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

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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*.

Acknowledgements

Dedication

This work is dedicated to

my parents

Guoming Sun and Peishuang Wu

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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^r 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 phospatase

CF   cystic fibrosis

CFTR   cystic fibrosis transmembrane conductance regulator gene

CFU   colony forming unit

CI   competitive index

CoASH   coenzyme A

COX   cyclooxygenase

DAG	diacyl-glycerol
DAP	diaminopimelic acid
DDW	double deionized water
DMG	dimethylglycine
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DMSO	dimethyl sulfoxide
Ec	*Escherichia coli*
EDTA	ethylendiamionotetraacetic acid
EPS	extracellular polymeric substance (EPS)
FA	fatty acid
FACS	fatty acyl-CoA synthetase
Fad	fatty acid degradation
FAD	flavin adenine dinucleotide
FADH$_2$	reduced flavin adenine dinucleotide
flp	gene encoding *Saccharomyces cerevisiae* recombinase
FRT	Flp recognition target
g	gram
g	gravitational force
GAP	GTPase-activating
Gm	gentamicin
Gm^r	gentamicin resistant/resistance
h	hour

HCD	high cell density
HS	heparan sulfate
HSPGs	heparan sulfate proteoglycans
IPTG	isopropyl β-D-1-thiogalactopyranoside
k	kilo
kb	kilobase
k_{cat}	enzyme catalytic efficiency
kDa	kilodalton
K_m	Michaelis constant
Km	kanamycin
Km^r	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
*ori*T	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*
PPi	pyrophosphate
QS	quorum sensing
s	second
*sac*B	*Bacillus subtilis* gene encoding levansucrase

SCFA	short chain fatty acid
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
Sp	streptomycin
Sp^r	streptomycin resistant/resistance
TAE	Tris base- acetate EDTA buffer
TCA	tricarboxylic acid cycle
T3SS	type III secretion system
Tet	tetracycline
Tet^r	tetracycline resistant/resistance
Tfp	Type IV pili
Tris-HCl	tris-hydrochloride
μ	micro
μg	microgram (s)
μl	microliter (s)
μM	micromolar
U	unit
UV	ultraviolet
v	volume
w	weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
°C	degrees Celsius
%	Percent

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 subtillis* (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 A_2 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 diacly-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 rhanmose-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 RhlI. LasR bound with N- (3-oxododecanoyl)-HSL regulates the transcription of several

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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-well 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 State 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-bing 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 mucocilliary 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 phosphorycholine 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 *fadD* homologues PA1617, PA2893, PA3860 and PA3924 out of 11 potential candidates, and PA1617 (*fadD4*) was found to be the major contributor to Fad [113].

PC metabolism could offer a very rich nutrient source upon which *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 *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.

1.6 Specific aims

The goal of the research described here is to demonstrate that *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 *fadBA*-operons, 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, involved in fatty acid degradation

2. Characterize individual pathway mutants *in vitro* by growth analysis and monitoring virulence factor production; determine the competitive fitness of individual pathway mutants by *in vitro* and *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 $C_{18:1}^{\Delta 9}$ (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 $37^{\circ}C$ 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

Oligonuceotides 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. *Pfu* and *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 *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^{TM} 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 de-phosphorylate 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 *ori*T 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 $37^{o}C$, the cells were scraped from the medium and twice washed in1 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

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(*fadAB1*: *Pst*I, *Bam*HI; *fadAB2*: *Stu*I, *Bam*HI; *fadAB3*: *Not*I, *Sma*I; *fadAB4*: *Eco*RV; *fadAB5*: *Sph*I, *Pst*I) and blunt-ended (except for *glpD*), the 1.1 kb *FRT*-Gm[R]-*FRT* cassette obtained from pPS856 digested with *Sma*I 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 *Xho*I and *Bam*HI. The *betBA* gene was sub-cloned in using *Sac*I and *Spe*I, yielding miniCTX2-*fadAB5-betBA*. *glpD* was first cloned into pUC19 by digesting the PCR product with *Hind*III and *Sac*I, which was then amplified using primers 896/519. P$_{lac}$-*glpD* fragment was amplified using primers 519/927 from pUC19-*glpD* and cloned into miniCTX2-*fadAB5* and miniCTX2-*fadAB5-betBA* to yield miniCTX2-*fadAB5-glpD* and miniCTX2-*fadAB5-betBA-glpD,* respectively.

The newly engineered mutant strains Δ*fadAB145*, Δ*fadAB145* Δ*glpD*, and Δ*fadAB145* Δ*betBA* Δ*glpD*, were complemented using the relevant gene(s) on the miniCTX2 single copy integration vector as previously described [119]. The resulting strains Δ*fadAB145/* complement, Δ*fadAB145* Δ*glpD/* complement, Δ*fadAB145* Δ*betBA/* complement, Δ*fadAB145* Δ*betBA* Δ*glpD/* 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, Δ*fadAB145*, Δ*fadAB145* Δ*glpD*, Δ*fadAB145* Δ*betBA*, Δ*fadAB145* Δ*betBA* Δ*glpD* 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

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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, Δ*fadAB145-mucA⁻*, Δ*fadAB145ΔglpD-mucA⁻*, Δ*fadAB145ΔbetBA-mucA⁻*, Δ*fadAB145ΔbetBAΔglpD-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

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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 $37^{o}C$, 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 $4^{o}C$ 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 $2x10^{8}$ 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, $C_{18:1}{}^{\Delta 9}$, 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 $37^{o}C$ with shaking for 1-2 days,

until the total cell densities reached $\sim 1x10^{9}$ 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 ($3x10^6$ 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 ($CFU_{mutant}/CFU_{complement}$ when grown in mouse lungs) was calculated [122]. Statistical analyses were performed in Graphpad Prism 5.0.

Chapter 3. Characterization of *fadBA*-operons Homologues in *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) *P. aeruginosa* growth and maintenance *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 *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].

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 *P. aeruginosa* are well characterized [97-101]. However, fatty acid degradation by *P. aeruginosa* and the genes involved in this process are yet to be elucidated.

P. aeruginosa has a larger genome (6.3 Mb) than *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 *P. aeruginosa* than in *E. coli* [7]. 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 long-chain fatty acids (LCFA, $C_{14:0} - C_{18:1}^{\Delta 9}$) 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 of LCFA [3,110]. Three *fadLs* have been investigated, but their exact role in fatty acid transport in *P. aeruginosa* is still unclear [111]. Two *P. aeruginosa* fatty acyl-CoA synthetase genes (*fadD1* and *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 *fadD* homologues PA1617, PA2893, PA3860 and PA3924 out of 11 potential candidates (Fig. 1), and PA1617 (*fadD4*) was found to be the major contributor to Fad [113].

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

3.2 Results

3.2.1 Identification of five *fadBA*-operons potentially involved in *P. aeruginosa* fatty acid degradation

The well-established aerobic fatty acid degradation (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. 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 $C_{16:0}$, with ~10 to 20 % of each of $C_{14:0}$, $C_{16:1}$, $C_{18:1}^{\Delta 9}$, and $C_{18:2}$ constituting the rest [94], growth analyses were performed in 1X M9 minimal medium supplied with each of $C_{12:0}$ (medium-chain fatty acid), $C_{14:0}$, $C_{16:0}$, and $C_{18:1}^{\Delta 9}$ (LCFAs) as sole carbon sources (Fig. 6). Accordingly, the PC we used in this experiment contains mostly LCFAs, approximately 33% $C_{16:0}$, 13% $C_{18:0}$, 31% $C_{18:1}^{\Delta 9}$, and 15% $C_{18:2}$.

Our previous work showed that *fadBA5*-operon is involved in LCFA utilization, but the Δ*fadBA5* mutant still grew on LCFAs as a carbon source, indicating the existence of other potential *fadBA*-operons in fatty acid degradation [3]. The *fadBA1*-operon was shown to be induced by medium-chain fatty acids (MCFAs) and to a lesser extent by LCFAs [110] suggesting that *fadBA1*-operon might have overlapping functions with the *fadBA5*-operon in the metabolism of different chain length fatty acids. However, the involvement of other *fadBA*-operons in Fad has not been characterized.

