NUMEROUS FATTY ACYL-COA SYNTHETASE HOMOLOGUES
ARE INVOLVED IN FATTY ACID DEGRADATION IN
Pseudomonas aeruginosa

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Abstract

Although fatty acid degradation pathway (Fad) is well established in *Escherichia coli*, is has not been fully characterized in *Pseudomonas aeruginosa*. Fatty acid catabolism in *P. aeruginosa* has been shown to contribute to degradation of major lung surfactant component phosphatidylcholine. Previously two fatty acyl-CoA synthetase genes (*fadD1* and *fadD2*), responsible for first step in Fad, have been characterized and determined to affect virulence factors expression.

Additional fatty acyl-CoA synthetase gene, *fadD3*, has been identified. Its role in Fad was determined through enzyme assay, growth curve studies, and induction studies. During the course of this work, all *fadD* homologues necessary for Fad were indentified. Through mutational analysis contribution of each fatty acyl-CoA synthetase gene to fatty acid catabolism was determined.

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Thank you to my parents and my younger brother for their love, support, and encouragement.

Dedication

This work

is dedicated to my

parents

Grazyna Zarzycka-Siek and Janusz Siek

and to my

brother Jerzy

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List of Abbreviations

aa	amino acid/amino acids
AMP	adenosine monophosphate
Ap	ampicillin
Ap^r	ampicillin resistant/resistance
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CAA	casamino acids
Cb	carbenicillin
CIP	calf intestinal alkaline phospotase
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator gene
CFU	colony forming unit
CoASH	coenzyme A
DAP	diaminopimelic acid
DDW	double deionized water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
Ec	*Escherichia coli*

EDTA	ethylendiamionotetraacetic acid
FA	fatty acid
FACS	fatty acyl-CoA synthetase
Fad	fatty acid degradation
FAD	flavin adenine dinucleotide
$FADH_2$	reduce flavin adenine dinucleotide
flp	gene encoding *Sacchaomyces cerevisiae* recombinase
FRT	Flp recognition target
g	gram
g	gravitational force
Gm	gentamicin
Gm^r	gentamicin resistant/resistance
h	hour
IPTG	isopropyl β-D-1-thiogalactopyranoside
k	kilo
kb	kilobase
k_{cat}	enzyme catalytic efficiency
kDa	kiliodalton
K_m	Michaelis constant
Km	kanamycin
Km^r	kanamycin resistant/resistance
l	liter
LB	Luria-Bertani

LCFA	long chain fatty acid
M	molar
Km	kanamycin
m	milli
Mb	mega base pair
MCAC	metal-chelate affinity chromatography
MCFA	medium chain fatty acid
m	milli
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide
n	nano
NADH	reduced nicotinamide adenine dinucleotide
nmol	nanomol
OD	optical density
ONPG	2-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
oriT	origin of transfer for conjugation
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine

PCR	polymerase chain reaction
PIA	Pseudomonas isolation agar
PIB	Pseudomnas isolation broth
pheS	gene encoding a mutated α-subunit of phenylalanyl tRNA synthase
Pa	*Pseudomoans aeruginosa*
PP_i	pyrophosphate
QS	quorum sensing
s	second
sacB	*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 three secretion system
Tet	tetracycline
Tet^r	tetracycline resistant/resistance
TNB	2-nitro-5-thiobenzoic acid
Tris-HCl	tris-hydrochoride
μ	micro

μg	microgram
μl	micoliter
μM	micromolar
U	unit
UV	ultraviolet light
v	volume
V_{max}	maximum activity
w	weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
°C	degrees Celsius
%	percentage

Chapter One: Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was first isolated as a pure culture by a French physician, Gessard, in 1882 from the cutaneous wounds of two patients that showed blue-green discoloration [1]. This blue-green color is due to two pigments made by this bacterium: pyoverdin, a fluorescent yellow pigment, and pyocyanin, a blue pigment [2]. Because of the blue-green color production, this microbe was first named *Bacillus* (rod) *pyo* (pus) *cyaneus* (blue) and has been also known as the "bacteria of blue pus" [1]. The current name of this bacterium comes from the historical term for an etiological unit, which caused infection: *monad* (derived from the Greek word meaning unit) and the prefix *pseudo* most likely was given because of variable cellular morphology observed in pseudomonads: which can be either curved or straight rods [3]. The term *aeruginosa* is derived from the Latin word for copper rust and refers to the green coloration produced by this bacterium [3].

P. aeruginosa is a Gram negative gamma proteobacteria, 1 to 3 μm long and 0.5 to 1 μm wide and is motile by means of a single flagellum [4, 5]. This aerobic bacillus is commonly found in soil, water, and plant surfaces [4 - 7], and it can grow at a variety of temperatures, ranging from 5°C to 42°C [7]. *P. aeruginosa* is capable of a dimorphic lifestyle meaning it can exist as planktonic, free swimming cells or as an aggregation of cells (mirocolonies) enclosed in slime like exopolysaccharides (a biofilm). In this biofilm form, *P. aeruginosa* can easily colonize various living and non-living surfaces and is protected from environmental stress (e.g. the host immune system or antibiotics) [8 - 10]. The ability to thrive in so many environments requires diverse metabolic capabilities [11]

and *P. aeruginosa* has a relatively large and complex genome (6.3 Mb [12] when compared to *Bacillus subtillis*, 4.2 Mb [13], *Escherichia coli*, 4.6 Mb [14], *Mycobacterium tuberculosis,* 4.4 Mb [15], and *Haemophilus influenzae*, 1.8 Mb [16]), with hundreds of genes responsible for transport and metabolism of a variety of nutrients [12]. *P. aeruginosa* is known to metabolize over 70 organic substances as sole carbon and energy sources (e.g. citrate, ribose, xylose, glycerol, fructose, glucose, mannose, acetate, as well as fatty acid of different length: $C_{4:0}$ - $C_{18:1}{}^{\Delta9}$) [7, 17]. Flexible nutritional requirements, a ubiquitous nature, the ability to form biofilms, and an array of virulence factors produced [2, 18], allows *P. aeruginosa* to be a opportunistic pathogen capable of infecting a broad range of hosts including microbes (e.g. amoeba [19]), plants (e.g. lettuce and thale cress [18, 20]), animals (e.g. *Caenorhabditis elegans* [21], minks and chinchillas [22 - 24], zebrafish [25], fruit flies [26]), and humans [27].

1.2 *P. aeruginosa* Virulence Mechanisms

P. aeruginosa produces multiple virulence factors which make this bacterium a successful opportunistic pathogen (e.g. pili, flagellum, exotoxins, biofilm, phospholipases, lipases, and proteases) [2, 18, 20, 28 - 31]. Many of its virulence factors (e.g. elastases, proteases, pyocyanin, exotoxin A, and biofilm maturation) are regulated by quorum sensing (QS) which allows coordinated expression of genes across a whole bacterial population when conditions are right for population. *P. aerugionsa* expresses virulence genes through QS only when a high-cell density is achieved, thus ensuring that any host immune response will not be able to destroy the whole bacterial population [2, 32].

P. aeruginosa possess flagella and type IV pili which allow it to invade a site of a host body and adhere to the cell membrane of healthy and unhealthy epithelial cells present at that site [2, 32]. Once attached, *P. aeruginosa* can produce other virulence factors (e.g. proteases and exotoxins) which have a localized effect, thereby increasing damage to the host [2] and, due to siderophores production (pyoverdin, and pyochelin), it has no problem in acquiring iron by sequestering it from the site of infection [33]. Furthermore, these pigments inhibit ciliary beating (mainly in the lung) reducing the host's ability to clear this microbe [2]. Impeded clearance of *P. aeruginosa* from the site of infection is also due to the establishment of a biofilm and the production of alginate which protects the bacteria from free radicals released by macrophages, providing a physical barrier against phagocytosis, and prevents chemotaxis of neutrophils and resulting complement activation [2, 32].

The type III secretion system (T3SS) is often activated by this bacterium once it is attached to epithelial cells. The *P. aeruginosa* T3SS is very similar to that of *Yersinia pestis* (the causative agent of Black Death) and is composed of a needle complex which facilitates the injection of effector toxins (ExoS, ExoT, ExoU, ExoY) into eukaryotic cells and is thought to have evolved as a defense mechanism against environmental predators [19, 34]. ExoU is a potent phospholipase which destroys cell membranes and causes rapid death of eukaryotic cells [34, 35]. ExoS and ExoT are bifunctional toxins that have both GTPase activity and ADP ribosyl-transferase activity and target many cytoplasmic enzymes/pathways resulting in cytoskeletal disruption, endocytosis prevention, DNA synthesis inhibition, and cell death [34]. ExoY is an adenyl cyclase

which interferes with gene expression resulting in further disruption of endocytosis, the cytoskeleton, and cell membranes [34].

P. aeruginosa also produces exotoxin A which has an ADP-ribosylation activity; utilizing a similar mechanism of action as the diphtheria toxin by blocking protein synthesis [36]. Another toxin produced by this microbe is leukocidin (a cytotoxin) which forms pores in cell membranes leading to neutrophil and lymphocyte death as well as tissue destruction [2, 37]. P. aeruginosa can cause further tissue damage with various proteolytic enzymes which degrade cellular tight junctions, fibroconectin, collagen, and elastin. Proteases (e.g. LasA, LasB, and alkaline protease) also cleave antibodies, complement components, and surface receptors of cells of the immune system blocking host immune responses [2]. The production of phospholipase C by P. aeruginosa results in additional tissue damage, hemolysis, and the cleavage of cell membrane phospholipids and the lung surfactant (e.g. PC, phosphatidylcholine) [2]. Rhamnolipids (heat stable hemolysins) have detergent like properties which cause hemolysis, increase the action of phospholipase C (by solubilizing the phospholipids and making them more accessible to the enzyme), inhibit ciliary beating, and are lethal to leukocytes [2, 38]. The destruction of the cell membrane is aided by lipases (LipA and LipC) that are produced by this microbe. These enzymes cleave down the diacylglycerol portions of cell membrane phospholipids and lung surfactants (e.g. PC) liberated by phospholipase C. This results in tissue damage and release of nutrients such as fatty acids and glycerol [2, 37].

1.3 *P. aeruginosa* Infections

P. aeruginosa is considered to be an important human pathogen [2, 32] known to cause various infections of the human body: dermatitis, soft tissue infections, gastrointestinal infections, respiratory tract infections, joint and bone infections, meningitis, otitis, ocular infections, endocarditis, and bacteremia [39 - 47]. As an opportunistic pathogen, *P. aeruginosa* normally does not cause disease in healthy individuals but mainly infects immuno-compromised hosts (with primary and acquired deficiencies). *P. aeruginosa* induced septicemia is common in patients with immunodeficiencies, cancer patients (e.g. leukemia), and AIDS patients [32, 48]. Following transplant surgeries (e.g. bone marrow or heart-lung transplants), patients have an increased chance of infection by this bacterium because of immunosuppressive drugs they take to prevent transplant rejection [32]. 8% to 9% of surgical wounds infections are caused by *P. aeruginosa* [1, 44] and this microbe is a leading agent of various nosocomial infections (e.g. pneumonia, septicemia, urinary tract infections) accounting for 11% - 13.8% cases [44]. *P. aeruginosa* is even more common in intensive care units (ICUs) with infection rates reported from 13% to 22 % [49]. Burn patients are frequently affected by *P. aeruginosa* [50] and 40% of infections in burn wound care units are caused by this microbe [51]. *P. aeruginosa* also plays an important role in cystic fibrosis patients due to their compromised lung environment. Chronic and recurrent infections of pulmonary tract with this bacterium affects most CF patients and result in high mortality [2, 32].

1.4 *P. aeruginosa* Treatment and Antibiotic Resistance

Infections caused by *P. aeruginosa* are difficult to treat because of the high intrinsic resistance of this bacterium to a broad spectrum of antibiotics [52] which is facilitated by several mechanisms. The outer cell membrane of this bacterium has low permeability, preventing the entrance of antibiotics (mostly β-lactams) in to the cell. *P. aeruginosa* is also known to express many different β-lactamases rendering many β-lactams ineffective [53]. Furthermore, this microbe has several multidrug efflux systems/pumps (*mexAB-oprM, mexCD-oprJ,* and *mexEF-oprN*), which can remove many structurally unrelated compounds (e.g. antibiotics) from the cell [54 - 56].

There are several classes of antibiotics that can be used against *Pseudomonas aeruginosa* such as β-lactams, quinolones, aminoglycosides, and polymixins [32, 57]. Several anitpseudomonal β-lactams are available including carboxypenicillins (carbenicillin and ticarcillin), ureidopenicillins (azlocillin and piperacillin), some 3rd generation cephalosporins (ceftazidime, cefsulodine, and cefoperazone), as well as 4th generation cephalosporins (monobactam aztreonam), carbapenems (meropenem, imipenem) and the more effective doripenem [32, 52]. β-lactams inhibit bacterial growth by preventing cross-linking of peptidoglycan thus stopping cell-wall synthesis [4]. Aminoglycosides are the main antibiotics used against *P. aeruginosa* including gentamycin, tobramycin, isepamicin, and amikacin [28]. These drugs bind irreversibly to the 30S ribosome subunit inhibiting protein synthesis and preventing bacterial growth [4, 28]. Quinolones, such as ciprofloxacin and norfloxacin, also exhibit anitpseudomonal activity [57] by inhibiting bacterial DNA gyrase, preventing the unwinding of DNA, thus interfering with DNA replication and transcription. Other less commonly used (due to

toxicity) antipseudomonal drugs are polymyxin B and polymixin E (colistin). These drugs belong to an older group of antimicrobial agents with antipseudomonal activity which work by disrupting the cell membrane and are useful against *P. aeruginosa* strains that are highly resistant to other drugs [32].

Even though antipseudomonal antibiotics are effective against many isolates of *P. aeruginosa*, there are a rising number of infections in which highly resistant or multi drug resistant *P. aeruginosa* are present [52, 58]. These highly resistant strains of *P. aeruginosa* overexpress their efflux systems, have structural alterations in DNA gyrase, express aminoglycoside-modifying enzymes, and have diminished levels of outer membrane proteins, further decreasing their membrane permeability [52, 58]. The search for new antipseudomonal drugs and new drug targets is a very important issue in managing this pathogen.

