test the hypothesis by investigating if pathway mutants experience differential virulence expression patterns due to genome manipulation. The production of protease, phospholipase, lipase (Fig 10), and rhamnolipid (Fig. 9) were monitored when all the strains are grown under the same condition *in vitro*, particularly different pathway mutants, their complement pairs and wild type strain. No apparent difference in the virulence factor expression pattern was observed among all of the strains. Therefore, reduced growth of the various mutants on PC is not due to the reduced expression of lipase and phospholipase, which decrease the cleavage rate of PC molecule into usable components and thus affecting the growth. I believe that the reduced growth on PC is attributed to the reduced ability to degrade individual components of PC, e.g., FAs, choline and glycerol.

Although the *in vitro* data support the hypothesis, the environment of the CF lung is much more complicated and poorly understood. The *in vivo* competition study was performed in a mouse lung infection model to evaluate the fitness of various pathway mutants in the lung environment. The link between the ability of *P. aeruginosa* to degrade lipids and FAs and the replication of this bacteria during infection of CF patients’ lung was previously established [112], i.e., greater defects in utilization of FAs and PC *in vitro* resulted in larger disadvantages during *in vivo* growth. The
ΔfadD1D2D3D4D5D6 mutant exhibited more growth defect in FAs and PC compared to ΔfadD1D2, correspondingly this mutant was less fit in mice lungs than ΔfadD1D2 mutant at 24h [112,128]. In this study, replication of various pathway mutants and their complement pairs was observed after 24h (Fig. 12). This could be explained by the fact that *P. aeruginosa* was still able to degrade other nutrients, e.g., amino acids and possibly DNA [127]. The *in vivo* study showed clearly that with more pathways blocked, greater reduced fitness in the lung was observed. The triple pathway mutant ΔfadAB145ΔbetBAΔglpD had a significantly reduced ability to survive and multiply in mice lung compared to its complement. With similar secretion levels of virulence factors (i.e., proteases, hemolysins, lipases) observed between these two strains (Fig. 9), the low CI is most likely due to its inability to utilize PC as a nutrient source as mentioned above. The CIs of the remaining three pathway mutants (ΔfadAB145, ΔfadAB145ΔbetBA, ΔfadAB145ΔglpD) groups were higher than that of the triple pathway mutant group (Fig. 11 B). ΔfadAB145ΔbetBA exhibited the greatest decrease in competitive fitness *in vivo* compared to its complement and the ΔfadAB145 single pathway mutant exhibiting the highest fitness. Clearly, the altered ability for the pathway mutants to metabolize PC as a nutrient source was mirrored by their competitive fitness within the lung. When the infections were allowed to persist for 48 h, with the exception of the triple pathway mutant group, mutant (ΔfadAB145, ΔfadAB145ΔbetBA, ΔfadAB145ΔglpD) groups exhibited the same trend of reduced fitness in the lung environment (Fig. 12 B and C). Surprisingly, none of the mice inoculated with the triple pathway mutant group (ΔfadAB145ΔbetBAΔglpD and complement pair) were able to survive for 48 h. It was noticed that at 24 h post-infection, the triple pathway mutant group reached $3.6 \times 10^8$
CFU/mouse, the highest average total CFU/mouse among all the mutants and complement pairs. Correspondingly, more severe lung damage was observed in mice inoculated with the triple pathway mutant group (data not shown). This unexpected and interesting phenotype needs to be explored in future research. The in vivo study provided direct evidence to support the role of PC as an important nutrient source during P. aeruginosa lung infection.

In this research, identification of the genes and pathways important in P. aeruginosa HCD replication in CF lungs provides insights into the pathogenesis of these bacteria. This provides fundamental knowledge that will help develop novel therapeutic strategies to directly inhibit pathogenic metabolism of lung surfactant PC.
**Tables**

Table 1. Bacterial strains used in this study$^a$.