In this study, triple mutants (Δ*fadBA125*, Δ*fadBA135, Δfadba145, ΔfadBA235, ΔfadBA245, ΔfadBA345*) and a quintuple mutant Δ*fadBA1-5* (Table 1) were generated for growth analysis on MCFA and LCFAs as sole carbon sources to further characterize the *fadBA*-operons. PCR confirmation of genotypes of all the *fadBA* mutants is shown in Fig. 4. Two colonies of individual mutants were picked for PCR verification. The PCR product of the wildtype *fadBA1* locus is 3.4 kb, and mutated it is ~ 1.3 kb with primers #1039 and #1040. The PCR product for the mutated *fadBA2*-operon is 1.4 kb, whereas for the wildtype it is 3.1 kb with primers #218 and # 219. For *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 *fadBA4*-operon is 3 kb, ~ 1.4 kb for the mutated product with primers #1496 and #1496. The mutated *fadBA5*-operon is 2.8 kb with the Gm resistance marker, the wildtype PCR product is ~ 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 $\Delta fadBA145$ triple mutant showed the same growth defect as $\Delta fadBA1$-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 $\Delta fadBA235$ triple mutant showed less growth defects than both the $\Delta fadBA245$ and $\Delta fadBA345$ mutants. In addition, with the absence of $fadBA5$ and $fadBA1$-operons, the different level of growth defects shown by $\Delta fadBA125$, $\Delta fadBA135$ and $\Delta fadBA145$ mutants unveiled the various roles that $fadBA2$, $fadBA3$ and $fadBA4$-operons played in Fad. On $C_{18:1}^{\Delta 9}$, $\Delta fadBA125$ and $\Delta fadBA135$ mutants had minimum growth rate reaching the final cell density of OD_{540} of ~0.7 after 20 h, while $\Delta fadBA145$ mutant shown the same growth pattern as $\Delta fadBA1$-5 quintuple mutant picking up slightly after 24 h and grew to an even lower final cell density of OD_{540} of ~0.4. The same growth pattern was detected on all the other fatty acids tested. Slight growth of the $\Delta fadBA145$ triple mutant and $\Delta fadBA1$-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 $\Delta fadBA145$ 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 $\Delta fadBA145$ mutant, because the $\Delta fadBA145$ 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

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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 *fadBA1,4,5*-operons in Fad and PC degradation.

It was also found that these three *fadBA*-operons are not equally involved in the metabolism of FAs, and have different substrate preferences. The *fadBA5*-operon plays the most significant role in LCFA degradation, while the *fadBA1*-operon is more important for MCFA ($C_{12:0}$). The *fadBA4*-operon displays less involvement in metabolizing the FAs tested compared to both *fadBA1* and *fadBA5*-operons. I complemented Δ*fadBA145* mutant strain by integrating miniCTX2-*fadBA5* as a single copy into the Δ*fadBA145* mutant background (Table 1). The complemented strain was fully restored to wild type growth on PC, further supporting the role of the *fadBA5*-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 Δ*fadAB145*Δ*betBA* and Δ*fadAB145*Δ*glpD*, and a triple pathway mutant Δ*fadAB145*Δ*betBA*Δ*glpD* (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 Δ*fadAB145,* the double pathway mutants Δ*fadAB145*Δ*betBA* and Δ*fadAB145*Δ*glpD*, and a triple pathway mutant Δ*fadAB145*Δ*betBA*Δ*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 Δ*fadAB145*Δ*betBA*Δ*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 ($\Delta fadAB145$, $\Delta fadAB145\Delta betBA$, $\Delta fadAB145\Delta glpD$, $\Delta fadAB145\Delta betBA\Delta glpD$) 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, $C_{18:1}{}^{\Delta 9}$, 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, $C_{18:1}{}^{\Delta 9}$, choline, or glycerol (Fig. 11B).

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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 (Δ*fadAB145*Δ*betBA*Δ*glpD* 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 (Δ*fadAB145*, Δ*fadAB145*Δ*betBA*, Δ*fadAB145*Δ*glpD*) were higher than that of the triple pathway mutant group (Fig. 11 B), with Δ*fadAB145*Δ*betBA* showing the greatest decrease in competitive fitness *in vivo* compared to its complement and the Δ*fadAB145* 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 (Δ*fadAB145*, Δ*fadAB145*Δ*betBA*, Δ*fadAB145*Δ*glpD*) 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 (Δ*fadAB145*Δ*betBA*Δ*glpD* 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, *fadD2, betA, glpK, and glpD,* were expressed by *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 *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 *P. aeruginosa* are well characterized. However, the Fad by *P. aeruginosa* and the genes involved in this process remain to be elucidated. Many genes involved in Fad were shown to be expressed by *P. aeruginosa,* e.g. *fadD1, fadD2, fadA5* and *fadB5* [127]. It was also reported that mutants defective in Fad had decreased fitness during mouse lung infection, and mutations in *fadD* genes influenced two modes of motility and virulence factor expression (proteases, phospholipases, rhamnolipids, and lipases) [112]. With a larger genome, *P. aeruginosa* could potentially have more FA degradation genes than *E. coli,* indicating possible redundancies and higher levels of complexity in this pathway [128]. Up to six *fadD* homologues in *P. aeruginosa* have been investigated [128]. Two *fadBA*-operons homologues, *fadBA1* and *fadBA5*, have been studied so far. The *fadAB1* (PA1736 and PA1737) operon was shown to be strongly induced by medium-chain FAs, and to a lesser extent, LCFA [110]. The *fadBA5* (PA3014 and PA3013) operon was determined to be involved in LCFA degradation and to be induced by LCFA [3]. Three additional *fadBA*-operons (*fadBA2, fadBA3, fadBA4*) in *P. aeruginosa* together with *fadBA1* and *fadBA5* are investigated here. Triple mutants (Δ*fadBA125*, Δ*fadBA135*, Δ*fadBA145*, Δ*fadBA235*, Δ*fadBA245,* Δ*fadBA345*) and a quintuple mutant Δ*fadBA1-5* (Table 1) were used for growth analysis on MCFA and LCFAs as sole carbon sources, to further characterize the

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fadBA-operons. Interestingly, the Δ*fadBA145* triple mutant showed the same growth defect as the Δ*fadBA1-5* 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 ($C_{12:0}$). The *fadBA4*-operon displays less involvement in metabolizing fatty acids tested compared to both *fadBA1* and *fadBA5*-operons. However, the lack of growth for Δ*fadBA145* on MCFAs and LCFAs strongly indicates that these three operons are significantly involved in MCFAs and LCFAs metabolism. Furthermore, the Δ*fadBA145* 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 Δ*fadAB145*Δ*betBA* and Δ*fadAB145*Δ*glpD*, and a triple pathway mutant Δ*fadAB145*Δ*betBA*Δ*glpD* (Table 1) were engineered to further determine whether these mutants were deficient in growth on PC. As expected, Δ*fadAB145*Δ*betBA* and Δ*fadAB145*Δ*glpD* exhibited reduced growth capability compared to Δ*fadAB145*. The triple pathway mutant, Δ*fadAB145*Δ*betBA*Δ*glpD*, 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

Δ*fadD1D2D3D4D5D6* mutant exhibited more growth defect in FAs and PC compared to Δ*fadD1D*2, correspondingly this mutant was less fit in mice lungs than Δ*fadD1D*2 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 Δ*fadAB145ΔbetBAΔglpD* 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 (Δ*fadAB145*, Δ*fadAB145ΔbetBA*, Δ*fadAB145ΔglpD*) groups were higher than that of the triple pathway mutant group (Fig. 11 B). Δ*fadAB145ΔbetBA* exhibited the greatest decrease in competitive fitness *in vivo* compared to its complement and the Δ*fadAB145* 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 (Δ*fadAB145*, Δ*fadAB145ΔbetBA*, Δ*fadAB145ΔglpD*) 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 (Δ*fadAB145ΔbetBAΔglpD* 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 3.6×10^8

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].