1.5 *P. aeruginosa* and Cystic Fibrosis

P. aeruginosa is a leading cause of mortality and morbidity in patients with cystic fibrosis (CF) [2] which is an autosomal, recessive genetic disease mostly occurring in individuals of Caucasian/European decent at a rate of 1 in 1000 births. In the USA and Canada, CF is present 1 in 3500 live births [59 - 61]. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) [59] encoding a membrane protein which mediates passive diffusion of bicarbonate and chloride through the membranes of epithelial cells [61]. CFTR belongs to the ABC transporter family and is composed of two transmembrane domains, two nucleotide binding domains, and one regulatory domain [59]. More than a thousand mutations have been found in the CFTR

gene which results in the CF phenotype. The deletion of phenylalanine at position 508 (ΔF508) in CFTR gene is the most common mutation and accounts for 66% of CF cases worldwide and 90% of CF cases in the USA [59, 62]. Defective CFTR proteins with the ΔF508 mutation are recognized as misfolded and degraded by the cell. Mutations that result in a absence of the functional CFTR channel lead to the presence of viscous secretions (due to abnormal fluid and electrolyte transport) which causes obstructions of the exocrine glands of the gastrointestinal tract, pancreatic ducts, genitourinary tract, and respiratory tract and lung infections [59, 61, 62].

Presence of this tenacious secretion impairs normal mucociliar lung clearance rendering CF patients susceptible to lung infections. Furthermore, the activities of antimicrobial peptides (eg. human β-defensin) are decreased in CF patients making them more prone to microbial infection. The main cause of death in adult CF patients is not the disease itself but chronic infections caused by *Stahpylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* and, predominantly, *P. aeruginosa* [2, 61, 63] resulting in the average life expectancy of 36 years [64]. In the initial stage of infection a mixed bacterial population exists in the CF lung but by three years of age, more than 97.5% of CF patients are infected with *P. aeruginosa*. Older CF patients are solely infected with *P. aeruginosa* and 10^8 colony forming units (CFU) of this bacterium are present in one milliliter of their sputa [32, 65, 66]. High susceptibility of CF patients to *P. aeruginosa* infection is linked to the defective CFTR protein, which serves as a receptor for this bacterium. Binding of *P. aeruginosa* to the CFTR is needed to coordinate clearing of the lung as part of the innate immunity response [63].

CF patient lungs cannot clear this microorganism which leads to persistence of *P. aeruginosa* in the lung and a 'vicious cycle' of lung inflammation induced by both host immune system and pathogen produced substances [2, 67]. Due to the overproduction of inflammation factors, host mucin, the action of bacterial virulence factors and the presence of extracelluar DNA from destroyed bacterial and host cells, patients' lungs are damaged and filled with large amounts of viscous sputum [2]. This causes difficulty breathing and the deterioration of lung functions resulting in the death of the CF patient [68]. Ant-inflammatory drugs can be used to reduce lung inflammation [68], and improved lung function can be achieved by mechanical clearance of mucus [69], however, no antibiotic therapy exists which completely eradicates *P. aeruginosa* from CF patients without remission [2, 68, 70].

1.5.1 Nutrient Acquisition During CF Lung Infection

Many studies have focused on the roles which virulence factors play in *P. aeruginosa* infections; however, little is known about the nutrient that *P. aeruginosa* uses during infection especially in the CF lung. Phosphatidylcholine (PC) (**Figure 1**) a major component of lung surfactant, which *P. aeruginosa* can metabolize *in vitro* (by cleaving it to is components: choline, glycerol-phosphate, and two long chain fatty acids), was suggested as a potential nutrient source during infection of the CF lung [71]. Work by Son *et al*. [71] showed that many fatty acid degradation (β-oxidation) genes (**Figure 2**) in *P. aeruginosa* are expressed during CF lung infection (e.g. acyl-CoA synthetases: *fadD1*: PA3299 and *fadD2*: PA3300 and *fadBA5*: PA3014 and PA3013). Other genes necessary for the degradation of PC such as phospholipase C and lipase (LipA) were also expressed

in vivo [71] and *P. aeruginosa* with mutated genes of fatty acid degradation (*fadBA5*) was shown to have a growth defect on PC *in vivo* [71].
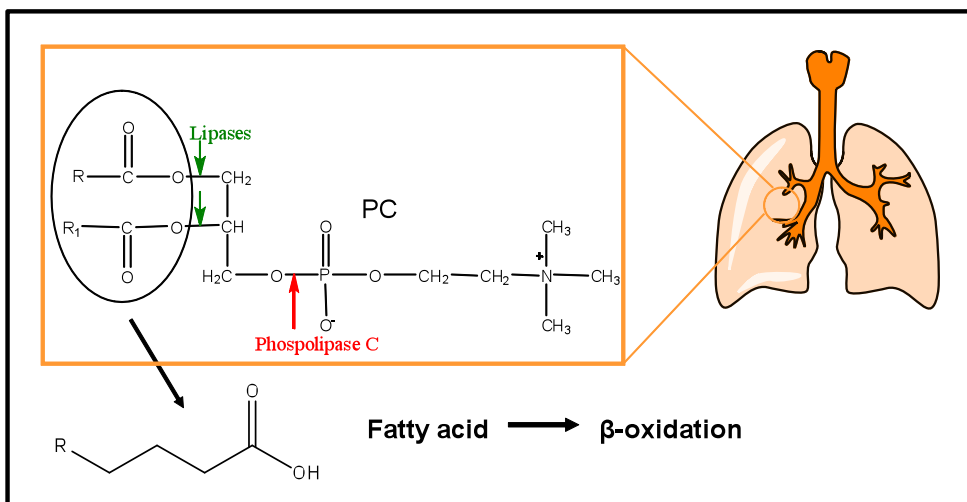
Figure 1. Molecule of phosphatidylcholine (PC), the major component of lung surfactant, and action of phospolipase and lipase. Sites of enzymatic action of *P. aeruginosa* phospholipase C and lipases on PC are indicated by arrows. R and R$_1$ represent different length carbon chains. After PC is cleaved by phospholipase C and lipases, free fatty acids are degraded via β-oxidation.

Interestingly, *P. aeruginosa* was reported to exhibit twitching-mediated chemotaxis towards phospholipids and unsaturated long chain fatty acids (LCFA) [72]. Thus, fatty acids might be a source of carbon and energy used by this bacterium during replication in the CF lung and the β-oxidation pathway could have implications in the pathogenesis of *P. aeruginosa* thereby allowing this bacterium to grow to high cell density *in vivo* (in the CF lung for example).

1.6 β-oxidation Pathway: Fatty Acid Degradation (Fad)

In *P. aeruginosa* the β-oxidation pathway has not been fully established; however, genes involved in this pathway have been predicted with bioinformatics and the pathway has been modeled [71] (**Figure 2**) based on the well established *Escherichia coli* β-

oxidation pathway [73]. There are five proteins responsible for transport, activation and degradation of long-chain fatty acids (LCFAs) in *E. coli.* For an exogenous LCFA to be degraded by this pathway it first must be transported by the membrane transporter (FadL) into the cell [74]. LCFA is then activated by FadD with the use of ATP and coenzyme A (CoA) (**Figure 2**) to yield fatty acyl-CoA [75]. The fatty acyl-CoA molecule can then proceed through the β-oxidation pathway (**Figure 2**). In the first step, FadE, which has acyl-CoA dehydrogenase activity, oxidizes the bond between the 2nd and 3rd carbons of a fatty acyl-CoA, and two electrons are transferred to flavin adenine dinucleotide (FAD) to form $FADH_2$ [76]. The FadBA multimeric, multi-functional enzyme complex is responsible for the remaining steps of fatty acid degradation (**Figure 2**) [76, 77]. FadB (which possesses *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase activities, and L-3-hydroxyacyl-CoA dehydrogenase activities) hydrates the double bond formed in the previous step and then oxidizes the hydroxyl group on the 3rd carbon while forming NADH from NAD^+ [76, 77]. Due to its four enzymatic activities, FadB can use both saturated and unsaturated fatty acyl-CoA as substrates [78]. Finally, FadA (3-ketoacyl-CoA thiolase) cleaves this newly formed molecule with the addition of CoASH resulting in acyl-CoA and a shorter coenzyme A thioester of fatty acid [76, 77]. Acyl-CoA can now be utilized in the TCA cycle and/or other pathways and fatty acyl-CoA is further degraded in the β-oxidation pathway and is shortened by two carbons with each successive cycle of this pathway (**Figure 2**). In *E. coli*, genes encoding enzymes needed for β-oxidation (*fadL, fadD, fadE, and fadBA*) are repressed in the absence of fatty acids by the FadR transcriptional regulator. Long chain acyl-CoA (length $\geq C_{12:0}$), can bind to FadR to induce Fad [76, 79, 80].

1.6.1 β-oxidation in *P. aeruginosa*

P. aeruginosa exhibits greater metabolic capabilities for fatty acid degradation than *E. coli* and can grow on short ($C_{4:0}$ - $C_{6:0}$), medium ($C_{8:0}$ - $C_{10:0}$), and long-chain fatty acids ($\geq C_{12:0}$) as sole carbon and energy sources [17]. With a genome of 6.3 Mb, *P. aeruginosa* could have 6-fold more β-oxidation genes than *E. coli* [12], suggesting possible redundancies and a high level of complexity in this pathway. Three potential *fadL*s have been investigated thus far in *P. aeruginosa* [72]. Multiple FadE homologues are present in *P. aeruginosa* but none have been shown to be involved in Fad. Only three *fadBA* operon homologues have been studied in *P. aeruginosa*: *fadAB1, fadAB4* and *fadBA5* (**Figure 2**). The *fadAB1* (PA1736 and PA1737) operon was shown to be strongly induced by medium chain fatty acids and, to a lesser extent, long chain fatty acids [81]. The *fadBA5* (PA3014 and PA3013) operon was determined to be involved in LCFA metabolism ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:1}^{\Delta 9}$) and can be induced by LCFA, especially oleate ($C_{18:1}^{\Delta 9}$). The regulation of the *fadBA5* operon was shown to be through PsrA, a TetR repressor, which binds and responds to LCFA to de-repress *fadBA5* [17]. Mutations in *fadAB5, fadAB1* and *fadBA4* (PA4785-6) almost completely abolish growth of *P. aeruginosa* on LCFA, palmitate ($C_{16:0}$) and oleate ($C_{18:1}^{\Delta 9}$), indicating that these *fadBA* homologues are mainly responsible for Fad [82]. Recently, two *fadD* homologues of *P. aeruginosa* have been identified in our laboratory: *fadD1* (PA3299) and *fadD2* (PA3300) [83]. Based on growth curve analysis of the *P. aruginosa* double *fadD1* and *fadD2* mutant, additional *fadD* homologues exist in this bacterium.
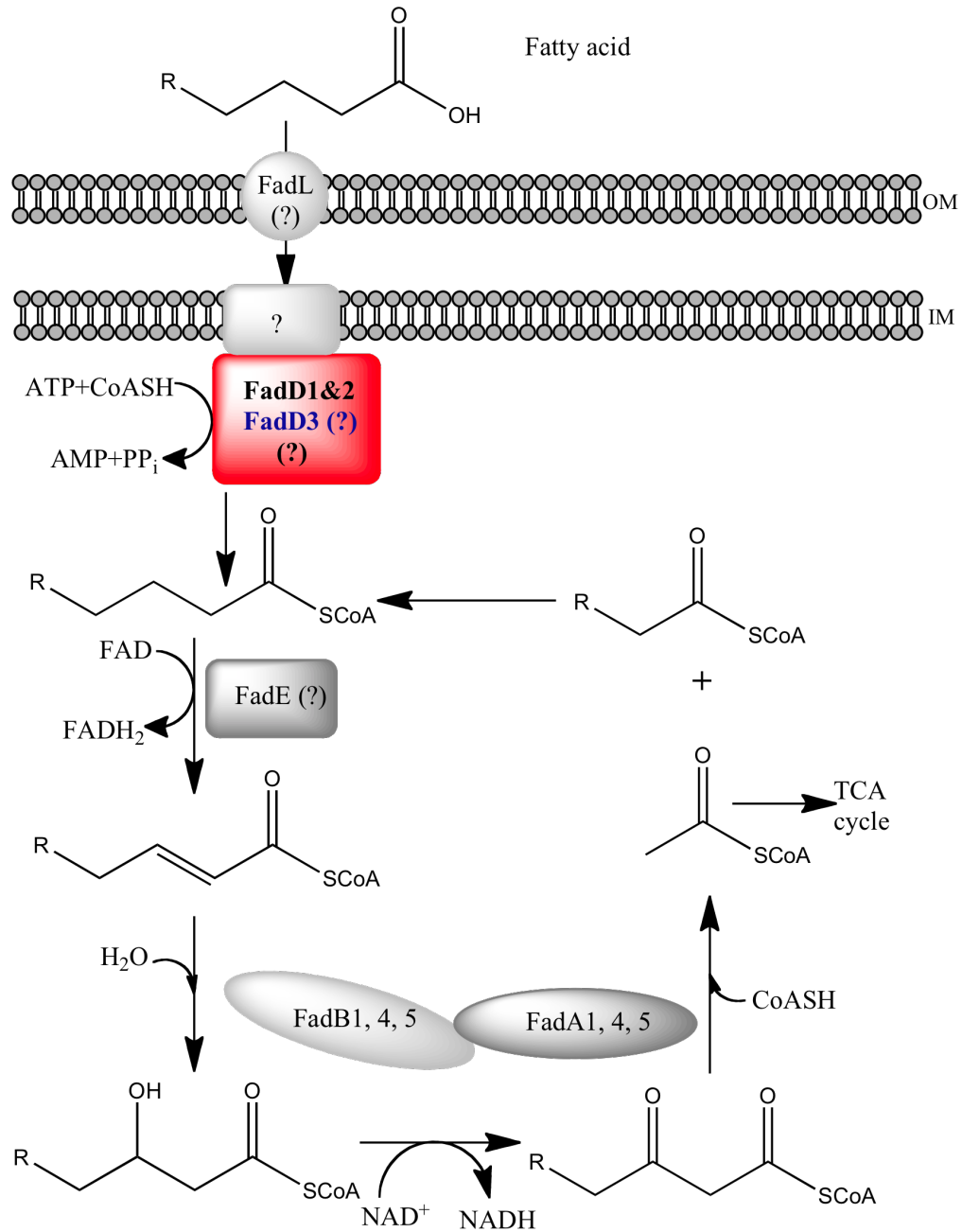
Figure 2. *P. aeruginosa* fatty acid degradation pathway (Fad) modeled on the *E. coli* β-oxidation pathway. Known *P. aeruginosa* Fad enzyme homologues are indicated by numbers: FadD1 (PA3299), FadD2 (PA3300), FadD3 (PA3860, highlighted in blue), FadAB1 (PA1736-7), FadBA4 (PA4785-6) and FadBA5 (PA3013-4). FadA, 3-ketoacyl-CoA thiolase; FadB, eonyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase; FadD, acyl-CoA synthetases; FadE, acyl-CoA dehydrogenase; FadL, outer membrane long-chain fatty acid translocase. OM- outer membrane, IN- inner membrane

1.7 Physiological Role of Fatty Acyl-CoA Synthetase (FadD)

Fatty acyl-CoA synthetase (FACS) (also known as fatty acid:CoA ligase, AMP forming; EC6.2.1.3) occupies a central role in many metabolic processes by the formation of fatty acyl-CoA [84]. Active fatty acid molecules (fatty acyl-CoAs), besides serving as a substrate for β-oxidation and phospholipid synthesis, are also involved in many processes inside prokaryotic and eukaryotic cells including protein transport [85, 86], protein acylation [87], cell signaling [88], enzyme activation [89, 90], and transcriptional control [80, 91-93]. Because of the multiple roles of fatty acyl-CoA in the cell, FACS plays an important role in cell homeostasis.