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<th>Lab ID</th>
<th>Genotype/Description</th>
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<tr>
<td>EP-Max10B</td>
<td>E1231</td>
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</tr>
<tr>
<td>SM10</td>
<td>E006</td>
<td>thi thr leu tonA lacY supE recA::RP4-2Tc::Mu Km$^R$</td>
<td>[129]</td>
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</tr>
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<tr>
<td>ΔfadAB145-mucA&lt;sup&gt;A&lt;/sup&gt;/</td>
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<td></td>
<td>P584 Gm&lt;sup&gt;+&lt;/sup&gt;, Cb&lt;sup&gt;+&lt;/sup&gt;, Tet&lt;sup&gt;+&lt;/sup&gt;; ΔfadAB145-mucA&lt;sup&gt;A&lt;/sup&gt; with pUC18 inserted in mucA</td>
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<tr>
<td>ΔfadAB145 ΔglpD-mucA&lt;sup&gt;A&lt;/sup&gt;/</td>
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<tr>
<td></td>
<td>P578 Gm&lt;sup&gt;+&lt;/sup&gt;, Cb&lt;sup&gt;+&lt;/sup&gt;, Tet&lt;sup&gt;+&lt;/sup&gt;; ΔfadAB145 ΔglpD-mucA&lt;sup&gt;A&lt;/sup&gt; with pUC18 inserted in mucA</td>
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<tr>
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<td>This study</td>
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<td>P580 Gm&lt;sup&gt;+&lt;/sup&gt;, Cb&lt;sup&gt;+&lt;/sup&gt;, Tet&lt;sup&gt;+&lt;/sup&gt;; ΔfadAB145 ΔbetBA-mucA&lt;sup&gt;A&lt;/sup&gt; with pUC18 inserted in mucA</td>
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<td>P582 Gm&lt;sup&gt;+&lt;/sup&gt;, Cb&lt;sup&gt;+&lt;/sup&gt;, Tet&lt;sup&gt;+&lt;/sup&gt;; ΔfadAB145 ΔbetBA ΔglpD-mucA&lt;sup&gt;A&lt;/sup&gt; with pUC18 inserted in mucA</td>
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<sup>a</sup> For strains constructed in this study, please see text for further details.
Table 2. Plasmids used in this study.

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<td>pFlp2</td>
<td>E0067</td>
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<td>pPS856</td>
<td>E0050</td>
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<td>Ap&lt;sup&gt;-&lt;/sup&gt;; cloning vector</td>
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<td>pUC19-&lt;i&gt;glpD&lt;/i&gt;</td>
<td>E1843</td>
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<td>pEX18T</td>
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<td>Ap&lt;sup&gt;-&lt;/sup&gt;, Gm&lt;sup&gt;-&lt;/sup&gt;; pEX18T with Δ&lt;i&gt;fadAB2&lt;/i&gt; operon with Gm&lt;sup&gt;+&lt;/sup&gt;-FRT-cassette insertion</td>
<td>This study</td>
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<td>pEX18TΔ&lt;i&gt;fadAB3&lt;/i&gt;::Gm</td>
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<td>E0461</td>
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<td>This study</td>
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<td>pEX18TΔ&lt;i&gt;glpD&lt;/i&gt;::Gm</td>
<td>E1066</td>
<td>Ap&lt;sup&gt;-&lt;/sup&gt;, Gm&lt;sup&gt;-&lt;/sup&gt;; pEX18T with Δ&lt;i&gt;glpD&lt;/i&gt; operon with Gm&lt;sup&gt;+&lt;/sup&gt;-FRT-cassette insertion</td>
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<td>E1070</td>
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<td>miniCTX2</td>
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<td>Tet&lt;sup&gt;-&lt;/sup&gt;; site-specific integration vector</td>
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<td>miniCTX2-&lt;i&gt;fadAB5&lt;/i&gt;/glpD</td>
<td>E2035</td>
<td>Tet&lt;sup&gt;-&lt;/sup&gt;; miniCTX2 with cloned fadAB5&lt;sup&gt;-&lt;/sup&gt;/glpD</td>
<td>This study</td>
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<td>E1953</td>
<td>Tet&lt;sup&gt;-&lt;/sup&gt;; miniCTX2 with cloned fadAB5&lt;sup&gt;-&lt;/sup&gt;/betBA</td>
<td>This study</td>
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<td>miniCTX2-&lt;i&gt;fadAB5&lt;/i&gt;/betBA/glpD</td>
<td>E1992</td>
<td>Tet&lt;sup&gt;-&lt;/sup&gt;; miniCTX2 with cloned fadAB5&lt;sup&gt;-&lt;/sup&gt;/betBA/glpD</td>
<td>This study</td>
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*For plasmids constructed in this study, please see text for further details.*
Table 3. Primers used in this study.