Strain	Lab ID	Genotype/Description	Reference
E.coli			
EP-Max10B	E1231	F⁻ λ⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ* Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galKrpsL nupG*	BioRad
SM10	E006	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu Km^R	[129]
HPS1-*mob* - Km/*Δasd* /*pir116*	E0464	Cm^r, Km^r, Tet^r; e14⁻ (*mcrA*) *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *Δ* (*lac-proAB*) *rif zxx* ::miniTn5 Lac4 (*lacI^q* lacZΔ M15) *Δasd*::FRT *uidA* ::*pir116 recA* ::RP4-2Tc ::*Mu* –Km^r	This study
P. aeruginosa			
PAO1	P007	Prototroph	[130]
Δ*fadAB125*	P122	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB2*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB135*	P124	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB3*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB145*	P319	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB4*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB235*	P317	Gm^r, PAO1-Δ*fadAB2*::*FRT*, Δ*fadAB3*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB245*	P130	Gm^r, PAO1-Δ*fadAB2*::*FRT*, Δ*fadAB3*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB345*	P126	Gm^r, PAO1-Δ*fadAB3*::*FRT*, Δ*fadAB4*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB1-5*	P102	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB2*::*FRT*, Δ*fadAB3*::*FRT*, Δ*fadAB4*::*FRT*, Δ*fadAB5*::Gm	This study
Δ*fadAB145* Δ*glpD*	P539	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB4*::*FRT*, Δ*fadAB5*::*FRT*, Δ*glpD*::Gm-*FRT1*	This study
Δ*fadAB145* Δ*betBA*	P555	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB4*::*FRT*, Δ*fadAB5*::*FRT*, Δ*betBA*::Gm-*FRT3*	This study
Δ*fadAB145* Δ*betBA* Δ*glpD*	P561	Gm^r, PAO1-Δ*fadAB1*::*FRT*, Δ*fadAB4*::*FRT*, Δ*fadAB5*::*FRT*, Δ*betBA*::*FRT3*, Δ*glpD*::Gm-*FRT1*	This study
Δ*fadAB145/* complement	P965	Gm^r, Tet^r*;* Δ*fadAB145* complemented with miniCTX2-*fadAB5*	This study
Δ*fadAB145* Δ*glpD/* complement	P1015	Gm^r, Tet^r*;* Δ*fadAB145* Δ*glpD* complemented with miniCTX2-*fadAB5/glpD*	This study
Δ*fadAB145*	P1017	Gm^r, Tet^r*;* Δ*fadAB145* Δ*betBA*	This study

Δ*betBA/* complement		complemented with miniCTX2-*fadAB5/betBA*	
Δ*fadAB145* Δ*betBA* Δ*glpD/* complement	P1019	Gm^r, Tet^r; Δ*fadAB145* ΔbetBA Δ*glpD* complemented with miniCTX2-*fadAB5/betBA/glpD*	This study
Δ*fadAB145-mucA⁻*	P576	Gm^r, Cb^r; Δ*fadAB145* with pUC18 inserted in *mucA* gene	This study
Δ*fadAB145* Δ*glpD-mucA⁻*	P570	Gm^r, Cb^r; Δ*fadAB145* Δ*glpD* with pUC18 inserted in *mucA* gene	This study
Δ*fadAB145* Δ*betBA-mucA⁻*	P572	Gm^r, Cb^r; Δ*fadAB145* Δ*betBA* with pUC18 inserted in *mucA* gene	This study
Δ*fadAB145* Δ*betBA* Δ*glpD-mucA⁻*	P574	Gm^r, Cb^r; Δ*fadAB145* Δ*betBA* Δ*glpD* with pUC18 inserted in *mucA* gene	This study
Δ*fadAB145-mucA⁻/* complement	P584	Gm^r, Cb^r, Tet^r; Δ*fadAB145-mucA⁻* complemented with miniCTX2-*fadAB5*	This study
Δ*fadAB145* Δ*glpD-mucA⁻/* complement	P578	Gm^r, Cb^r, Tet^r; Δ*fadAB145* Δ*glpD-mucA⁻* complemented with miniCTX2-*fadAB5/glpD*	This study
Δ*fadAB145* Δ*betBA-mucA⁻/* complement	P580	Gm^r, Cb^r, Tet^r; Δ*fadAB145* Δ*betBA-mucA⁻* complemented with miniCTX2-*fadAB5/betBA*	This study
Δ*fadAB145* Δ*betBA* Δ*glpD-mucA⁻/* complement	P582	Gm^r, Cb^r, Tet^r; Δ*fadAB145* Δ*betBA* Δ*glpD-mucA⁻* complemented with miniCTX2-*fadAB5/betBA/glpD*	This study

[a] For strains constructed in this study, please see text for further details.

Table 2. Plasmids used in this study[a].

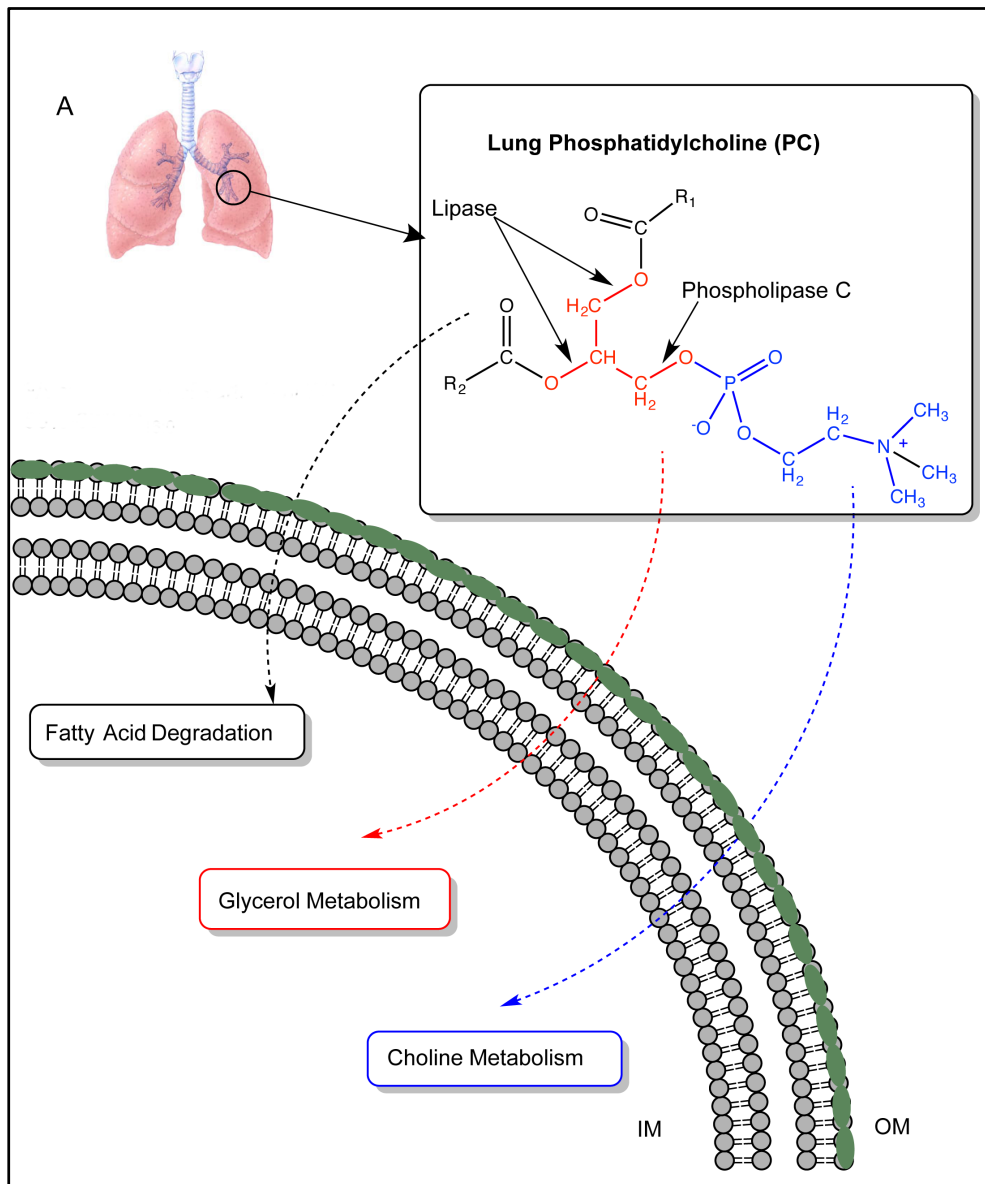| Plasmids	Lab ID	Relevant properties	Reference
pFlp2	E0067	Ap^r, *sacB*$^+$; Flp-containing plasmid	[118]
pPS856	E0050	Ap^r; Gm^r; plasmid with Gm^r-*FRT*-cassette	[118]
pUC18	E0135	Ap^r; cloning vector	[131]
pUC18-'*mucA*'	E1907	Ap^r; *mucA* internal region cloned into pUC18	This study
pUC19	E0014	Ap^r; cloning vector with P$_{lac}$	[131]
pUC19-*glpD*	E1843	Ap^r; pUC19 with *glpD* gene cloned in downstream of P$_{lac}$	This study
pEX18T	E0055	Ap^r, *ori*T$^+$, *sacB*$^+$; gene replacement vector	[118]
pEX18TΔ*fadAB1*::Gm	E0202	Ap^r, Gm^r; pEX18T with *ΔfadAB1 operon with* Gm^r-*FRT*-cassette insertion	This study
pEX18TΔ*fadAB2*::Gm	E0224	Ap^r, Gm^r; pEX18T with *ΔfadAB2 operon with* Gm^r-*FRT*-cassette insertion	This study
pEX18TΔ*fadAB3*::Gm	E0225	Ap^r, Gm^r; pEX18T with *ΔfadAB3 operon with* Gm^r-*FRT*-cassette insertion	This study
pEX18TΔ*fadAB4*::Gm	E0226	Ap^r, Gm^r; pEX18T with *ΔfadAB4 operon with* Gm^r-*FRT*-cassette insertion	This study
pEX18TΔ*fadAB5*::Gm	E0461	Ap^r, Gm^r; pEX18T with *ΔfadAB5 operon with* Gm^r-*FRT*-cassette insertion	This study
pEX18TΔ*glpD*::Gm	E1066	Ap^r, Gm^r; pEX18T with *ΔglpD operon with* Gm^r-*FRT*-cassette insertion	This study
pEX18TΔ*betBA*::Gm	E1070	Ap^r, Gm^r; pEX18T with *ΔbetBA operon with* Gm^r-*FRT*-cassette insertion	This study
miniCTX2	E0076	Tet^r; site-specific integration vector	[119]
miniCTX2-*fadAB5*	E1765	Tet^r; miniCTX2 with cloned *fadAB5*	This study
miniCTX2-*fadAB5/glpD*	E2035	Tet^r; miniCTX2 with cloned *fadAB5/glpD*	This study
miniCTX2-*fadAB5/betBA*	E1953	Tet^r; miniCTX2 with cloned *fadAB5/betBA*	This study
miniCTX2-*fadAB5/betBA/glpD*	E1992	Tet^r; miniCTX2 with cloned *fadAB5/betBA/glpD*	This study