In *E. coli,* the model organism of bacterial β-oxidation, FadD (FACS) is a 62-kD enzyme with highest activates for FAs of chain length between $C_{12:0}$ and $C_{18:1}{}^{\Delta 9}$ [94]. In this bacterium, FadD is thought to catalyze the activation of LCFA and facilitate the transport of exogenous LCFA into the cell in conjunction with FadL in a process defined as vectorial esterification [84, 93, 95]. FadD is found associated with the inner membrane as well as in the cytoplasm [96] suggesting the movement of this enzyme between the membrane and cytoplasm for LCFA transport. Because of the concentrated activities of outer membrane fatty acid transporter (FadL) and FadD, the transport of LCFA into the cell is essentially unidirectional [95] (**Figure 2**).

1.7.1 FadD as an Enzyme

The FadD enzyme catalyzes the formation of fatty acyl-CoA in two steps involving hydrolysis of ATP and the release of pyrophosphate (PP_i) (**Figure 3 A**) [97]. During this reaction, an enzyme bound adenylated fatty acyl intermediate is formed [97].

In the first step of activation of the fatty acid, the carboxyl group of FA is linked by an acyl bond to the phosphoryl group of AMP and pyrophosphate is released. In the subsequent step, a fatty-acyl group is transferred to the sulfhydryl group of CoA which results in the formation of free AMP and the release of fatty acyl-CoA (**Figure 3 B**). This reaction most likely proceeds through the Bi-Uni, Uni-Bi, tri-molecular ping-pong mechanism [84, 98].
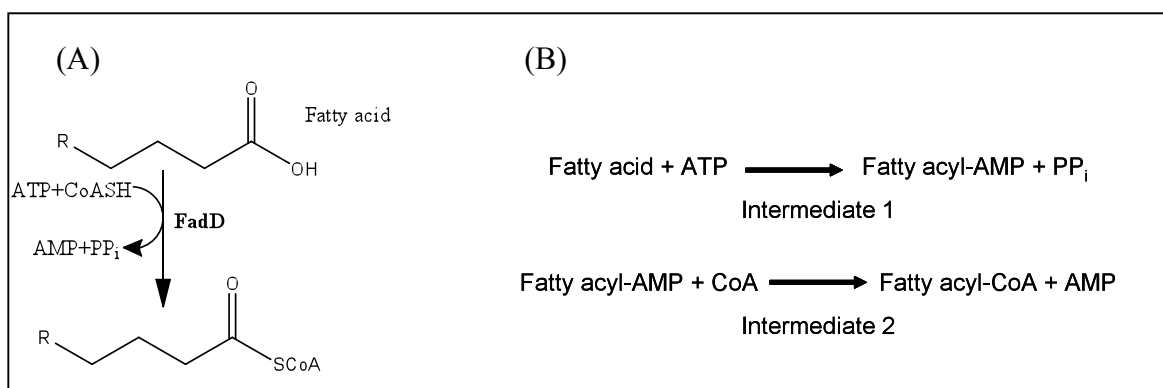
Figure 3. Function of FadD enzyme. (A) Reaction catalyzed by fatty acyl-CoA synthtetase: FadD. (B) Details of the two step reaction carried out by FadD resulting in the formation of fatty acyl-CoA.

The *E. coli* FadD enzyme possesses an ATP/AMP binding signature motif ([213]YTSGTTGXPKGV[224] and [356]GYGLTE[361]), which is similar to the same motif found in other acyl-CoA synthetase and adenyl forming enzymes [75, 84, 99, 100]. The *E. coli* FadD also has a second region ([431]NGWLHTGDIAVMDEEGFLRIVDRKK[455]) which is similar to fatty acyl-CoA synthetases (FACS) signature motifs present in all known prokaryotic and eukaryotic fatty acyl-CoA synthetases [101]. Mutations in this region were found to altere the specificity of the *E. coli* FadD enzyme for fatty acids of various chain length or abolished enzyme activity indicating that this region is essential for the function of FadD [101]. Presence of both motifs in a protein is a strong indicator that this protein posses

acyl-CoA synthetase activity. Alignment of the ATP/AMP and FACS motifs of the *E. coli* FadD to potential *P. aeruginosa* FadD homologues was used to identify most probable FadD homologues, specifically PA3860 (*fadD3*).

1.7.2 Fatty Acyl-CoA Synthetase (FadD) in *P. aeruginosa*

As previously mentioned, the β-oxidation pathway in *P. aeruginosa* has not been fully elucidated but enzymes involved in Fad have been studied in other *Pseudomonas* species. In *Pseudomonas fragi*, Fad proteins including a broad substrate specificity fatty acyl-CoA synthetase have been characterized [102 - 104]. *Pseudomonas putida* was initially found to have one FACS with broad substrate specificity [105]. Further characterization of the role of this enzyme in *P. putida* led to the identification of a second FACS and these two acyl-CoA synthetases were named FadD1 and FadD2, respectively [106, 107]. It was determined that in this double FadD system, FadD1 has a dominant role in metabolism of FA whereas FadD2 is activated only when FadD1 is not present [107].

Two *fadD* homologues of *P. aeruginosa*, *fadD1* (PA3299) and *fadD2* (PA3300), were previously shown to be induced *in vivo* during infection of the lung CF [71]. Recently, our laboratory has characterized these two *fadD* homologues of *P. aeruginosa* [83] (**Figure 2**). Both genes were functionally confirmed by their ability to complement growth of the *E. coli fadD⁻/fadR⁻* strain on various fatty acids ($C_{10:0}$ - $C_{18:1}^{\Delta 9}$) while the biochemical characterization of FadD1 and FadD2 indicated that both enzymes have broad specificity for fatty acids of different chain lengths. FadD1 prefers LCFAs as a substrate, whereas FadD2 exhibits a preference for MCFAs. *P. aerugionsa fadD1*, *fadD2*,

and *fadD2D1* mutants showed growth defects when grown in FAs of different length (especially long chain) as sole carbon sources. Furthermore, the double mutant of *fadD2D1* displayed an impaired ability to grow on PC as a sole carbon source. This growth defect translated into decrease fitness *in vivo* in mouse lung infection model indicating that FadD1 and FadD2 may be also important for *P. aeruginosa* replication *in vivo* (CF lung). The double *fadD2D1* mutant was still able to grow on FA (to approximately half of the optical density of the wildtype in LCFA), suggesting the involvement of other acyl-CoA synthetases in fatty acid degradation. Hence, identification and characterization of the additional *fadD* homologues will provide more insight into the metabolism of FAs in *P. aeruginosa.*

1.7.3 FadD and Virulence

Studies of FadD in many bacterial species have shown a connection between FA metabolism and the expression of virulence factors. In *M. tuberculosis*, 36 *fadD* homologues have been identified [15] and a mutation of the *fadD28* gene severely impaired replication of this bacterium in mouse lungs possibly due to the inability to produce complex cell-wall associated lipids [108]. Another *fadD* homologue, *fadD33*, was shown to be involved in the ability of *M. tuberculosis* strain H37Ra to replicate in mouse's liver [109]. In the plant pathogen *Xanthomonas campestris*, a mutation of the *rpfB* gene (homologue of fatty acyl-CoA synthetase) resulted in the decreased production of protease, endoglucanase, and polygalacturonate lyase due to the inability to produce a diffusible extracellular factor which is thought to contain a FA moiety [110]. Transposon mutagenesis studies in *Salmonella enterica* serovar Typhimurium resulted in the isolation

of a *fadD* mutant that had reduced expression of the *hilA* gene (which is thought to be a transcriptional activator of the T3SS [111]) as well as invasion genes [112]. A mutation in the *fadD* gene of *Sinorhizobium meliloti*, a nitrogen fixing bacterium, resulted in a swarming phenotype previously not observed in the wild type as well as a decreased ability to nodulate alfalfa plant roots [113].

Interestingly, the connection between FACS and virulence has recently been observed in *P. aeruginosa* [83]. Increased swarming phenotype was displayed by the *fadD1* mutant; on the other hand, the *fadD*2 mutant had impaired swimming and swarming abilities. Furthermore, this *fadD2* mutant shows decreased production of lipases, phospholipases, proteases, and rhamnolipids. These virulence phenotypes expressed by *fadD*s mutants indicate that *fadD*s may play a more pivotal role in the pathogenicity of *P. aeruginosa* beyond providing nutrients during infection.

1.8 Research Specific Aims

The goal of this research is to characterize the newly identified fatty acyl-CoA synthetase homologue, PA3860 (*fadD3*), which is thought to take part in Fad though its exact contribution to fatty acid metabolism in *P. aeruginosa* is unknown. Deciphering the role of *fadD3* in fatty acid degradation will broaden our knowledge of the β-oxidation pathway in this bacterium. This was achieved by completing the following specific aims:

1. Purification of FadD3 and performance of biochemical analysis of its acyl-CoA synthetase function

2. Genetical characterization of *fadD*3 (PA3860) of *P. aeruginosa* through mutational analysis and gene fusion studies

During the course of this research additional discoveries about the β-oxidation pathway of *P. aeruginosa* were made: 1) additional acyl-CoA synthetase homologues were found, i.e. *fadD4*, *fadD5*, and *fadD6* 2) and their role in Fad was determined along with *fadD3*.

Chapter Two: Materials and Methods

2.1 Media and Growth Conditions

Luria-Bertani (LB) medium (Difco) was used to culture *Escherichia coli* strains. Pseudomonas Isolation Agar and Broth (PIA, PIB; Difco) and LB were used to culture *Pseudomonas aeruginosa* strain PAO1 and its derivatives. *E. coli* and *P. aeruginosa* strains were grown at 37°C unless indicated otherwise. 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 supplier. *E. coli* Δ*asd* (E464, E1353) and Δ*dapA* (E2057) strains were grown in media supplemented with 100 μg/ml diaminopimelic acid (DAP, Sigma) prepared as previously described [114]. Minimal media used for various experiments with *E. coli* and *P. aeruginosa* strains contained 1x M9 salts [115] + 0.5 mM $MgSO_4$ + 0.02 mM $CaCl_2$ + 1% (w/v) Brij-58 and was supplemented with different fatty acids, glucose (Sigma), or casamino acids (CAA, Fisher Scientific) as carbon sources. When making agar (plates Fisher Scientific) was added to the various 1x M9 based minimal media at a concentration of 1.5% (w/v).

2.2 Reagents

All restriction enzymes, T4 DNA polymerase, T4 DNA ligase, calf intestinal alkaline phospotase (CIP), deoxynucleoside triphosphates (dNTPs), and DNA markers were purchased from New England Biolabs (NEB) and used as recommended by the

supplier. *Pfu* DNA polymerase was purchased from Stratagene. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), isopropyl β -D-1-thiogalactopyranoside (IPTG), *2*-nitrophenyl-β-*D*-galactopyranoside (ONPG), and agarose were purchase from Reaserch Products International (RPI). 30% (w/v) acrylamide and bis-acrylamide mix (29:1, respectively), protein standards were obtained from Bio-Rad. FAs of different chain lengths and other chemicals were obtained from Sigma. 3% (w/v) FAs stocks were prepared by neutralizing FA with equal-molar amount of potassium hydroxide and dissolving in 1% (w/v) Brij-58 [17]. Prior to addition to liquid or solid media, FAs stocks were warmed to 80°C until FAs were completely dissolved.

2.3 Bacterial Strains

Bacteria strains used and constructed in this study are listed in **Table 1**.

Table 1. Strains utilized in this study.

Strains	Lab ID	Relevant Properties	Reference/ Source
E. coli			
EPMax10B-Δ*dapA*:: *lacI*^{q}-*pir* /*leu*$^+$	E2057	F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 galU galK rpsL nupG* Δ*dapA*::*lacI*^{q}- *pir* -FRT8	Kang Y
EPMax10B-lacI^q/pir	E1869	F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139 Δ(ara, leu)galU galK rpsL nupG lacI*^{q}-FRT8 pir-FRT4	Kang Y
EPMax10B-*pir116*/Δ*asd*/ *mob*-Km	E1353	Km^r; F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL nupG* Tn-*pir116*-FRT2 Δ*asd*::FRT *recA*::RP4-2Tc::*Mu*-Km^r	Hoang TT
HPS1-*mob*-Km/Δ*asd*/ *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*::miniTn5Lac4 (*lacI*^{q} *lacZ*ΔM15) Δ*asd*::FRT *uidA*::*pir116* *recA*::RP4-2Tc::*Mu*-Km^r	Hoang TT
K-12	E0577	Prototroph	ATCC #23740
K27-*fadR*$^-$	E2011	Km^r; *fadD*$^-$ (*oldD88*) *fadR*::Km^r	[83]
K27-*fadR*$^-$/*att*Tn7::Gm^r	E2387	Gm^r, Km^r; E2011 with miniTn7 vector inserted at *attTn7* site	This study