<table>
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<th>Sequencea</th>
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<td>186; fadAB1-upstream</td>
<td>5'-CGAAAGCTTGCAATGGTATCTCTTCC-3'</td>
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<td>187; fadAB1-downstream</td>
<td>5'-GCGGAAATGCCTACCCGAGTGAGCG-3'</td>
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<tr>
<td>218; fadBA2-upstream</td>
<td>5'-CGGTAGGCTTCCGACC-3'</td>
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<td>219; fadBA2-downstream</td>
<td>5'-GGGGAATCGGTGTCATCGGCAGCGGC-3'</td>
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<td>220; fadBA3-upstream</td>
<td>5'-CGGAAGCTTTGAGGAAGAAACAGACG-3'</td>
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<td>221; fadBA3-downstream</td>
<td>5'-TGCAGGAATTCGAGGATAGTCTGCGCTAC-3'</td>
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<tr>
<td>221; fadBA4-upstream</td>
<td>5'-CGTAAAGCTTCCGGGGAGTCAAGGGGC-3'</td>
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<td>221; fadBA4-downstream</td>
<td>5'-CCCAGAATTCGACGGCACCAGCCCAGA-3'</td>
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<tr>
<td>272; fadBA5-HindIII</td>
<td>5'-AGTTCAAGCTTTCCATATAACG-3'</td>
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<td>273; fadBA5-EcoRI</td>
<td>5'-CCCCGAATTCCCTCCCGAGAACTTAG-3'</td>
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<td>518; glpK-BamHI</td>
<td>5'-AGCTGAAGTGATCCGACAAA-3'</td>
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<td>519; glpKD-SacI</td>
<td>5'-CTGCGACGGCTCAAGCCCGCATGACCC-3'</td>
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<td>522; betA-SacI</td>
<td>5'-CAACGAGCTCGCGATATCTACCGCGG-3'</td>
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<td>523; betB-HindIII</td>
<td>5'-GCCAAAGCTTCCAGGAAGAACGGCT-3'</td>
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<td>888; Xho-fadB5</td>
<td>5'-CCTGCGACAGGGCGCTCGAGGAGGC-3'</td>
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<td>5'-GGGCAAGGAGTCCCGGAGTTGGTC-3'</td>
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<tr>
<td>895; Spe-betB</td>
<td>5'-CGGATTTGAGCTAGTACCTGCTCG-3'</td>
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<tr>
<td>896; Hind-glpD</td>
<td>5'-GCCTGCTGGAAGCTTCGCGCTTGAGTC-3'</td>
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<td>927; SacI-Puc-glpD</td>
<td>5'-CGCTCGCCGGAGCTGAACGGGAGACG-3'</td>
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<td>1039; fadAB1-XbaI</td>
<td>5'-GGACGTCTAGAAGGATAAGGAGGCC-3'</td>
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<td>1040; fadAB1-HindIII</td>
<td>5'-GAGTCCGTCAAGGTCAAGGTCAAGG-3'</td>
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<td>1881; fadA5 down</td>
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<td>5'-GGCCTGACAAATAGAGACC-3'</td>
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aRestriction enzyme sites utilized in this study are underlined.
Figures

A

Lung Phosphatidylcholine (PC)