[a] For plasmids constructed in this study, please see text for further details.
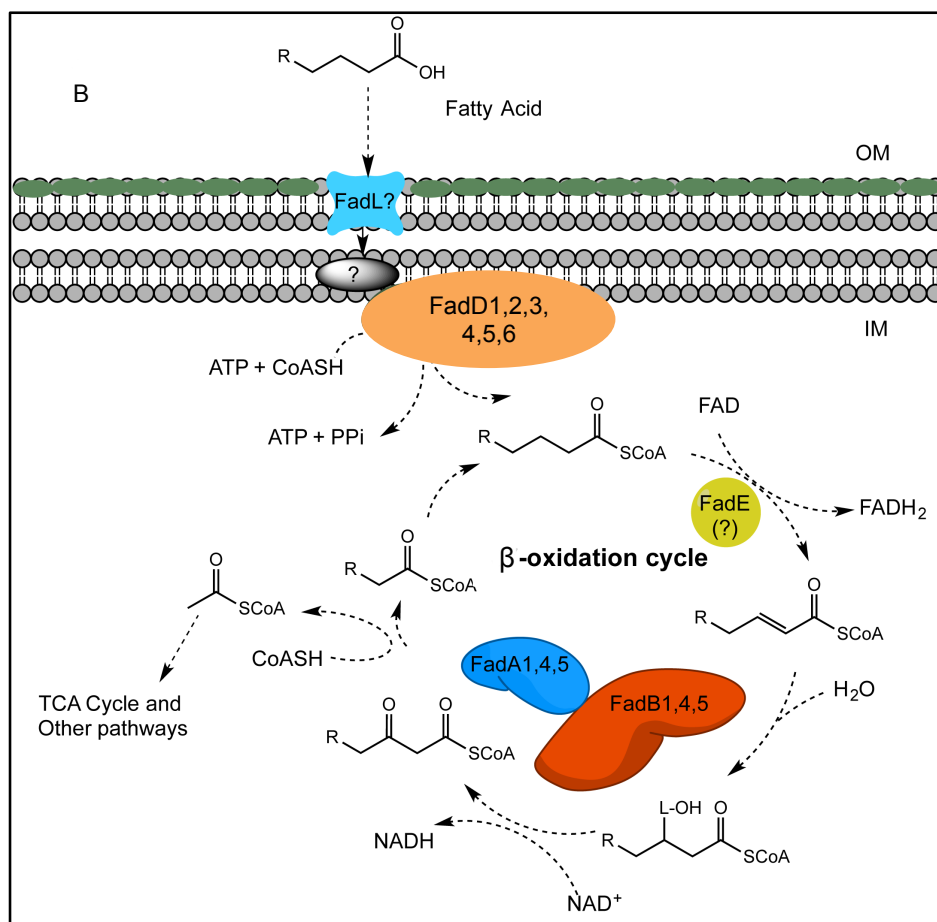
Table 3. Primers used in this study.

Primer number and name	Sequence[a]
186; *fadAB1*-upstream	5'-CGA<u>AAGCTT</u>GCATGGTGCTATCTTCC-3'
187; *fadAB1*-downstream	5'-GCG<u>GAATTC</u>GCCCTACCCGTGGCG-3'
218; *fadBA2*-upstream	5'-CGGTG<u>AAGCTT</u>TCGCGCACC-3'
219; *fadBA2*-downstream	5'-GGG<u>GAATTC</u>GGTGTCTCATCGGCAGCGC-3'
220; *fadBA3*-upstream	5'-GCG<u>AAGCTT</u>ATTCAGCAGGAGAAAACGACG-3'
221; *fadBA3*-downstream	5'-TGC<u>GAATTC</u>GACGGATAGTCGCCGCTAC-3'
211; *fadBA4*-upstream	5'-CGT<u>AAGCTT</u>GCCGGGGAGTCAGGGGC-3'
212; *fadBA4*-downstream	5'-CCC<u>GAATTC</u>GCACGGCACCGCCCAAG-3'
272; *fadBA5*-HindIII	5'-AGTC<u>AAGCTT</u>CCATAATAGC-3'
273; *fadBA5*-EcoRI	5'-CCC<u>GGAATTC</u>CCCCTTCGAGAACGCTTAG-3'
518; *glpK*-BamHI	5'-AGCTGAAGT<u>GGATCC</u>TCGACAA-3'
519; *glpKD*-SacI	5'-CTGGC<u>GAGCTC</u>AGGCCGCATGCACCCG-3'
522; *betA*-SacI	5'-CAAC<u>GAGCTC</u>GGCGATATCTACGGCGG-3'
523; *betB*-HindIII	5'-GCCA<u>AAGCTT</u>CCAGGACAAGAACGGCT3'
888; Xho-*fadB5*	5'-CCTGCGCAGAGGGC<u>CTCGAG</u>AGGGC-3'
889; *fadB5*-Bam	5'-GGGCACGA<u>GGATCC</u>CCGGCTTTCCCC-3'
895; Spe-*betB*	5'-CGGATTCAG<u>ACTAGT</u>ACCTGCTCG-3'
896; Hind-*glpD*	5'-GCCTGGTG<u>AAGCTT</u>CGGGCTGGTC-3'
927; SacI-P$_{lac}$-*glpD*	5'-CGCTCGCCG<u>GAGCTC</u>GAACGACCGAGC-3'
1039; *fadAB1*-XbaI	5'GGACGTCTAGAACAGGATAAGAGAGACC-3'
1040; *fadAB1*-HindIII	5'-GAGTCCTGACGTCGCATGGAAGCTTAGCC-3'
1495; FadA4-SacI-SpeI	5'AGTGGAGCTCACTAGTCAAGTCACGGAGTTGCTTACATC-3'
1496; FadB4-SacI	5'-AAGCGAGCTCGGTGCAGGGGCAGATAACG-3'
1881; *fadA5* down	5'-CGTCATTCGCTCGATACAGCC-3'
1882; *fadB5* up	5'-GGCCTCGACAATAGAGACCC-3'

[a]Restriction enzyme sites utilized in this study are underlined.

Figures

Lung Phosphatidylcholine (PC)

Lipase

Phospholipase C

Fatty Acid Degradation

Glycerol Metabolism

Choline Metabolism

IM

OM

Fatty Acid

OM

FadL?

?