Strains	Lab ID	Relevant Properties	Reference/ Source
K27-*fadR*[-]/*att*Tn7::Gm[r]-P*lac*	E2665	Gm[r], Km[r]; E2011 with miniTn7- Gm[r]-P*lac* vector inserted at *attTn7* site	This study
K27-*fadR*[-]/*att*Tn7:: *fadD3*	E2666	Gm[r], Km[r]; E2011 with *fadD3*-His$_6$ inserted at *attTn7* site	This study
K27-*fadR*[-]/*att*Tn7:: *fadD4*	E2667	Gm[r], Km[r]; E2011 with *fadD4*- His$_6$ inserted at *attTn7* site	This study
K27-*fadR*[-]/*att*Tn7:: *fadD$_{Ec}$*	E2385	Gm[r], Km[r]; E2011 with *fadD$_{Ec}$* inserted at *attTn7* site	This study
K27-T7	E1063	Gm[r]; K27 with T7 expression system; *fadD*[-] (*oldD88*) λ*attB*::T7(*pol-lysS*)-*lacI^q*- Gm[r]	[83]
SM10/*lacI^q*	E0052	Km[r], Cm[r], Tet[r]; *thi thr leu tonA lacy supE recA*::RP4-2-Tc::*Mu*-Km[r] *lacI^q*	Hoang TT
P. aeruginosa			
PAO1	P007	Prototroph	[116]
PAO1-Δ*fadD2D1*::*FRT*	P177	PAO1 with *fadD2D1* deletional mutation	[83]
PAO1-Δ*fadD3*::*FRT*	P677	PAO1 with *fadD3* deletional mutation	This study
PAO1-Δ*fadD3*::*FRT-lacZ/ attB*::miniCTX2-*fadD5*	P683	Tet[r], Gm[r]; *fadD3* complement strain with *fadD3-FRT-lacZ* fusion	This study
PAO1-*attB*::miniCTX2-P*fadD4*-*lacZ*	P713	Tet[r], PAO1 with P*fadD4*-*lacZ* fusion inserted at *attB* site	This study
PAO1- *fadD4*:: Gm[r]-*pheS$_{Pa}$*- m*FRT*	P685	Gm[r], *pheS$_{Pa}$*[+]; PAO1 with *fadD4* gene inactivated by Gm[r]-*pheS$_{Pa}$*-m*FRT* cassette	This study
PAO1-Δ*fadD2D1*::*FRT/* Δ*fadD3*::*FRT*	P678	PAO1-Δ*fadD2D1*::*FRT* with *fadD3* deletional mutation	This study
PAO1-Δ*fadD2D1*::*FRT/* *fadD6*::m*FRT*	P696	PAO1-Δ*fadD2D1*::*FRT* with insertional mutation of *fadD6*	This study
PAO1-Δ*fadD2D1*::*FRT/* Δ*fadD3*::*FRT-fadD4*::m*FRT*	P698	PAO1-Δ*fadD2D1*::*FRT-*Δ*fadD3*::*FRT* with insertional mutation of *fadD4*	This study
PAO1-Δ*fadD2D1*::*FRT/* *fadD4*::m*FRT/attB*::mini CTX2-*fadD4*	P703	Tet[r]; PAO1-Δ*fadD2D1*::*FRT-fadD4*::m*FRT* complemented with miniCTX2-*fadD4*	This study
PAO1-Δ*fadD2D1*::*FRT/* Δ*fadD3*::*FRT/* *fadD4*::m*FRT/* *attB*::miniCTX2-*fadD4*	P705	Tet[r]; PAO1-Δ*fadD2D1*::*FRT-*Δ*fadD3*::*FRT-fadD4*::m*FRT* complemented with miniCTX2-*fadD4*	This study
PAO1-Δ*fadD2D1*::*FRT/* *fadD5*::Gm[r]	P239	Gm[r]; PAO1-Δ*fadD2D1*::*FRT* with *fadD5* gene inactivated by Gm[r]-*FRT* cassette	Son M
PAO1-Δ*fadD2D1*::*FRT/* *fadD5*::*FRT/* *fadD6*::Gm[r]	P416	Gm[r]; PAO1-Δ*fadD2D1*::*FRT/ fadD5*::*FRT* with *fadD6* gene inactivated by Gm[r]-*FRT* cassette	Norris MH
PAO1-Δ*fadD2D1*::*FRT/* *fadD5*::*FRT/ fadD6*::*FRT*	P722	PAO1 with *fadD1, fadD2, fadD5,* and *fadD6* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT/* *fadD3*::*FRT/ fadD5*::*FRT*	P768	PAO1 with *fadD1, fadD2, fadD3,* and *fadD5* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT/* *fadD3*::*FRT/ fadD6*::*FRT*	P769	PAO1 with *fadD1, fadD2, fadD3,* and *fadD6* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT/* *fadD4*::*FRT/ fadD5*::*FRT*	P770	PAO1 with *fadD1, fadD2, fadD4,* and *fadD5* genes mutated	This study

Strains	Lab ID	Relevant Properties	Reference/ Source
PAO1-Δ*fadD2D1*::*FRT*/ *fadD4*::m*FRT*/ *fadD6*::*FRT*	P771	PAO1 with *fadD1*, *fadD2*, *fadD4*, and *fadD6* genes mutated	This study
PAO1-Δ*fadD3*::*FRT*/ *fadD4*::m*FRT*/ *fadD5*::*FRT*/ *fadD6*::*FRT*	P781	PAO1 with *fadD3*, *fadD4*, *fadD5*, and *fadD6* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT*/ Δ*fadD3*::*FRT*/ *fadD4*::m*FRT*/ *fadD5*::*FRT*	P772	PAO1 with *fadD1*, *fadD2*, *fadD3*, *fadD4*, and *fadD5* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT*/ Δ*fadD3*::*FRT*/ *fadD4*::m*FRT*/ *fadD6*::*FRT*	P773	PAO1 with *fadD1*, *fadD2*, *fadD3*, *fadD4*, and *fadD6* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT*/ Δ*fadD3*::*FRT*/ *fadD5*::*FRT*/ *fadD6*::*FRT*	P726	PAO1 with *fadD1*, *fadD2*, *fadD3*, *fadD5*, and *fadD6* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT*/ *fadD4*::m*FRT*/ *fadD5*::*FRT*/ *fadD6*::*FRT*	P766	PAO1 with *fadD1*, *fadD2*, *fadD4*, *fadD5*, and *fadD6* genes mutated	This study
PAO1-Δ*fadD2D1*::*FRT*/ Δ*fadD3*::*FRT*/ *fadD4*::m*FRT*/ *fadD5*::*FRT*/ *fadD6*::*FRT*	P767	PAO1 with *fadD1*, *fadD2*, *fadD3*, *fadD4*, *fadD5*, and *fadD6* genes mutated	This study

2.4 Bacterial Plasmids

Plasmids used and constructed in this study are listed in the **Table 2**.

Table 2. Plasmids utilized in this study.

Plasmids	Lab ID	Relevant Properties	Reference/ Source
pCD13SK-*flp-oriT*	E0783	Sp^r; suicidal Flp-expressing plasmid	[81]
pET15b	E0047	Ap^r; T7 expression vector	Novagen
pET15b-*fadD3*	E2658	Ap^r; pET15b with *fadD3* gene	This study
pET28a	E0158	Km^r; T7 expression vector	Novagen
pET28a-*fadD4*	E2644	Km^r; pET28a with *fadD4* gene	This study
pEX18T	E0055	Ap^r, *sacB$^+$*; gene replacement vector	(2)
pEX18T-*fadD3*::Gm-*pheS$_{Pa}$*	E2438	Ap^r, Gm^r, *pheS$_{Pa}$$^+$*, *sacB$^+$*; Gm-*pheS$_{Pa}$*-*FRT* cassette inserted into *fadD3*	This study
pEX18T-*fadD4*	E2484	Ap^r, *sacB$^+$*; pEX18T with *fadD4*	This study
pEX18T-*fadD4*::Gm-*pheS$_{Pa}$*	E2506	Ap^r, Gm^r, *pheS$_{Pa}$$^+$*, *sacB$^+$*; Gm-*pheS$_{Pa}$*-m*FRT* cassette inserted into *fadD4*	This study
pFRT1-*lacZ*-Gm	E0790	Gm^r; *FRT-lacZ* fusion vector	[81]
pFLP2	E0067	Ap^r, *sacB$^+$*; broad-host range Flp expressing plasmid	(2)

Plasmids	Lab ID	Relevant Properties	Reference/ Source
pmFRT-*pheS_Pa*	E2382	Ap^r, Gm^r, *pheS_Pa$^+$*; plasmid with Gm^r- *pheS_Pa* -*mFRT* cassette	Zarzycki-Siek J
pRK2013	E0298	Km^r; helper plasmid for mobilization of plasmids containing *oriT*	[117]
pTNS2	E1189	Ap^r; helper plasmid for Tn7 transposition system	[118]
pTn7T- *FRT*8-Gm^r	E1467	Ap^r, Gm^r; miniTn7 integration vector	Kang Y
pwFRT-*pheS_Pa*	E2380	Ap^r, Gm^r, *pheS_Pa$^+$*; plasmid with Gm^r- *pheS_Pa* -*FRT* cassette	Zarzycki-Siek J
pUC19	E0014	Ap^r; cloning vector	[119]
pUC19-PA0996	-	Ap^r; PAO1 PA0996 gene cloned into pUC19	This study
pUC19-PA1221	-	Ap^r; PAO1 PA1221 gene cloned into pUC19	This study Videau P
pUC19-PA1617	E2472	Ap^r; PAO1 PA1617 gene cloned into pUC19	This study
pUC19-PA1997	-	Ap^r; PAO1 PA1997 gene cloned into pUC19	This study
pUC19-PA2555	-	Ap^r; PAO1 PA2555 gene cloned into pUC19	This study
pUC19-PA2557	-	Ap^r; PAO1 PA2557 gene cloned into pUC19	This study Videau P
pUC19-PA3568	-	Ap^r; PAO1 PA3568 gene cloned into pUC19	This study
pUC19-PA3860	E2356	Ap^r; PAO1 PA3860 gene cloned into pUC19	This study Videau P
pUC19-PA3860:: Gm-*pheS_Pa*	E2422	Ap^r, Gm^r, *pheS_Pa$^+$*; Gm-*pheS_Pa*-*FRT* cassette inserted into *fadD3*	This study
pUC19-PA4198	-	Ap^r; PAO1 PA4198 gene cloned into pUC19	This study Videau P
miniCTX2	E0076	Tet^r; *P. aeruginosa* site specific integration vector	[120]
miniCTX2-P_*fadE*-*lacZ*	E0690	Tet^r; miniCTX2 with P_*fadE*-*lacZ*	Nguyen DT
miniCTX2-P_*fadD4*-*lacZ*	E2610	Tet^r; P_*fadD4* cloned into miniCTX2-P_*fadE*-*lacZ* in place of P_*fadE*	This study
miniCTX2-*fadD3*	E2401	Tet^r; PA3860 gene cloned into mini-CTX2	This study
miniCTX2-*fadD4*	E2589	Tet^r; PA1617 gene cloned into mini-CTX2	This study
miniTn7-Gm^r-P_*lac* -*fadD3*	E2377	Ap^r, Gm^r; P_*lac* -*fadD3* from pWKS30-*fadD3*subcloned into pTn7T- *FRT*8-Gm^r	This study
miniTn7-P_*lac*	E2643	Ap^r, Gm^r; pTn7T- *FRT*8-Gm^r with P_*lac*	This study
miniTn7-*fadD3*	E2645	Ap^r, Gm^r; *fadD3*-His$_6$ cloned into miniTn7 -P_*lac*	This study
miniTn7-*fadD4*	E2647	Ap^r, Gm^r; *fadD4*-His$_6$ cloned into miniTn7 -P_*lac*	This study
miniTn7-*fadD_Ec*	E2378	Ap^r, Gm^r; *E. coli fadD* cloned into miniTn7-Gm^r-P_*lac* -*fadD3* in place of *fadD3*	This study
pWKS30	E0013	Ap^r; low-copy-number cloning vector	[121]
pWKS30-*fadD3*	E2368	Ap^r; PA3860 gene cloned into pWKS30	This study
pViet	E0082	Ap^r; low copy-number T7 expression plasmid	[122]
pViet-*fadD3*	E2663	Ap^r; *fadD3* gene cloned into pViet	This study

2.5 DNA Techniques

2.5.1 Plasmid DNA Isolation

Plasmid DNA was isolated from *E. coli* using Zyppy plasmid miniprep I kit (Zymo Research Corporation) according to supplier protocol.

2.5.2 Isolation of Bacterial Chromosomal DNA

Chromosomal DNA of *E. coli* and *P. aeruginosa* was isolated from 3 ml of overnight culture using a phenol-chloroform extraction protocol as previously described [115].

2.5.3 Restriction Enzymes Digests

Restriction enzyme digestions were performed using enzymes from NEB and buffers provided by the manufacturer. The digestion reactions were incubated at 37°C for at least 1 h. De-phosphorylation of vectors was done by addition of CIP directly to the digestion mixture and incubation at 37°C for 30 min. To blunt-end DNA fragments, 1 μl of 2 mM dNTPs and 3U of T4 DNA polymerase were added directly to the digestion mixture followed by incubation at 37°C for 30 min. To allow subsequent enzyme digest, T4 DNA polymerase was deactivated by incubation at 75°C for 20 min.

2.5.4 Agarose Gel Electrophoresis

Various DNA samples were separated on 1 - 2% agarose with 1x TAE buffer. Visualization of the DNA was performed by staining with ethidium bromide and exposure to UV.

2.5.5 Extraction of DNA from Agarose Gels

DNA bands of desired sizes were excised from agarose gels and DNA was extracted using Zymo Gel Recovery kit following supplier's protocol.

2.5.6 Ligations

Generally for ligations a 3:1 molar ratio of insert to vector DNA was used. Ligation reactions were performed with 1U T4 DNA ligase (NBE) in 1x ligation buffer in 10 μl final volume at 16°C for at least 4 h. Ligation mixtures were routinely transformed into strain EPMax10B/*pir*/*lacI*^{q} (E1869).

2.5.7 Preparation of Competent Cells and Transformation

Chemically competent *E. coli* cells were prepared via a $MgCl_2$ /$CaCl_2$ method and used for transformation as described previously [115]. Electro competent *E. coli* cells were prepared and used as described previously [114].

2.6 Polymerase Chain Reaction (PCR)

The polymerase chain reaction amplifications (PCR) were generally performed by initial deanturation at 94°C for 2 min and 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1min kb^{-1} at 72°C, with a final step of 10 min at 72°C. 10 - 30 pmol of forward and reverse primers, 10 ng of DNA template and 5 U of *pfu* DNA polymerase were used per each 50 μl reaction. For some of the GC rich *P. aeruginosa* chromosomal fragments PCR reactions were supplemented with 5 - 10% (v/v) of dimethyl sulfoxide (DMSO).

2.6.1 Oligonucleotides

Oligonucleotide primers utilized in this research were synthesized by Integrated DNA Technologies (IDT) and are listed in **Table 3**.

Table 3. Oligonucleotide primers utilized in this study. Restriction sequences are underlined.