Lipase

Phospholipase C

Fatty Acid Degradation

Glycerol Metabolism

Choline Metabolism
Figure 1. Phosphatidylcholine (PC) degradation pathway in *P. aeruginosa* (A), and the proposed FA degradation pathway in *P. aeruginosa* based on *E. coli* β-oxidation (B). (A) PC is the main components of lung surfactant and can be cleaved by phospholipase C and lipase producing free fatty acids, glycerol and phosphorylcholine. Three different pathways then further metabolize each component: the *bet* pathway for choline head group metabolism, the *glp* pathway for glycerol metabolism, and the beta-oxidation pathway for the degradation of the FAs. (B) FAs are transported through the outer membrane (OM) aided by an unidentified *P. aeruginosa* FadL. FA may be transported through the inner membrane (IM) via an unknown mechanism coupled to a single peripheral membrane FadD protein in *E. coli*. There are up to six FadDs.
homologues that have been studied thus far [111,113]. Although there are a dozen potential FadE homologues in the *P. aeruginosa* genome, the specific enzyme that catalyzes this reaction has not been identified. FadB catalyzes the next two steps followed by cleavage of the 3-keto-acyl-CoA by FadA. Up to five *fadBA*-operons have been identified in *P. aeruginosa* in this study. Three of them *fadBA1, 4, 5* operons play the most important role in Fad.
Figure 2. Five potential *fadBA*-operon homologues of *P. aeruginosa*. Each gene of the operon is shown in light purple. (A) *fadAB1* is 3.363 kb, (B) *fadBA2* is 2.760 kb, (C) *fadBA3* is 2.346 kb, (D) *fadAB4* is 2.887 kb, and (E) *fadBA5* is 3.353 kb.
Figure 3. Comparison of *P. aeruginosa* FadBAs with *E. coli* FadBA. *P. aeruginosa* FadAs and FadBs are aligned with *E. coli* FadA and FadB. Amino acids with similar properties are assigned the same colors using CLC Sequence Viewer 6 sequence FadBA5 shows the greatest similarity to the *E. coli* FadBA.
Figure 4. PCR confirmation of \textit{fadBA} mutant strains. The PCR results for Δ\textit{fadBA125}, Δ\textit{fadBA235}, Δ\textit{fadBA345}, Δ\textit{fadBA145} are shown. Two independent isolates of each mutant strain were picked for PCR confirmation as shown in lane 1 and lane 2. Five \textit{fadBA}-operons (1,2,3,4,5) were amplified with primers 1039/1040, 218/219, 220/221, 1495/1496, 272/273, respectively. The wildtype PCR products for each operon were 3.4 kb, 3.1 kb, 2.6 kb, 3 kb, 3.4 kb with the corresponding mutant PCR products 1.3 kb, 1.4 kb, 1.2 kb, 1.4 kb, 2.9 kb.
Figure 5. PCR verification of the mutant strains at the end of growth analysis. All of the mutants were confirmed, a representative gel picture was shown here for ΔfadBA125. Lane 1 and 2 represent two independent colonies. Primers 1881/1882 were used for amplifying fadBA5-operon: wldtype ~ 3.4 kb, mutant ~ 2.9 kb; fadBA1-operon: wldtype ~ 3.4 kb, mutant ~ 1.3 kb; fadBA2-operon: wldtype ~ 3.1 kb, mutant ~ 1.4 kb; fadBA3-operon: wldtype ~ 2.6 kb, mutant ~ 1.2 kb; fadBA4-operon: wldtype ~ 3.0 kb, mutant ~ 1.4 kb. The primers used for PCR amplification were (fadBA1) 1039/1040, (fadBA2) 218/219, (fadBA3) 220/221, (fadBA4) 1495/1496.
Figure 6. Growth analysis of different fadBA mutants on medium and long chain-length fatty acid (C\textsubscript{12:0}, C\textsubscript{14:0}, C\textsubscript{16:0} and C\textsubscript{18:1}\textsuperscript{Δ9}). Along with wild type PAO1, mutants were grown in 1X M9 minimal medium supplemented with 1% CAA or 0.4% different FAs as sole carbon source. Although fadBA mutants showed various defects when grown with FAs of different chain-lengths, no growth defects were observed for any of the mutants when grown with the casamino acids (CAA) as a control.
Figure 7. Growth analysis of ΔfadBA1,4,5 mutant on PC as a sole carbon source.

The mutant ΔfadBA145 exhibited growth defects on PC as a sole carbon source. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wild type PAO1 strain.
Figure 8. Growth analysis of various pathway mutants on PC. The Fad pathway mutant ΔfadAB145, the double pathway mutants ΔfadAB145ΔbetBA and ΔfadAB145ΔglpD, and a triple pathway mutant ΔfadAB145ΔbetBAΔglpD were used in this study. The mutants exhibited various growth defects on PC as a sole carbon source. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wild type PAO1 strain.
Figure 9. Analyses of protease, hemolysin, lipase, and rhamnolipid production by *P. aeruginosa* triple pathway mutant. The triple pathway mutant did not display significant decrease in production of protease (A), rhamnolipid (B), hemolysin (C), and lipase (D), and no growth defects in LB (E) were observed.
Figure 10. Detection of virulence factors produced by *P. aeruginosa*. The production of virulence factors were monitored for all the pathway mutants and their complement strains. Representative plates are shown, performed in triplicate. Diameters of the clear zone were measured for skim milk agar plate and blood agar plate. The diameter of the orange halo was measured by exposing the rhodamine B agar plate a UV transilluminator.
Figure 11. *In vitro* competition studies of the various mutants and their complemented strains in different growth media. A. Flow chart demonstrating *in vitro* competition study. B. n equals the number of independent *in vitro* competition experiments performed with each carbon source, The solid red line indicates the geometric mean of the competitive indices (CI) in each competition group. CI<1 indicates the mutant was less competitive than its complemented strain in various growth media.
Figure 12. *In vivo* competition study. *In vivo* lung competition of the various mutants and their complemented strains after 24 h (B) and 48 h (C). The experiment was carried out by following the flow chart (A). n equals the number of mice in each group that were inoculated with a total of $6 \times 10^6$ CFU/mouse. The solid red line indicates the geometric mean of the competitive indices (CI) in each competition group. CI<1 indicates the mutant was less competitive than its complemented strain within the lungs (B and C). Numbers above the red line represent the average total recovered CFU/mouse for each competition group.
Reference Cited


size without altered composition and surface tension function in \textit{cfr}^{-1}\text{HRGU/mHRGU} mice. Thorax 52: 723-730.


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acyl-CoA synthetases contribute to differential fatty acid degradation and 

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74: 4498-4508.


tellurite-resistant genetic tools for single-copy chromosomal analysis of 
*Burkholderia* spp. and characterization of the *Burkholderia thailandensis betBA* 