FadD1,2

ATP + CoASH

ATP + PPi

FAD

R   SCoA

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 phospharylcholine. 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.
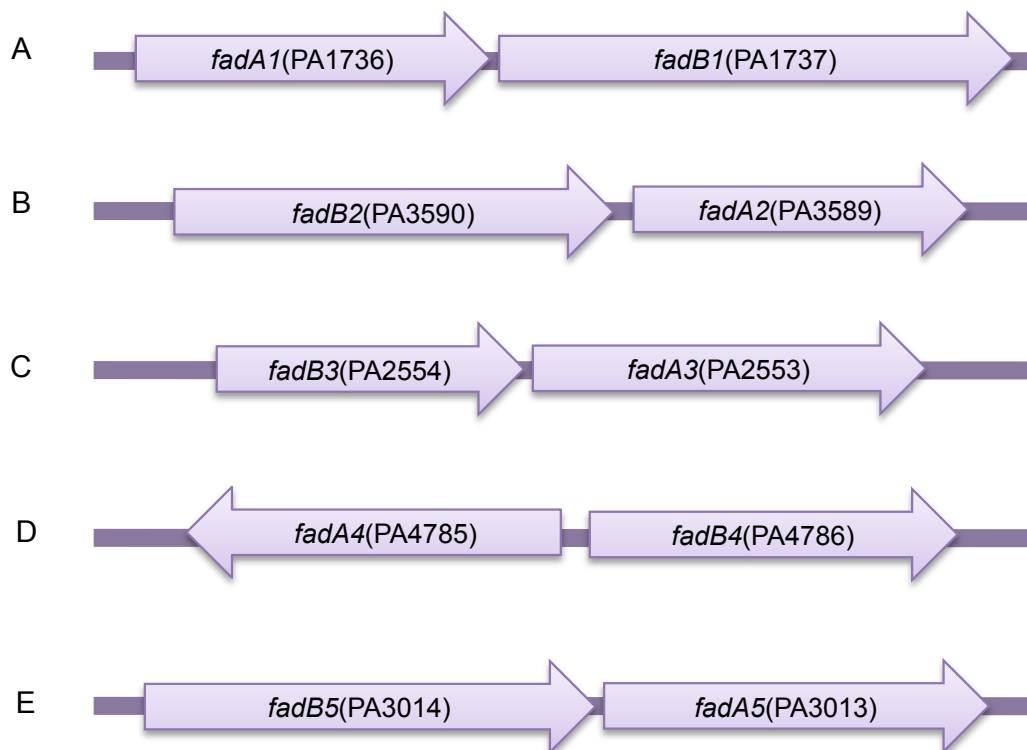
Figure 2. **Five potential *fadBA*-operon homologues of *P. aeruginosa*.** Each gene of the operon is shown in light purple. (A*) fadAB1* is 3.363 kb, (B*) fadBA2* is 2.760 kb, (C) *fadBA3* is 2.346 kb, (D) *fadAB4* is 2.887 kb, and (E) *fadBA5* is 3.353 kb.
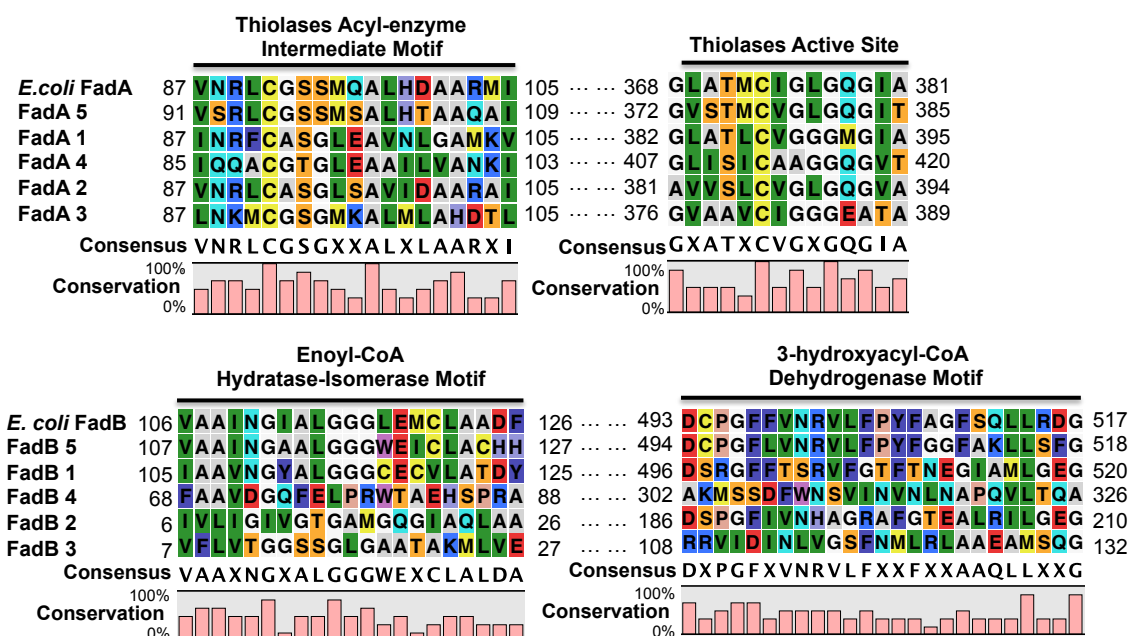
Figure 3. **Comparison of *P. aeruginosa* FadBAs with *E. coli* FadBA.** *P. aeruginosa* FadAs and FadBs are aligned with *E. coli* FadA and FadB. Amino acids with similar properties are assigned the same colors using CLC Sequence Viewer 6 sequence FadBA5 shows the greatest similarity to the *E. coli* FadBA.
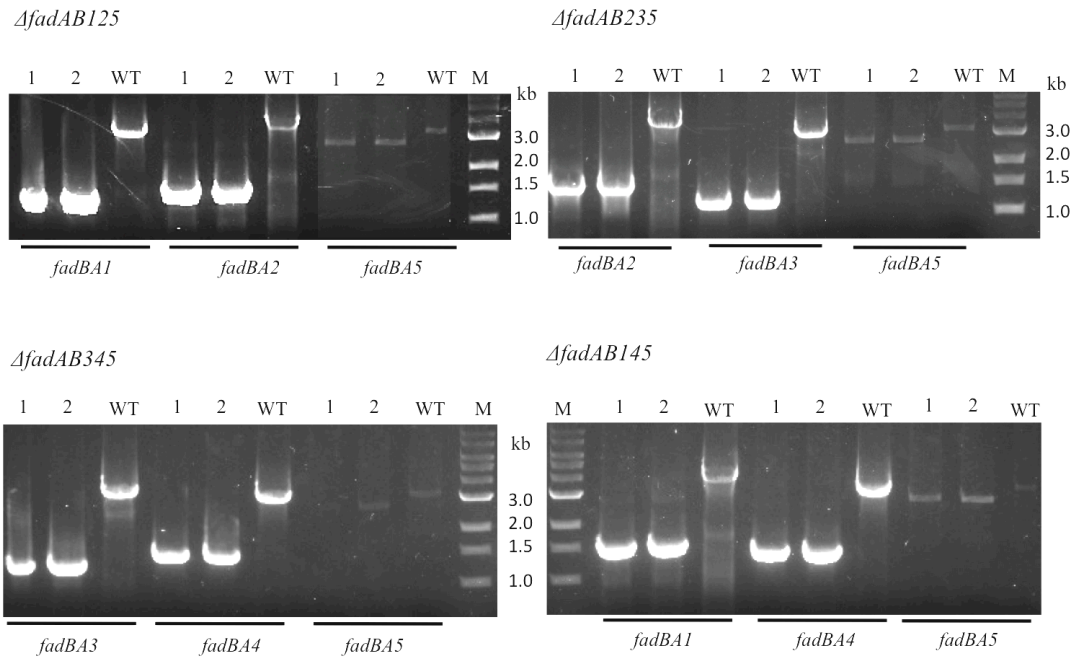
Figure 4. PCR confirmation of *fadBA* mutant strains. The PCR results for Δ*fadBA125*, Δ*fadBA235,* Δ*fadBA345,* Δ*fadBA145* are shown. Two independent isolates of each mutant strain were picked for PCR confirmation as shown in lane 1 and lane 2. Five *fadBA*-operons (1,2,3,4,5) were amplified with primers 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.
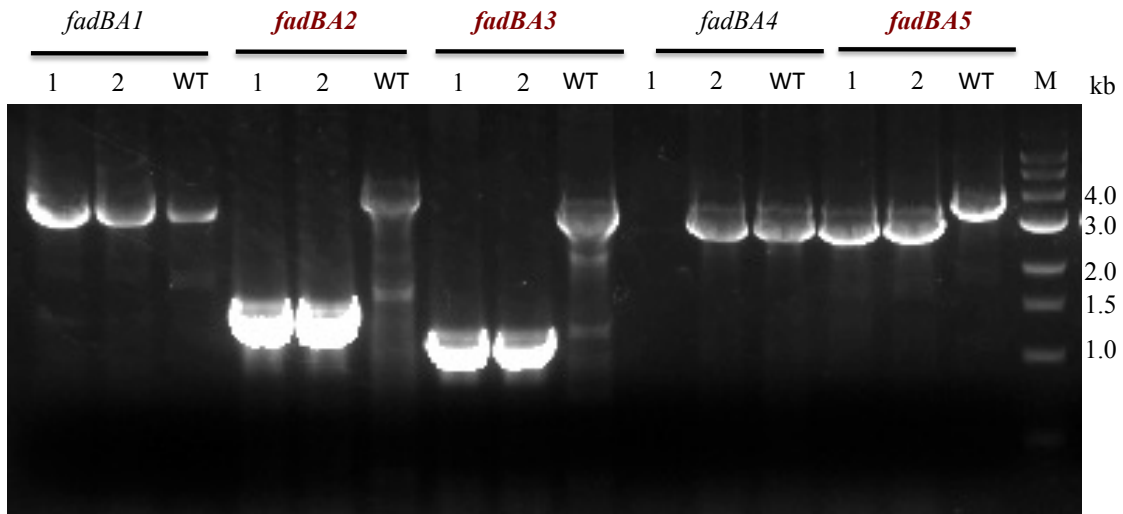
Figure 5. PCR verification of the mutant strains at the end of growth analysis. All of the mutants were confirmed, a representative gel picture was shown here for Δ*fadBA125*. Lane 1 and 2 represent two independent colonies. Primers 1881/1882 were used for amplifying *fadBA5*-operon: wldtype ~ 3.4 kb, 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.
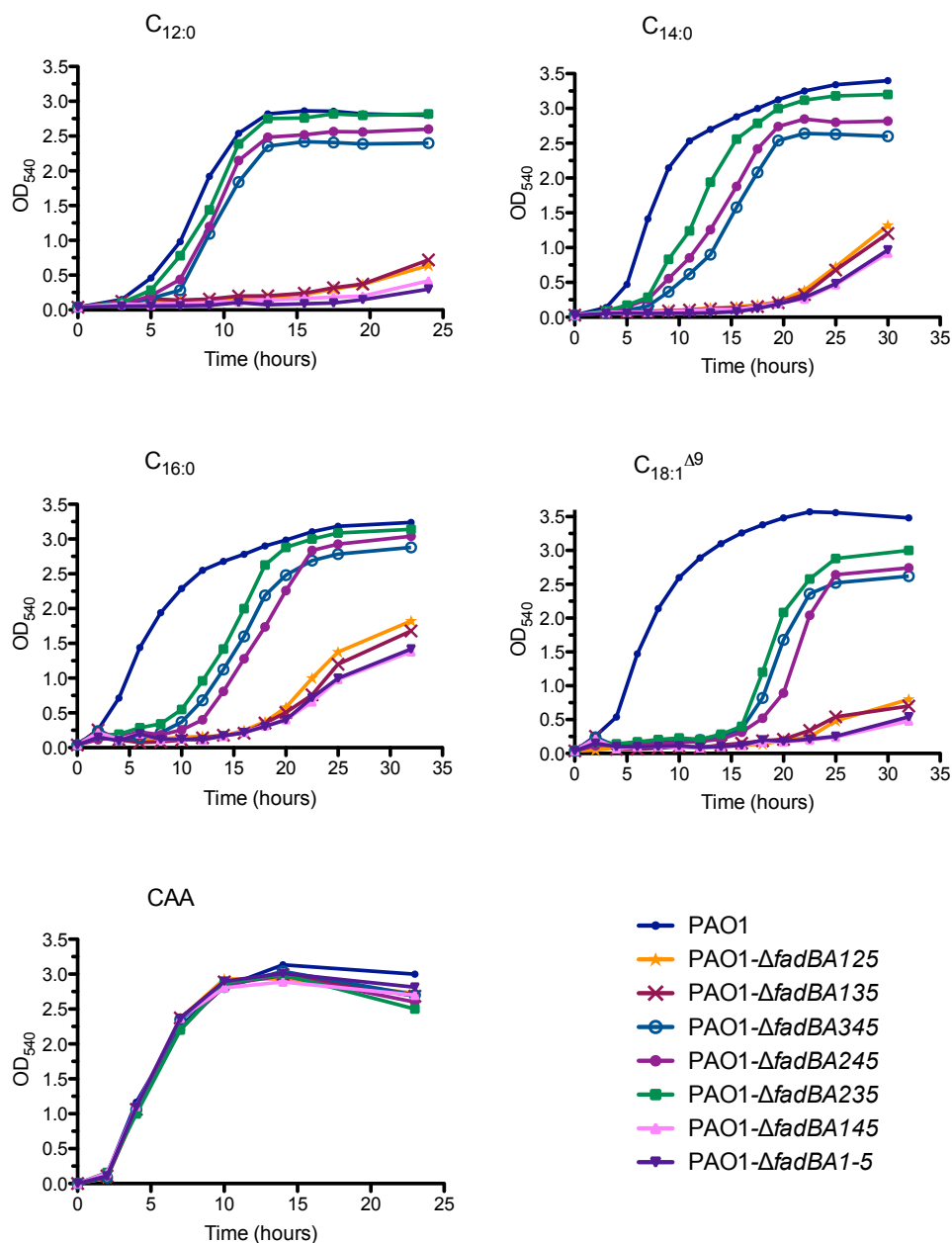
Figure 6. **Growth analysis of different *fadBA* mutants on medium and long chain-length fatty acid ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$ and $C_{18:1}^{\Delta 9}$).** Along with wild type PAO1, mutants were grown in 1X M9 minimal medium supplemented with 1% CAA or 0.4% different FAs as sole carbon source. 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.