Primer number and name	Sequence
302; FadD-up-Hind	5'-ATCGG<u>AAGCTT</u>CCGGGTGCTGCTGGCGGAT-3'
342; fadD1-BamHI	5'-GGGGC<u>GGATCC</u>AGGCAACGGCGGACTTACTTC-3'
437; fadD5-NdeI	5'-CGAGA<u>CATATG</u>ACCCACGCCGACCTG-3'
512; fadD4-BamHI	5'-TGCTTG<u>GGATCC</u>GGGCGTTTCGGCGGTGTA-3'
876; TN7L	5'-ATTAGCTTACGACGCTACACCC-3'
1093; EcfadD-down-BamHI	5'-AACG<u>GGATCC</u>TCAGGCTTTATTGTC-3'
1109; FadD6-NdeI	5'-GTGTACGC<u>CATATG</u>CTGAATACCC-3'
1134: fadD5-BamHI	5'-CTGGAA<u>GGATCC</u>GAGTTGCAGTTCGAGTTC-3'
1151; PA1221 BamHI-up	5'-ACCGT<u>GGATCC</u>ATTCTCATCGCTTTTCTCTC-3'
1152; PA1221 BamHI-down	5'-AGCGCGTTTTCGTCGGCGAA<u>GGATCC</u>GACT-3'
1153; PA2557 BamHI-up	5'-TGGGC<u>GGATCC</u>GCCTCTTGCGTTTACCTT-3'
1154; PA2557 BamHI-down	5'-GAAAGCGAAGCTGCCACTCTTCA<u>GGATCC</u>GCGAGT-3'
1155; PA3860 BamHI-up	5'-GAACG<u>GGATCC</u>AGTGTAAAGCATGTTGCCAG-3'
1156; PA3860 BamHI-down	5'- CTGGAGGAAATCCACGACATC<u>GGATCC</u>TGGCT G-3'
1157; PA4198 BamHI-up	5'-CCAGA<u>GGATCC</u>AGCCGTTTTCGACGCAGT-3'
1158; PA4198 BamHI-down	5'-CGAACACGTCGTTGAGCA<u>GGATCC</u>GCATG-3'
1216; PA3860-up	5'-CGGCGATCTCGTAGTG-3'
1217; PA3860-down	5'-TGGGCTGGCACGACTA-3'
1218; fadDEc-HindIII-up	5'-TCAT<u>AAGCTT</u>GGGGGTTGCGATGAC-3'
1238; glmS-Dn	5'-GTAGCCGAAGATGACGGTTTG-3'
1251; fadD3-NdeI	5'-AACC<u>CATATG</u>AATCCGTCCCCATCG-3'
1252; PA3568-Up-HindIII	5'-ACTCC<u>AAGCTT</u>CACTCACTGCTTCATC-3'
1253; PA3568-Down-SalI	5'-GGCTG<u>GTCGAC</u>GAAGGCGTGTTGAA-3'
1254; PA1997-Up-BamHI	5'-CCTGT<u>GGATCC</u>AGCAGATGCAGGA-3'
1255; PA1997-Down-SmaI	5'-CTGAAGATGGCATTGTCG-3'
1256; PA0996-Up-BamHI	5'-CTTCTTGCTTGGTTGCC-3'
1257; PA0996-Down-BamHI	5'-CCAGC<u>GGATCC</u>TCCAGACACACATAGGA-3'

Primer number and name	Sequence
1258; PA2555-Up-HindIII	5'-GCGTG<u>AAGCTT</u>CCGGCTACTCCATACA-3'
1259; PA2555-Down-KpnI	5'-CCGCC<u>GGTACC</u>CAGGAACACTCGATTT-3'
1260; PA1617-Up-HindIII	5'-CTAGG<u>AAGCTT</u>CTGGCGCAACGACTACAA-3'
1261; PA1617-Down-EcoRI	5'-GTTCAGTTGCTCCAGGTC-3'
1271; fadD3-up-HindIII	5'-GGGT<u>AAGCTT</u>GCCTTCCTGCGGTT-3'
1387; PA1617-up	5'-CTGGTCTCGCTGTATCAC-3'
1441; PA1614-HindIII	5'-G<u>AAGCTT</u>CATGACAGAGCAGCAAC-3'
1442; PA1617-dw-SmaI	5'-ATTT<u>CCCGGG</u>CAGTGACCACGTACC-3'
1443; PA1617-up-L-HindIII	5'-GACGAAGAGCCG<u>AAGCTT</u>GTGAAGCTCTGG-3'
1444; PA1617-NdeI	5'-ATGC<u>CATATG</u>GTCACTGCAAATCGTCT-3'

2.7 Genetic Techniques

2.7.1 Bacterial Matings

To introduce vectors containing *oriT* into *P. aeruginosa,* matings were performed with *E. coli* mobilizable strains E464 or SM10/*lacI^q^*. Conjugation of plasmids into *E. coli* was performed with E464 or E1353 *E. coli* strains. Alternatively, helper plasmid pRK2013 was utilized to mobilize plasmids into *P. aeruginosa* and *E. coli*. Generally, donor(s) and recipient(s) strains were grown to mid-log phase in LB. 0.5 ml of each culture was then mixed in the same 1.5 ml microfuge tube and spun down at 7,000 *g*. Supernatant was decanted and the cell pellet was resuspended in 50 µl of LB. The cell suspension was spotted on a pre-warmed LB plate and incubated from 4 h to 12 h at 37°C. Cells were scraped and resuspended in 1 ml of 1x M9 salts buffer, washed in 1 ml of 1x M9 salts buffer and dilutions were plated on selective media.

2.7.2 Construction of Transcriptional Reporter Gene Fusions of *fadD3* (PA3860) and *fadD4* (PA1617) in *P. aeruginosa*

2.7.2.1 *fadD3* Transcriptional Fusion

The native locus transcriptional fusion of *fadD3* with *lacZ* reporter gene was constructed utilizing methods described previously [81]. Briefly, suicidal *FRT-lacZ* fusion plasmid, pFRT1-*lacZ*-Gm, and suicidal Flp expressing plasmid, pCD13SK-*flp-oriT*, were co-conjugated with PAO1-Δ*fadD3*::*FRT*. Flp mediated integration of pFRT1-*lacZ*-Gm into *fadD3* gene at the *FRT* 'scar' locus was selected on PIA + Gm150 + 80 µg/ml of X-Gal, yielding PAO1-Δ*fadD3*::*FRT-lacZ*. Next, miniCTX2-*fadD3* was introduced via triparental mating with pRK2013, to complement the *fadD5* mutation resulting in PAO1-Δ*fadD3*::*FRT-lacZ*/ *attB*::miniCTX2-*fadD3*.

2.7.2.2 *fad4* Transcriptional Fusion

To construct *lacZ* transcriptional fusion for *fadD3* (PA1617), an ~2 kb fragment upstream of *fadD4* was amplified from PAO1 chromosomal DNA with oligos #1441 and #1442. PCR product was digested with HindIII and SmaI and cloned into miniCTX2-P*fadE*-*lacZ* digested with the same enzymes. The resulting vector miniCTX2-P*fadD6*-*lacZ* has the upstream region of *fadD4* gene cloned upstream of promoterless *lacZ* reporter gene. This vector was conjugated into PAO1 via triparental matting with helper plasmid, pRK2013, and integration into PAO1 chromosomal *attB* site was selected on PIA + Tet 100 µg/ml + X-Gal 80 µg/ml.

2.7.3 Gene Replacement and Mutant Strains Construction

PA3860 (*fadD3*) and PA1617 (*fadD4*) genes were mutated in various PAO1 backgrounds utilizing pEX18T gene replacement vectors [123] to obtain several strains (P677, P678, P685, P696, P698, P726, and P767, **Table 1**). The *fadD3* gene replacement vector, pEX18T-*fadD3*::Gm^r-*pheS$_{Pa}$*, was constructed as follows. pUC19-PA3860 was digested with MscI and SgrAI, blunt ended with T4 DNA polymerase and ligated with Gm^r-*pheS$_{Pa}$*-FRT cassette which was SmaI excised from pwFRT-*pheS$_{Pa}$*. The resulting plasmid, pUC19-PA3860::Gm^r-*pheS$_{Pa}$*, was digested with BamHI and the PA3860::Gm^r-*pheS$_{Pa}$* fragment was cloned into pEX18T digested with BamHI, yielding pEX18T-*fadD3*::Gm^r-*pheS$_{Pa}$*. The *fadD4* gene replacement vector pEX18T-*fadD4*::Gm-*pheS$_{Pa}$* was obtained as follows. *fadD4* gene was excised from pUC19-PA1617 with HindIII/EcoRI digest and cloned into pEX18T digested with the same enzymes, yielding pEX18T-*fadD4*. Next, Gm^r-*pheS$_{Pa}$*-mFRT cassette was excised from pmFRT-*pheS$_{Pa}$* by SalI digest and cloned into pEX18T-*fadD4* digested with XhoI, resulting in pEX18T-*fadD4*::Gm^r-*pheS$_{Pa}$*.

These gene replacement vectors were utilized as previously described to mutate *fadD3* and *fadD4* genes using *sacB* and sucrose counterselction to select for second homologous recombination [123]. Successful mutation of *fadD3* and *fadD4* genes was confirmed via PCR with primer pairs # 1216 and # 1217, and #1261 and # 1387, respectively, which anneal outside the regions used for initial cloning into gene replacement vectors. Unmarked mutations of *fadD3* and *fadD4* genes in various strains were obtain in one step via Flp mediated excision of Gm^r -*pheS$_{Pa}$* cassettes. Mutant version of *P. aeruginosa pheS* gene [114] present in the Gm^r-*pheS$_{Pa}$*-FRT cassettes allow

for couterselection on minimal media containing chlorinated phenylalanine (cPhe). Briefly, strains containing *fadD* genes inactivated with Gm^r-*pheS$_{Pa}$*-*FRT* cassettes were conjugated with *E. coli* strain E464 harboring suicidal Flp expressing plasmid, pCD13SK-*flp-oriT*. Transconjugants, which had Gm^r-*pheS$_{Pa}$*-*FRT* cassette successfully excised, were selected on 1x M9 + 20 mM glucose + 0.1% (w/v) cPhe. The resulting *fadD3* and *fadD4* mutant strains have *FRT* 'scars' left in the *fadD3* or *fadD4* genes.

Other PAO1 *fadD* homologues mutants were constructed as described previously [124]. Briefly, chromosomal DNA of PAO1strains containing Gm^r labeled mutations of *fadD4* (P685), *fadD5* (P239), and *fadD6* (P416) was electroporated into various PAO1 strains (P678, P696, P698, and P722, Table 1) and the transfer of mutation via homologues recombination was selected on LB + Gm 150 μg/ml. Strains containing unmarked mutations were obtained through Flp mediated excision of Gm^r-*FRT* cassettes utilizing pCD13SK-*flp-oriT* and cPhe as described above (P766, **Table 1**) or pFLP2 (P722, P768, P769, P770, P771, P772, and P773, **Table 1**) as described previously [123]. Strain PAO1-Δ*fadD3*::*FRT*/*fadD4*::m*FRT*/*fadD5*::*FRT*/*fadD6*::*FRT* (P781) was constructed in PAO1-Δ*fadD3*::*FRT* background by subsequent electroporation of chromosomal DNA of PAO1 strains P685, P239, and P416 followed by Flp mediated excision of Gm^r-*FRT* cassettes. Mutations in *fadD5* and *fadD6* genes were confirmed by PCR with oligo pairs #437/#1134 and # 512/#1109, respectively. Presence or absence of deletion of *fadD1D2* region was reconfirmed in various strains by PCR with oligos #302 and #342.

2.8 Identification of Potential PAO1 *fadD* Homologues

Potential *P. aeruginosa fadD* homologues were indentified through BLAST alignment of PAO1 protein sequences to *E.coli* FadD amino acid sequence and alignment to the ATP/AMP and FACS motifs of the *E. coli* FadD. The candidate *fadD* homologues PA0996, PA1221, PA2557, PA3860, and PA4198 were PCR amplified with primers pairs: #1256/#1257, #1151/#1152, #1153/#1154, #1155/#1156, and #1157/#1158, respectively, and cloned into pUC19 as a BamHI fragments. The following *fadD* homologues: PA1617, PA1997, PA2555, and PA3568, were PCR amplified with primers pairs: #1260/#1261, #1254/#1255, #1258/#1259, and #1252/#1253, respectively, and cloned into pUC19 as HindIII/EcoRI, BamHI/SmaI, HindIII/KpnI, and HindIII/SalI fragments, respectively. For functional testing, pUC19 vectors containing PAO1 *fadD* homologues were then transformed into *E. coli fadD⁻/fadR⁻* strain (E2011) and the resulting transfromants were patched onto 1x M9 + 1% Brij-58 (w/v) + Ap 100 µg/ml supplemented with 20 mM glucose 0.2% (w/v) of decanoate ($C_{10:0}$) or oleate ($C_{18:1}{}^{\Delta 9}$). Plasmids were isolated from isolates that showed growth on fatty acid and retransformed into E2011 and repatched on 1x M9 + 1% Brij-58 (w/v) + Ap 100 µg/ml + FA to confirm phenotype.

2.9 Construction of Complementation Vectors for *E. coli* and *P. aeruginosa*
2.9.1 *E. coli*

Initially, pWKS30-*fad3* was constructed by digesting pUC19-PA3860 with EcoRI and HindIII and the *fadD3* fragment was subcloned into pWKS30 digested with the same enzymes. pWKS30-*fad3* was digested with BglII, ScaI and SpeI and the 2.7 kb fragment

of P$_{lac}$-*fadD3* was ligated into the miniTn7 delivery vector, pTn7T-*FRT*8-Gm^r, digested with BamHI and SpeI, resulting in miniTn7-Gm^r-P$_{lac}$-*fadD3*. T his construct was not utilized directly in complementation studies, instead it was used to construct other miniTn7 complementation vectors containing *lac* operon promoter. miniTn7-P$_{lac}$ vector was constructed by digesting miniTn7-Gm^r-P$_{lac}$-*fadD3* with BamHI to delete *fadD3* gene and self-ligated. Final *fadD3* complementation vector miniTn7-*fadD3* was constructed as follow. pET15b-*fadD3* was digested with BamHI and XbaI and the *fadD3*-His$_6$ fragment was cloned into miniTn7-P$_{lac}$ digested with the same enzymes.

To construct miniTn7-*fadD$_{Ec}$*, the *fadD$_{Ec}$* gene was amplified from *E.coli* K-12 chromosomal DNA with oligos #1093 and #1218. The PCR product was digested with HindIII, blunt ended with T4 DNA polymerase, digested with BamHI and cloned into miniTn7-Gm^r-P$_{lac}$-*fadD3* digested XbaI, blunt ended with T4 DNA polymerase, and digested BamHI, yielding miniTn7-*fadD$_{Ec}$*.

To construct miniTn7-*fadD4*, first the *fadD4* gene was amplified with oligos #1261 and #1444 from PAO1 chromosomal DNA. The PCR product was digested with NdeI and EcoRI and cloned into pET28a digested with the same enzymes. Next, pET28a-*fadD4* was digested with EcoRI, blunt-ended with T4 DNA polymerase and digested with XbaI. The *fadD4*-His$_6$ fragment was cloned into miniTn7-P$_{lac}$ digested with the BamHI, blunt-ended with T4 DNA polymerase and digested with XbaI.