PC Growth Curve
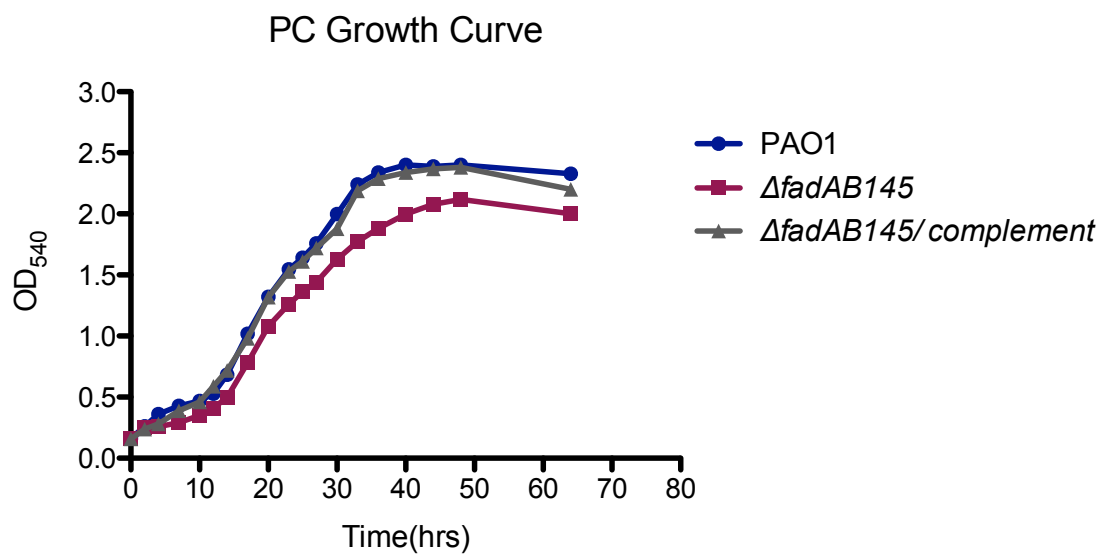
Figure 7. **Growth analysis of *ΔfadBA1,4,5* mutant on PC as a sole carbon source.**
The mutant *ΔfadBA145* exhibited growth defects on PC as a sole carbon source. The
growth defects were fully recovered in complemented strains, as they had identical
growth rates compared to the wild type PAO1 strain.

PC Growth Curve

Legend:
- PAO1
- ΔfadAB145
- ΔfadAB145 ΔglpD
- ΔfadAB145 ΔbetBA
- ΔfadAB145 ΔbetBA ΔglpD
- ΔfadAB145/ complement
- ΔfadAB145 ΔglpD/ complement
- ΔfadAB145 ΔbetBA/ complement
- ΔfadAB145ΔbetBAΔglpD/ complement
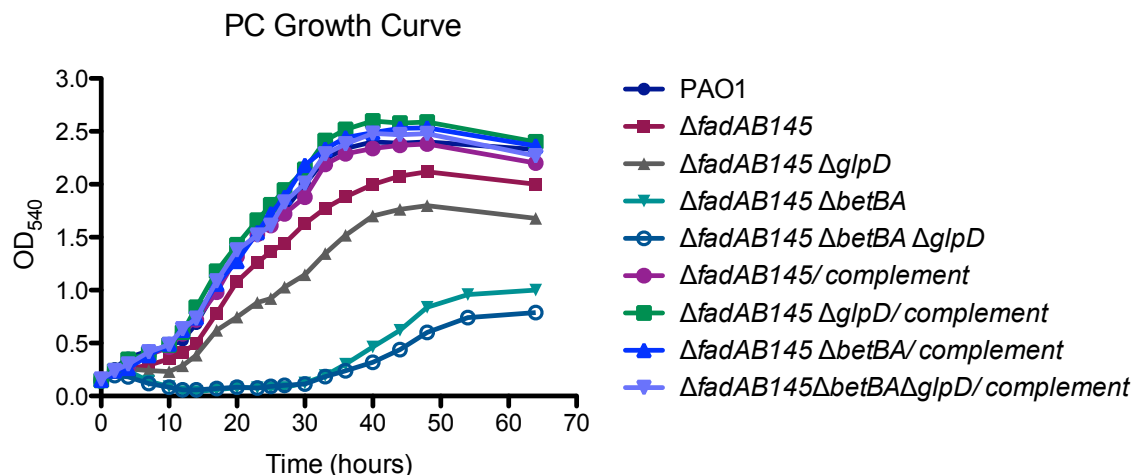
Figure 8. Growth analysis of various pathway mutants on PC. The Fad pathway mutant Δ*fadAB145,* the double pathway mutants Δ*fadAB145ΔbetBA* and Δ*fadAB145ΔglpD*, and a triple pathway mutant Δ*fadAB145ΔbetBAΔglpD* were used in this study. The mutants exhibited various growth defects on PC as a sole carbon source. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wild type PAO1 strain.

A Protease Production

B Rhamnolipid production

C Hemolysin Production

D Lipase production

E

LB Growth Curve

PAO1
ΔfadAB145
ΔfadAB145 ΔglpD
ΔfadAB145 ΔbetBA
ΔfadAB145 ΔbetBA ΔglpD
ΔfadAB145/ complement
ΔfadAB145 ΔglpD/ complement
ΔfadAB145 ΔbetBA/ complement
ΔfadAB145ΔbetBAΔglpD/complement
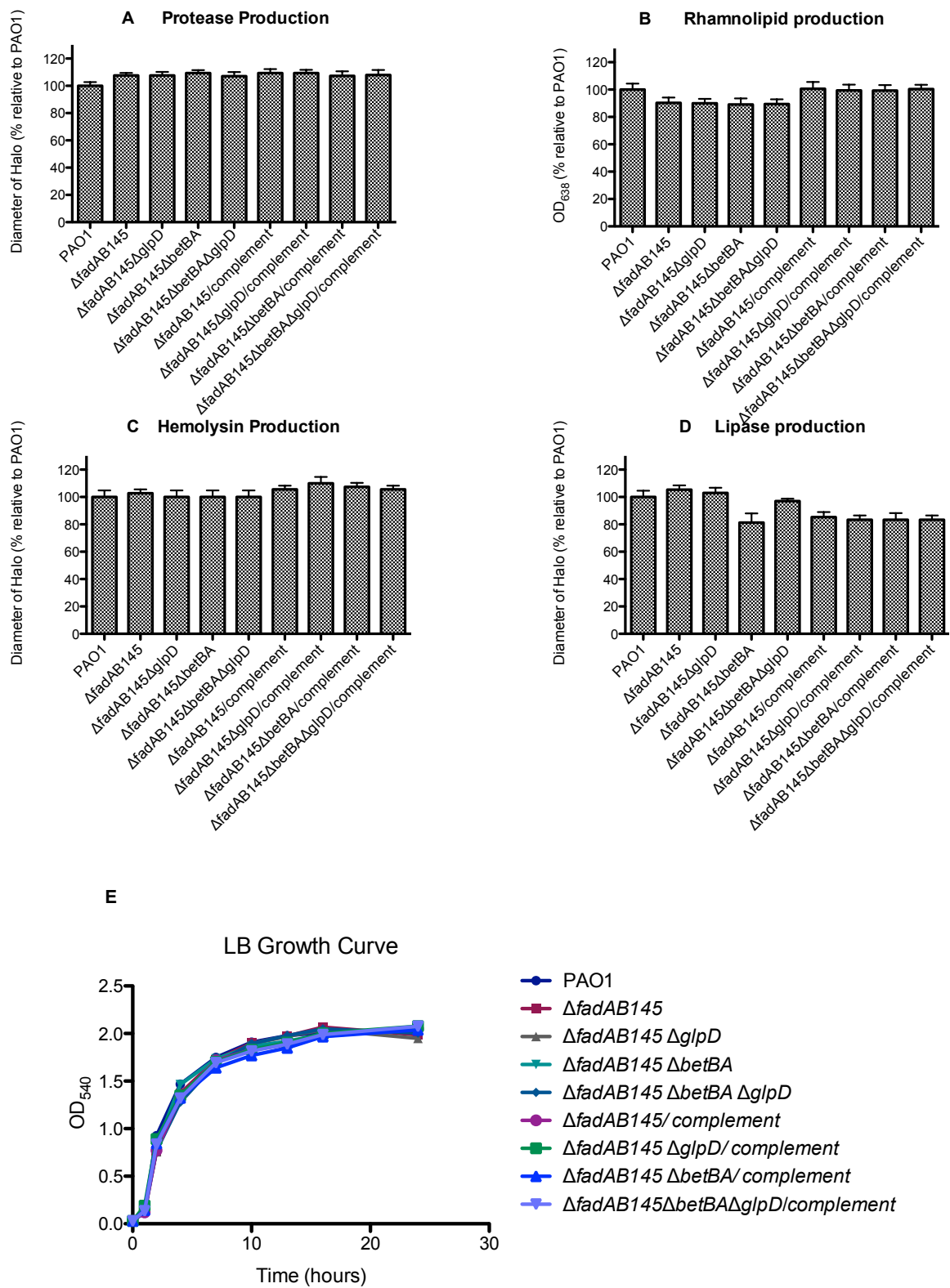
Figure 9. Analyses of protease, hemolysin, lipase, and rhamnolipid production by *P. aeruginosa* triple pathway mutant. The triple pathway mutant did not display significant decrease in production of protease (A), rhamnolipid (B), hemolysin (C), and lipase (D), and no growth defects in LB (E) were observed.