2.9.2 *P. aeruginosa*

Single copy complementation vector miniCTX2-*fadD3* was constructed as follows. PA3860 gene with ~650 bp upstream sequence was amplified from PAO1

chromosomal DNA with oligo #1156 and #1271. The PCR product was digested with BamHI and HindIII and cloned into miniCTX2 digested with the same enzymes. This complementation vector was introduced into *fadD3* mutant strain via triparental conjugation with helper plasmid, pRK2013.

Construction of single copy complementation vector miniCTX2-*fadD4* was accomplished as follows. PA1617 gene with 600 bp upstream sequence was amplified from PAO1 chromosomal DNA with oligos #1261 and #1443. The fragment was digested with HindIII and EcoRI and cloned into miniCTX2 digested with the same enzymes. miniCTX2-*fadD4* was introduced into *fadD4* mutant strains through triparental mating with pRK2013.

2.10 Single Copy Complementation Study of *E. coli fadD⁻/fadR⁻* on Different Fatty Acids.

Various miniTn7 vectors (**Table 2**) were introduced into E2011 via four parental mating with pTNS2, pRK2013, and the miniTn7 vectors, utilizing *E. coli* DAP auxotrophic cloning (E2057) and mobilizable (E464 and E1353) strains. Alternatively, miniTn7 vectors were transformed into E1353 and conjugated into E2011 along with pTNS2. Transposition of miniTn7 vectors was selected on LB + Gm 15 μg/ml. Integration at Tn7 attachment site was confirmed by PCR with oligos # 846 and #1238. For the complementation study, 4 colonies of K-12, E2011, E2011*attTn7*::miniTn7, E2011*attTn7*::*fadD_{Ec}*, E2011*attTn7*::*fadD5*, E2011*attTn7*::*fadD6* were patched onto 1x M9 + 1% (w/v) Brij-58 + 1mM IPTG solid medium supplemented with 0.2% (w/v) fatty acids ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:1}^{\Delta 9}$). 1x M9 + 1% Brij-58 (w/v) +

1mM IPTGs solid medium, supplemented with 20mM glucose was used as positive control. Plates were incubated for 3 days at 37°C and bacterial growth was scored from +1 to +6. Very little growth was marked as +1 and very heavy growth on a patch was marked as +6.

2.11 Expression and Purification of and PA3860 (FadD3)

FadD3 expression vector, pET15b-*fadD3*, was constructed as follows. *fadD3* gene was amplified with oligos #1251 and #1156 from PAO1 chromosomal DNA. The PCR product was digested with NdeI and BamHI and cloned into pET15b digested with the same enzymes. pViet-*fadD3*, a low copy-number FadD3 expression vector, was obtained by excising *fadD3* gene from pET15b-*fadD3* by NdeI/BamHI digest and sucbloning into pViet digested with the same enzymes.

FadD3 was expressed from pViet in the *E. coli fadD⁻* T7 expression strain, E1063, at 25°C after 12 h induction with 1 mM IPTG. Purification of histidine-tagged protein was performed with Ni^+-NTA column (Qiagen) as described previously [122]. Proteins were resolved on 10% (v/v) SDS PAGE and visualized with coomassie stain [115]. FadD3 was dialyzed twice against 500 ml of 10 mM Tris-HCl. The Bradford assay [125] was used to determine protein concentration of with BSA as standard.

2.12 Enzyme Assay for Fatty-Acyl-CoA Synthetase Activity

Fatty acyl-CoA synthetase activity was measured as previously described using Ellman's reagent [83]. The assay reactions were conducted in total volumes of 375 µl in thin walled glass tubes in a buffer that was composed of 150 mM Tris-HCl (pH 7.2), 10

mM $MgCl_2$, 2 mM EDTA, 0.1% Triton X-100, 5 mM ATP to which an individual FA (30 to 200 µM, prepared as 2 mM stock in 0.1% (v/v) Trition X-100), to which 80 µg - 160 µg of purified FadD3 protein was added. Reaction mixtures were pre-incubated at 37°C for 3 min and the reaction was started with the addition of CoASH to final concentration of 0.5mM (5 mM stock of CoASH was prepared in 20 mM Tris-HCl, pH 6.7) that was pre-incubated at 37°C for 3 min. The reaction was incubated at 37°C for the duration of the experiment. At time zero 75 µl of the 375 µl reaction mix was removed and mixed with 600 µl of 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, dissolved in 0.1 M potassium phosphate at pH 8.0) and the A_{412} were measured. Subsequent 75 µl aliquots of the reaction were taken at 1 min intervals and mixed with DTNB for further measurements. Reactions with each FadD were repeated to obtain triplicate data for each FA at each concentration. Decreases in A_{412} values (loss of CoASH), for each FA substrate, over time were used to calculate the initial velocity (V_0) for each FA concentration. The maximum velocity (V_{max}) of the enzymes and affinity for the different substrates (Michaelis constant, K_m) were then determined using the Hanes-Woolf plot. The V_{max} and K_m for the substrate ATP, was determine with the same procedure, except that the concentration of $C_{18:1}^{\Delta 9}$ was constant (1 mM) and varying concentrations of ATP (0.03 to 0.2 mM) were used.

2.13 Growth Curve Experiments

To characterize phenotypes of various *fadD* mutants' stains of *P. aeruginosa* growth curve studies were performed using FAs as sole carbon source. Strains were grown overnight (14 - 16 h) in PIB and were washed twice with one volume of 1x M9

salts buffer and resuspended in the same volume of 1x M9 salts buffer. Starter cultures were diluted 200-fold into 1x M9 + 1% Brij-58 minimal medium supplemented with either 20mM glucose or 0.2% (w/v) fatty acids ($C_{4:0}$ - $C_{16:0}$, and $C_{18:1}^{\Delta9}$). To measure the bacterial growth at each time point, cultures were diluted four fold in pre-warmed 4% Brij-58 and the OD_{540} was measured.

2.14 Induction Studies

PAO1-$\Delta fadD3$::FRT-$lacZ$/$attB$::miniCTX2-$fadD3$ and PAO1/$attB$::miniCTX2-P_{fadD4}-$lacZ$ fusion strains were grown overnight in PIB medium, washed twice with one volume of 1x M9 salts buffer, resuspended in the same volume of 1x M9 salts buffer and inoculated at 200-fold dilution into 1x M9 + 1% (w/v) Brij-58 + 1% (w/v) casamino acids (CAA) medium. This master mix was then aliquoted into 125 ml Erlenmeyer flasks and various fatty acids ($C_{4:0}$ - $C_{16:0}$, and $C_{18:1}^{\Delta9}$) were added at concentrations of 0.1% (w/v). Culture growth was measured as described above in **section 2.13**. 1 ml of these cultures was harvested at each time point and β-galactosidase assays were performed in triplicate and Miller units were determined as previously described [126].

2.15 Growth Study of Various *P. aeruginosa* *fadD*s Mutants on Different Fatty Acids

To assess involvement of *P. aeruginosa fadD* homologues in FAs degradation various strains: PAO1, double, triple, quadruple, quintuple, and sextuple *fadD* mutants were streaked out on LB. After 24 h incubation at 37°C, 2 colonies of each strain were patched onto 1x M9 + 1% (w/v) Brij-58 solid medium supplemented with 0.2% (w/v)

fatty acids ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:1}{}^{\Delta 9}$) or 20 mM glucose. Plates were incubated for at 37°C for 4 days. Every 24 h growth of each strain was scored from +1 (little growth) to + 6 (very heavy growth).

Chapter 3. Identification of Fatty Acyl-CoA Synthetase Homologues in

P. aeruginosa **and Biochemical Characterization of PA3860 (FadD3)**

Acknowledgments:

3.1 Introduction

In a previous study, two of *P. aeruginosa* fatty acyl-CoA synthetase genes were identified and their role in Fad was investigated [83]. FadD1 and FadD2 biochemical characteristics indicate that both enzymes are involved in catalysis of $C_{4:0}$ - $C_{18:1}{}^{\Delta 9}$ to fatty acyl-CoA. Each enzyme has different substrate preference: FadD1 longer chain FAs whereas FadD2 shorter chain FAs. *fadD1* and *fadD2* were determined to be involved in utilization of $C_{4:0}$ - $C_{18:1}{}^{\Delta 9}$ as carbon and energy source. However, the double $\Delta fadD2D1$ mutant still has half the ability of wildtype PAO1 strain to grow on FAs. This indicates that additional *fadD* gene (s) is/are present in PAO1. The *P. aeruginosa* genome was predicted to contain 6 - 10 times more genes for Fad then *E. coli* [12]. Identification of homologues of fatty acyl-CoA synthetase is complicated since 20 potential genes, identified by BLAST, have low values of identity (best score is 31%) and homology (highest score is 52%). In comparison FadD1 and FadD2 have high homology (72%) and identity (53%) to *E. coli* FadD.

A screening method based on the presence of FadD's motifs and functional testing was utilized to identify *P. aeruginosa* fatty acyl-CoA synthetase homologue (s).

Initially indentified homologue, FadD3, was hypothesized to be last FadD present in

P. aeruginosa and was characterized for its biochemical properties. Since FadD1 and

FadD2 both activate various FAs, FadD3 was hypothesized to also be involved in

activating of various chain length FAs.

3.2 Results

3.2.1 Identification of *fadD* Homologues of *P. aeruginosa*

To identify potential *fadD* homologues of *P. aeruginosa*, *E. coli* FadD amino acid

(aa) sequence was compared to PAO1 ORFs via BLAST [127]. The potential

homologues obtained in the initial search (20 genes) were further analysed for presence

of ATP/AMP [75, 84, 99, 100] and FACS (Fatty Acyl-CoA Synthase) motifs [101].

Proteins that contain amino acid sequences most similar to those of *E. coli* FadD's motifs

(**Table 4** and **Figure 4**) were chosen for complementation testing in *E. coli*. Homologues

selected initially were PA1221, PA2557, PA3860, and PA4198. All but PA1221 were

predicted to be AMP binding enzymes (**Table 4)** and contained the ATP/AMP binding

motif **(Figure 4)**. All of the homologues have similar aa length when compared to *E. coli*

FadD (561 aa) and their identities range from 24% to 31% and homologies from 40% to

52% and (**Table 4**). When cloned into pUC19 vector (as described in **Section 2.8** of

Chapter 2) only PA3860 was found to complement *E. coli fadD⁻/fadR⁻* (E2011) strain on

FAs $C_{10:0}$ and $C_{18:1}{}^{\Delta 9}$ (**Table 5**). PA3860 gene which is 31% identical and 47%

homologous to the *E. coli* FadD on aa level was designated as PAO1 *fadD3*. After the

successful identification of *fadD3* and preliminary growth analysis of *fadD3* mutant

strains (**Chapter 4, section 4.2.1**) further search for more PAO1 *fadD* homologues was performed utilizing the same approach as describe above.

Table 4. *P. aeruginosa fadD* potential homologues indentified through BLAST.

Gene	Identity/ Homology to FadD$_{Ec}$	Length (amino acids)	Possible AMP/ATP Binding Motif	Possible FACS Motif	Predicted function [c]
PA1221[a]	24%/40%	618	+	+	hypothetical protein
PA2557[a]	31%/52%	564	+	+	probable AMP-binding enzyme
PA3860[a]	29%/47%	632	+	+	probable AMP-binding enzyme
PA4198[a]	26%/43%	540	+	+	probable AMP-binding enzyme
PA0996[b]	28%/43%	517	+	+	probable coenzyme A ligase
PA1617[b]	24%/40%	555	+	+	probable AMP-binding enzyme
PA2555[b]	25%/44%	555	+	+	probable AMP-binding enzyme
PA1997[b]	21%/37%	651	+	+	probable AMP-binding enzyme
PA3568[b]	24%/39%	628	+	+	probable acetyl-CoA synthetase

[a] Homologues selected for initially complementation test. [b] Homologues selected for second complementation test. [c] From www.pseudomonas.com.

Proteins chosen (PA0996, PA1617, PA2555, PA1997, and PA3568) for complementation test in *E. coli* had less overall identity and homology (21% - 28% and 37% - 44%, respectively) (**Table 4**) and their motifs (especially FACS motif, **Figure 4**) have less identity to *E. coli* FadD then homologues selected in the previous bioinformatic screen. Selected genes were predicted to be AMP binding enzymes or to have CoA ligase activity (**Table 4**). Out of these five genes, growth was observed for *E. coli fadD* mutant (E2011) complemented with PA1617 and PA1997 (**Table 5**). However, the amount of growth for PA1997 was minimal on $C_{18:1}^{\Delta 9}$, less then growth observed for PA3860 (*fadD3*) and PA1617 on $C_{10:0}$, indicating that PA1997 has minimal fatty acyl-CoA activity even when

Protein	ATP/AMP Binding Motif	FACS Motif
E. coli FadD	213 YTGGTTGVAKGA 224 - 256 GYGLTE 361	431 NG WLHTGD I AVMDEEGFL RIVDRKK 455
PA1221 [a]	168 F SSGTTGRP KA I 179 - 306 GYGPTE 312	398 RGR L RTGDRARYDEQGR L RFIG RGD 417
PA2557 [a]	208 YTSGTTGFP KGA 220 - 349 AYGMTE 354	429 PA- WMHTGDLAVMDDDGYVRIVGRSK 451
PA3860 [a]	223 HTGGTTGTP KLA 235 - 366 GYG LTE 372	447 DG WFNTGD LGR I DED GYIWLTGRSK 472
PA4198 [a]	188 YTSGTTGNP KGV 199 - 225 VYGLTE 230	414 GG WFHTGDL AV CHP DG I E I R DRLK 435
PA0996 [b]	163 YTSGSTGAP KGV 174 - 300 GI GATE 305	375 GG WYRTGDLFER DE SGAY RHCGRED 399
PA1617 [b]	173 YTSGTTGVP KGV 184 - 334 VYGMTE 339	399 DG F LR TGDKGEQDADGNL RLTGRMK 423
PA1997 [b]	271 YSSGTTGVP KC I 282 - 420 GTD I VS 425	499 GV WAH- GDY A EETVH GGLV I H - RSD 522
PA2555 [b]	198 FTSGTTGL AKP V 207 - 337 HYGQTE 342	415 DY YL-S GDTVEL-N EDGSISF VG RAD 338
PA3568 [b]	238 YTSGTTGKP KG 248 - 392 GWPVT 398	469 N- GYYHTGDGGY- LDEDGFVYIMGRTD 495

Figure 4. Comparison of *P. aeruginosa* potential FadD homologues' motifs to *E. coli* FadD motifs. In green are highlighted identical amino acids (aa) whereas in yellow are highlighted similar aa. Underlined represents aa pextra in the motifs. [a] homologues selected in initially search. [b] homologues selected in second search. FACS; Fatty Acyl-CoA Synthetase.

present at high copy number (300 - 500 of copies of gene per *E. coli* cell). Only PA1617 was able to restore growth of E2011 on $C_{10:0}$ and $C_{18:1}^{\Delta 9}$. PA1617 (24% identity and 40% homology to *E. coli* FadD) was named as *fadD4* of PAO1.

Table 5. Complementation test of potential *P. aeruginosa fadD's* homologues in *E. coli*.

Potential PAO1 *fadD* homologue[c]		PA1221[a]	PA2557[a]	PA3860[a]	PA4198[a]	PA0996[b]	PA1617[b]	PA2555[b]	PA1997[b]	PA3568[b]
Growth on FA	$C_{10:0}$	-	-	+1	-	-	+1	-	-	-
	$C_{18:1}^{\Delta 9}$	-	-	+4	-	-	+4	-	+1/2	-

[a] Genes tested initially. [b] Second set of genes tested. [c] Genes were cloned into pUC19 (as described in **Chapter 2, Section 2.8**) and tested on 1x M9 + 1% Brij-58 + Ap 100 µg/ml + 0.2% (w/v) of different fatty acids. +1 indicates very little growth and +6 means heavy growth.

3.2.2 Single Copy Complementation study of *E. coli fadD⁻/fadR⁻* on Different Fatty Acids with *P. aeruginosa fadD* Homoluges

Both genes *fadD3* and *fadD4* were tested further for their ability to support growth of *E. coli fadD⁻/fadR⁻* (E2011) on various FAs at single copy number levels. mini-Tn7 based complementation vectors were constructed and integrated into E2011 chromosome at the Tn7 attachment site (as described in **Chapter 2**, **Section 2.9.1** and **2.10**) and resulting strains were tested for growth on FAs (**Table 6**). Wild type *E. coli* control strain K12 show growth on longer chain fatty acids ($C_{12:0}$ - $C_{18:1}^{\Delta 9}$) but not on the medium or short chain length FAs. E2011, *E. coli fadD* mutant, with and without mini-Tn7 insertion, was not able to grow on all of the FAs as expected. E2011 complemented with *E. coli fadD* (*fadD_{Ec}*) was able to grow on $C_{12:0}$ - $C_{18:1}^{\Delta 9}$ as well as or even better than K12. *fadD3* and *fadD4* allowed the E2011 strain to grow on $C_{12:0}$ - $C_{18:1}^{\Delta 9}$ to very similar level as K12. Both *fadD3* and *fadD4* supported growth of E2011 on $C_{10:0}$ to the same level as *fadD_{Ec}*. E2011 complemented with *fadD_{Ec}*, *fadD3*, and *fadD4* did not grow

on short FAs $C_{4:0}$ - $C_{8:0}$ which was in agreement with previous observations that other *E. coli* Fad enzymes do not support metabolism of shorter FA [128].

Table 6. Single copy complementation of *E.coli fadD* mutant with *P. aeruginosa fadD* homologues.

Strain	Growth on different carbon sources*								
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:1}{}^{\Delta 9}$	Glu
K12	-	-	-	-	+3	+5	+5	+5	+6
E2011 (*fadD⁻ fadR⁻*)	-	-	-	-	-	-	-	-	+6
E2011/*attTn7*::miniTn7	-	-	-	-	-	-	-	-	+6
E2011/*attTn7*::fadD$_{Ec}$	-	-	-	+1	+5	+5	+5	+5	+6
E2011/*attTn7*::fadD3	-	-	-	+1	+3	+5	+5	+5	+6
E2011/*attTn7*::fadD4	-	-	-	+1	+4	+5	+5	+5	+6

Strains were grown on 1x M9 +1% Brij-58 medium supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu) + 1mM IPTG for 3 days at 37°C. * - indicates no growth on a patch; + denotes growth: +1 is very little growth whereas +6 is heavy growth

3.2.3 Purification of PA3860 (FadD3)

To characterize FadD3 protein as an enzyme *fadD3* gene was cloned into T7 polymerase expression vector with N-terminal poly-histidine tag as described in **Chapter 2 Section 2.11**. FadD3 with N-terminal His-tag was determined to be functional in *E. coli* at single copy levels (**Table 7**, see **Section 2.9.1** of **Chapter 2**). The FadD3 was expressed and purified as described in **Section 2.11** from *E. coli fadD⁻* to ~70% homogeneity (**Figure 5**) using nickel ion chromatography. FadD3 elutes at low concentration of imidazole (20 mM) as can be seen in lane one and additional bands of the same size are also present in 200 mM imidazole eluent (lane two) (**Figure 5**). Attempts at obtaining cleaner protein preparation were unsuccessful (i.e. increasing concentration of nonionic surfactant in binding and wash buffers, gradient elution, and ion exchange chromatography with weak anion exchange support, data no shown). Since FadD3 was purified from an *E. coli fadD⁻* strain*,* all of acyl-CoA synthetase activity of the
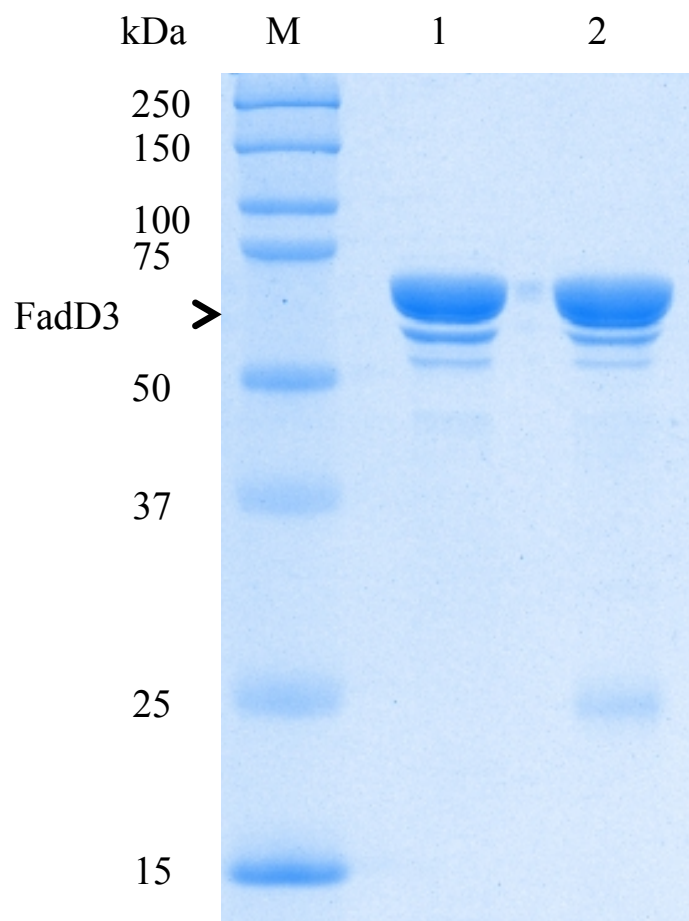
Figure 5. SDS PAGE of purified FadD3. FadD3 with N-terminal His-tag (71,115 kDA) was purified from *E. coli fadD*⁻. Lane 1; 20 µg of FadD3 eluted with 20 mM imidazole (10 µg). Lane 2; 20 µg of FadD3 eluted with 200 mM imidazole.

protein preparation will come from the expressed recombinant enzyme and not from contaminating *E. coli* FadD protein. Therefore, this FadD3 preparation was used in the enzyme assay.

3.2.4 Biochemical characterization of PA3860 (FadD3)

Fatty acyl-CoA synthetase activity of FadD3 was determined using an assay based on Ellman's Reagent (**Figure 6 A** and **B**) as described in **Section 2.12** of **Chapter 2**. Enzyme assay curves of FadD3 with different substrates (ATP and FAs) are presented in **Figure 6 C**. FadD3 can activate all of the FAs tested to fatty acyl-CoA with different efficiencies. FadD3 is most active towards longer chain FA ($C_{16:0}$, $C_{18:1}$, and $C_{14:0}$,) and least active towards shorter chain FAs ($C_{8:0}$, $C_{6:0}$, and especially $C_{4:0}$). Moderate activity was observed towards ATP and medium chain length FAs ($C_{10:0}$ and $C_{12:0}$). Variability in the measurements of activities (presented as S.E.M., **Figure 6 C**) was in agreement with variability observed previously for this method of assaying fatty acyl-CoA synthetase activity [83].

The kinetic parameters of FadD3 were determined for different substrate and are presented in **Table 7**. Kinetic parameters of for ATP indicate that ATP is a good substrate for FadD3. Maximum velocity (V_{max}) was highest for long chain FAs (with greatest value of 53.2 acyl-CoA nmol/min/mg of protein for $C_{16:0}$) and smallest for short chain FAs (V_{max} for $C_{4:0}$ equals to 17.8 acyl-CoA nmol/min/mg of protein). In general greater chain length of fatty acids corresponds to higher activity of FadD3 towards that substrate. Based on the turn over numbers (K_{cat}) for different FAs, FadD3 is faster at converting long chain FAs to fatty acyl-CoA, then short chain FAs. Michaelis constant's, K_m, for
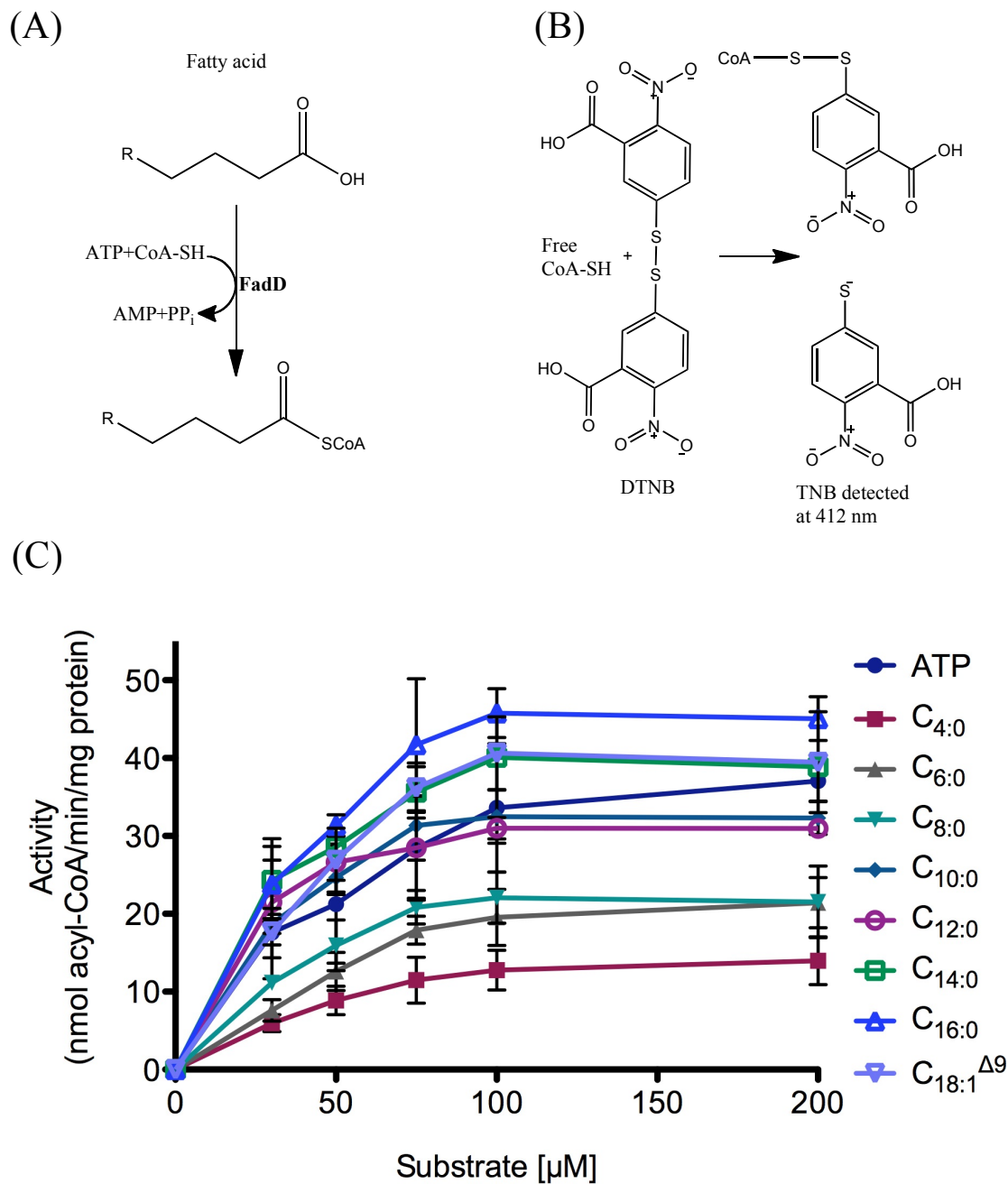
Figure 6. Determination of activities of FadD3 with different substrates. (A) FadD actives fatty acids in the presence of ATP and CoASH. (B) Ellman's Reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), reacts with sulfhydryl group of CoASH producing 2-nitro-5-thiobenzoic acid (TNB) which can be detected at 412 nm. (C) Activities of FadD3 towards different substrates were determined using Ellman's Reagent and are presented as average ± S.E.M.

tested FAs ranged from 12.9 μM to 66.5 μM. FadD3 has highest affinity towards $C_{12:0}$, $C_{10:0}$ and $C_{14:0}$ (K_m= 12.9 μM - 20.4 μM) and lowest for $C_{4:0}$ and $C_{6:0}$ (K_m= 49.0 μM and K_m= 66.5 μM, respectively).

Table 7. Kinetic parameters of FadD3.

FadD3 Kinetic Parameter[a]				
Substrate	V_{max}[b]	K_m[c]	K_{cat}[d]	K_{cat}/K_m[e]
ATP	46.1	48.7	0.0546	1.12
$C_{4:0}$	17.8	49.0	0.0211	0.430
$C_{6:0}$	29.7	66.5	0.0352	0.529
$C_{8:0}$	25.1	25.4	0.0298	1.17
$C_{10:0}$	36.4	20.3	0.0431	2.12
$C_{12:0}$	33.3	12.9	0.0395	3.06
$C_{14:0}$	43.9	20.4	0.0520	2.55
$C_{16:0}$	53.2	28.7	0.0631	2.20
$C_{18:1}^{\Delta 9}$	48.8	37.3	0.0578	1.55

[a] Kinetic constants (V_{max} and K_m) were determined using Hanes-Woolf plot
[b] nmole of acyl-CoA formed/min/mg of protein.
[c] μM of substrate (ATP or FA).
[d] s^{-1}; determined using MW of recombinant FadD3 (71,115).
[e] $mM^{-1}s^{-1}$; enzyme catalytic efficiency.