A. Protease Production

Skim Milk Agar Plate

B. Hemolysin Production

Blood Agar Plate

C. Lipase Production

Rhodamine B Agar Plate
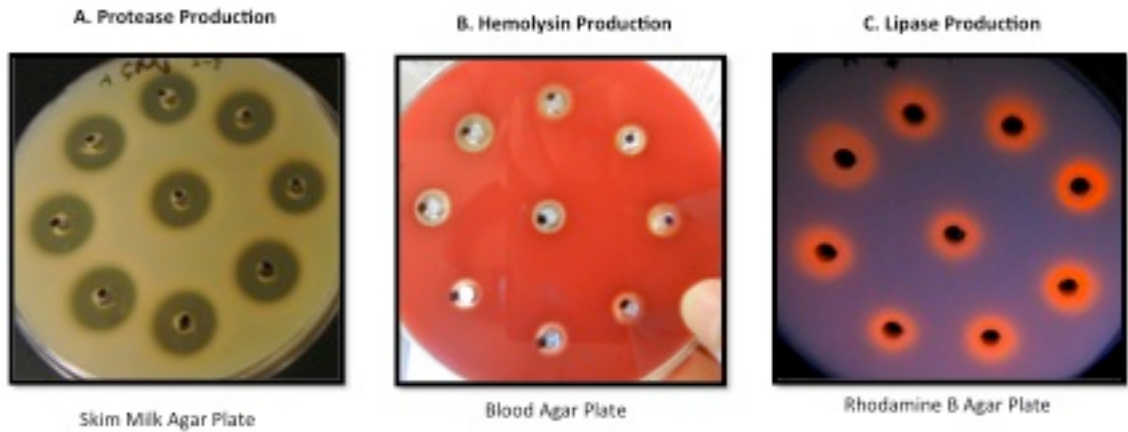
Figure 10. Detection of virulence factors produced by *P. aeruginosa.* The production of virulence factors were monitored for all the pathway mutants and their complement strains. Representative plates are shown, performed in triplicate. Diameters of the clear zone were measured for skim milk agar plate and blood agar plate. The diameter of the orange halo was measured by exposing the rhodamine B agar plate a UV transilluminator.
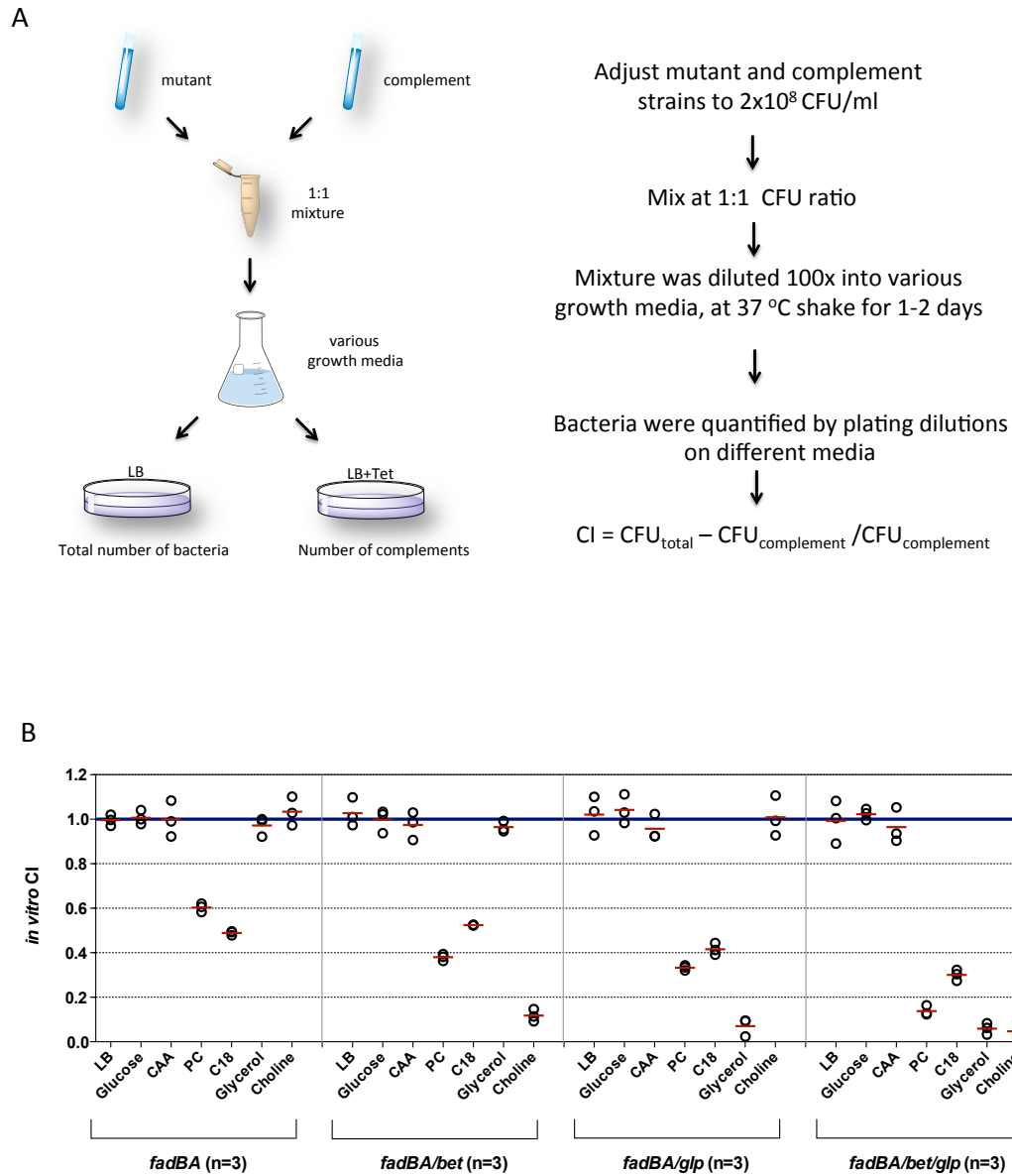
Figure 11. *In vitro* **competition studies of the various mutants and their complemented strains in different growth media.** A. Flow chart demonstrating *in vitro* competition study. B. n equals the number of independent *in vitro* competition experiments performed with each carbon source, The solid 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.
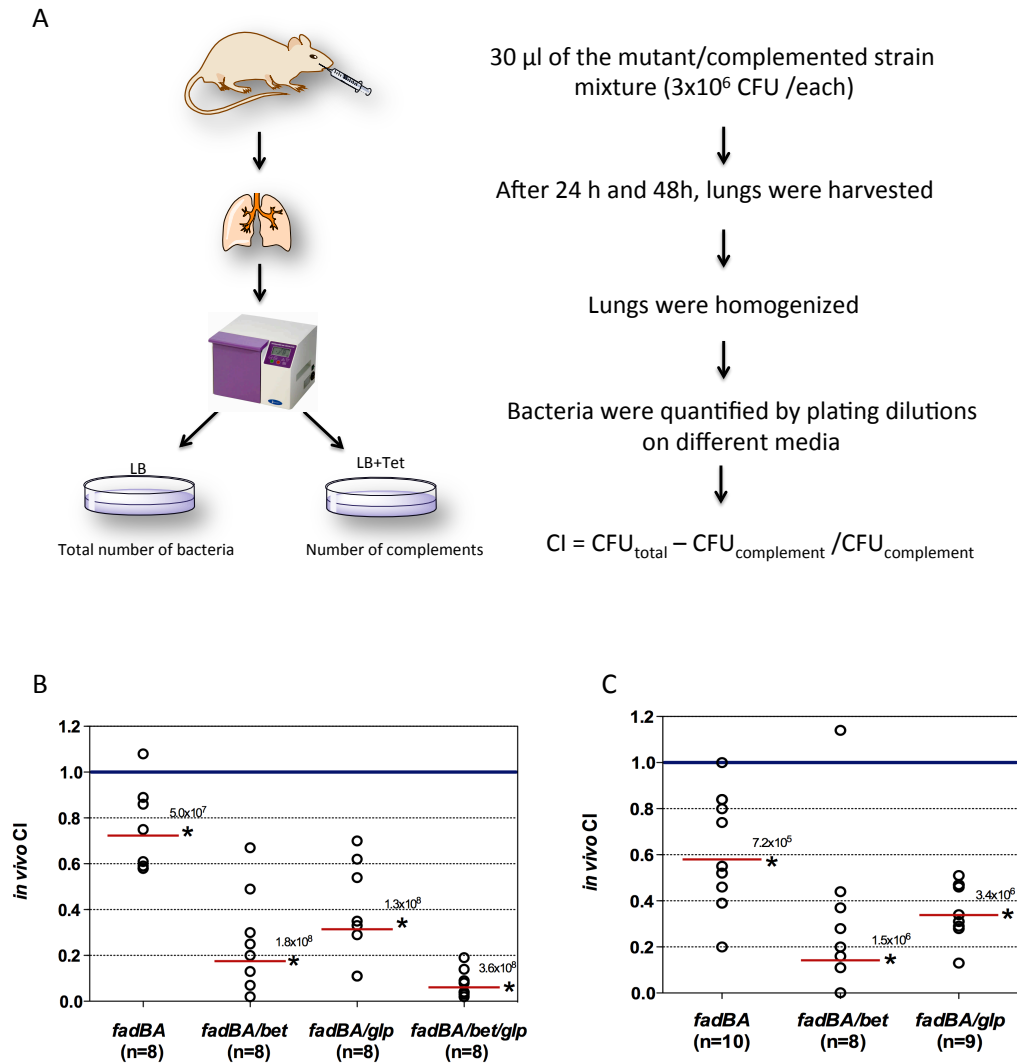
Figure 12. *In vivo* competition study. *In vivo* lung competition of the various mutants and their complemented strains after 24 h (B) and 48 h (C). The experiment was carried out by following the flow chart (A). n equals the number of mice in each group that were inoculated with a total of 6×10^6 CFU/mouse. 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.

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