FadD3's catalytic efficiency (K_{cat}/K_m) increases with the length of FAs to reach maximum at $C_{12:0}$ and then decreases as chain length of FAs increase (**Table 7**). Catalytic efficiency is on average ~ 5 times higher for FAs $C_{10:0}$ through $C_{16:0}$ when compare to $C_{4:0}$ and $C_{6:0}$. Taken together, this indicates that FadD3 is most efficient at activating $C_{10:0}$ through $C_{16:0}$ to fatty acyl-CoA.

Chapter 4. Contribution of *fadD3* and *fadD4* to Fatty Acid Degradation in

P. aeruginosa

4.1 Introduction

Previous investigation of two *P. aeruginosa* fatty acyl-CoA syntheatase genes, *fadD1* and *fadD2*, indicated that additional *fadD* gene(s) is/are present in *P. aeruginosa* and contribute(s) to ~ 50% of fatty acid degradation [83]. PA3860 (*fadD3*) gene was identified as fatty acyl-CoA syntheatase and was biochemically characterized (**Chapter 3**). It was the only homologue found in the first search thus was hypothesized to be the last *fadD* gene of *P. aeruginosa*. Preliminary growth curve studies with *fadD3* mutant strains (see detail below in **section 4.2.1**) indicated existence of more homologues of *fadD*. PA1617 (*fadD4*) was identified in second search (**Chapter 3**). Contribution of both *fadD* genes in Fad was investigated through growth curves studies with mutant strains of *fadD3* and *fadD4* in this chapter. Roles of *P. aeruginosa* genes, *fadBA1*, *fadBA5*, *fadD1* and *fadD2*, in Fad were studied previously through reporter fusions with informative outcomes [17, 81, 83]. Thus, *fadD3* and *fadD4* reporter fusions were also utilized in determining involvement of these homologues in Fad.

4.2 Results

4.2.1 Growth Curve Experiments with *fadD3* and *fadD4* Mutants

To determine level of involvement in Fad of *fadD3* unmarked mutant strains: PAO1Δ*fadD3* and PAO1Δ*fadD2D1D3* were created as described in **Chapter 2, section 2.7.3**. PCR confirmation of genotypes of both strains is presented in **Figure 7.** PCR product of wildtype *fadD2D1* locus is 4.1 kb and mutated is ~1.9 kb with primers #302
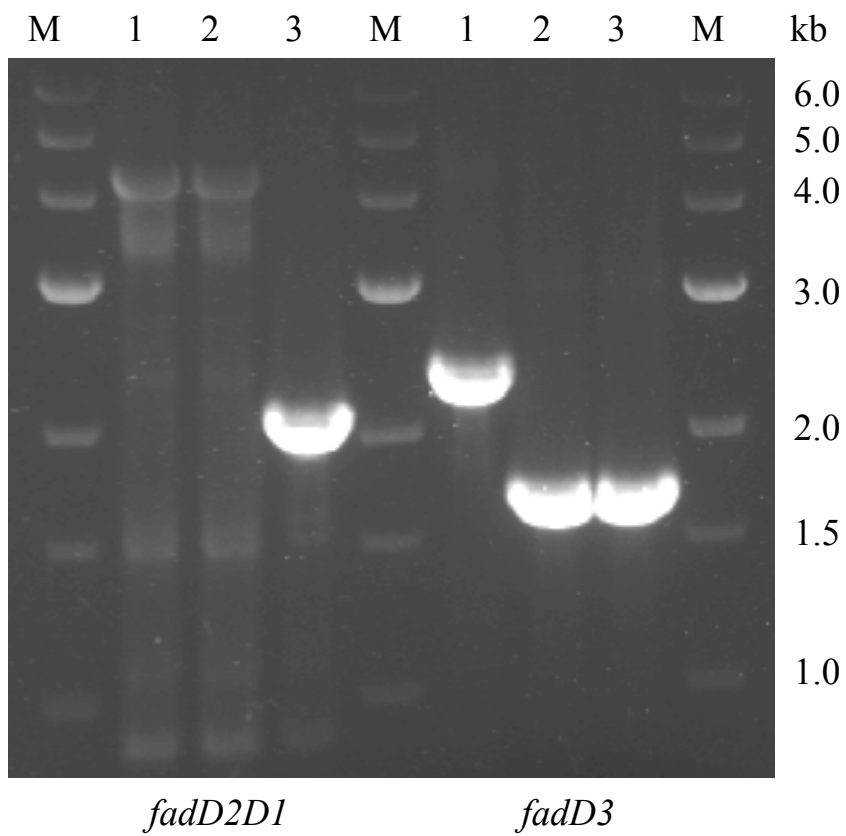
fadD2D1   *fadD3*

Figure 7. PCR confirmation of unmarked *fadD3* mutant strains. Lane: (1) PAO1, (2) PAO1Δ*fadD3*, and (3) PAO1Δ*fadD2D1D3*. Primers #302 and #342 were used for PCR of *fadD2D1* region: wildtype ~4.1 kb, mutant ~1.9 kb. Primers #1216 and #1217 were used for PCR of *fadD3* region: wildtype ~2.1 kb, mutant ~1.6 kb.
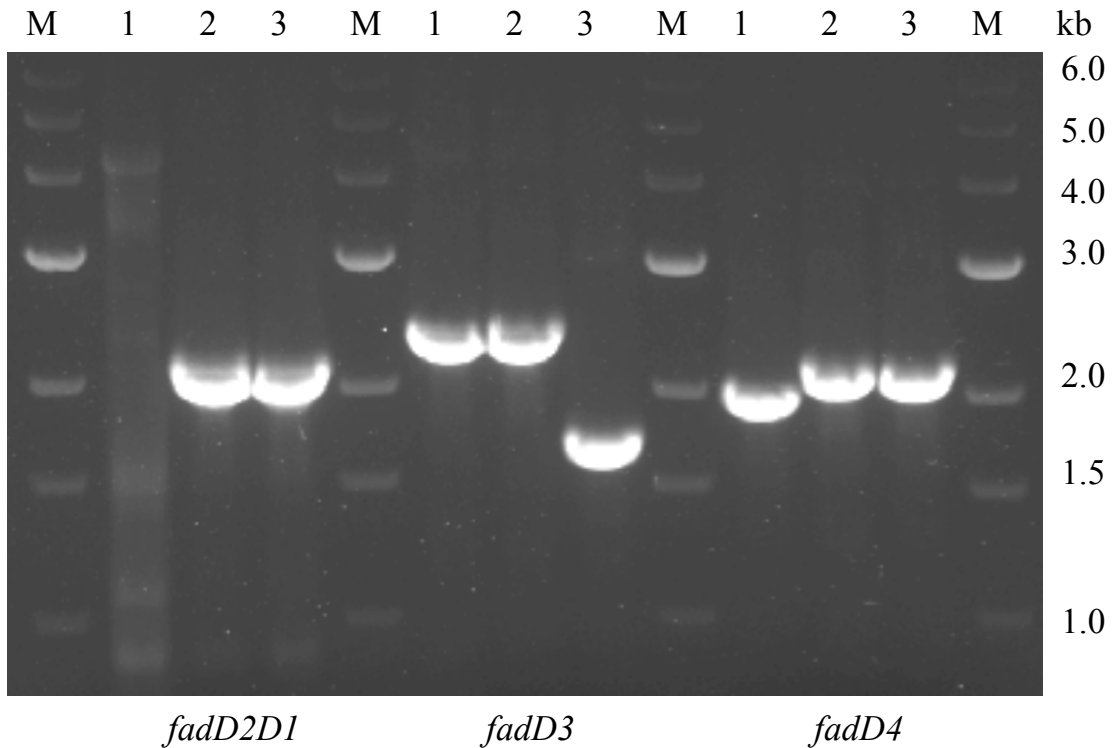
Figure 8. PCR confirmation of unmarked *fadD4* mutant strains. Lane: (1) PAO1, (2) PAO1Δ*fadD2D1D4*, and (3) PAO1Δ*fadD2D1D3D4*. Primers #302 and #342 were used for PCR of *fadD2D1* region: wildtype ~4.1 kb, mutant ~1.9 kb. Primers #1216 and #1217 were used for PCR of *fadD3* region: wildtype ~2.1 kb, mutant ~1.6 kb. Primers #1261 and #1387 were used for PCR of *fadD4* region: wildtype ~ 1.9 kb, mutant ~2.0 kb.

and #342 (**Figure 7**). PCR product for mutated *fadD3* gene is 1.5 kb whereas for wiltype it is 2.1 kb with primers #1216 and #1217 (**Figure 7**). Initial growth curves in FAs were performed on PAO1Δ*fadD3* and PAO1Δ*fadD2D1D3* along with PAO1 and PAO1Δ*fadD2D1*. No growth differences were observed between the *fadD3* mutant strain and PAO1 and PAO1Δ*fadD2D1D3* and PAO1Δ*fadD2D1*, respectively (data not shown). Since an additional gene encoding fatty acyl-CoA synthetase, *fadD4* (PA1617), was identified (**Chapter 3, section 3.2.1**) strains with *fadD4* mutations were constructed: PAO1Δ*fadD2D1D4* and PAO1Δ*fadD2D1D3D4* (as described in **Chapter 2, section 2.7.3**). PCR confirmation of genotypes of both strains is presented in **Figure 8.** As it can be seen in **Figure 8**, PCR product for mutated *fadD4* gene is ~100 bp larger then for wildtype because of presence of FRT 'scar'.

Growth curves with *fadD4* mutants' strains along with PAO1, Δ*fadD2D1*, and Δ*fadD2D1D3* were performed to determine the role of *fadD3*, *fadD4,* as well as the combined role of *fadD3* and *fadD4* in Fad (**Figure 9**). The rationale for use of triple and quadruple mutants was that the contribution of *fadD3* and *fadD4* to Fad could be difficult to observe in the presence of the major fatty acyl-CoA synthetase genes, *fadD1* and *fadD2*, or the other identified fatty acyl-CoA synthetase (i.e. *fadD3* or *fadD4*). All strains: Δ*fadD2D1*, Δ*fadD2D1D3*, Δ*fadD2D1D4*, Δ*fadD2D1D3D4*, and PAO1 grew the same in minimal medium with glucose as carbon source (**Figure 9 A**). In $C_{4:0}$ PAO1 achieved higher OD than the mutant strains and no growth difference was observed between all mutants (**Figure 9 B**). In minimal media supplemented with $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ FAs PAO1 grown faster and denser than all the mutants whereas Δ*fadD2D1* and Δ*fadD2D1D3* exhibited the same growth characteristic, i.e. increased lag phase, the same growth rate
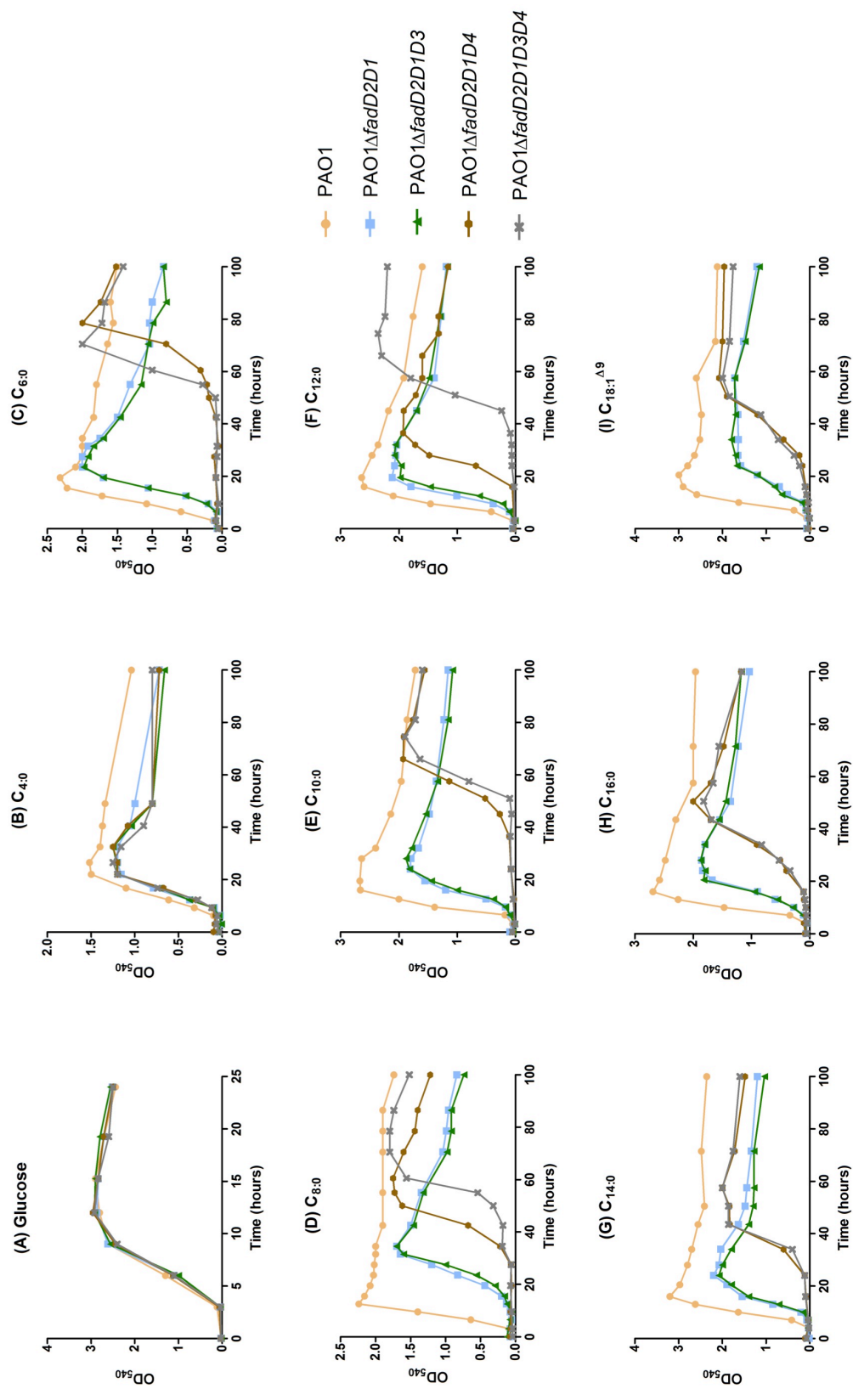
Figure 9. Growth curves of *fadD3* and *fadD4* mutants in glucose and different chain length fatty acids.

and reduced final OD compared to PAO1 (**Figure 9 C - I**). Growth of triple and quadruple *fadD4* mutants was characterized by much longer lag phase, 20 h - 50 h, in comparison to 10 h - 12 h for $\Delta fadD2D1$ and $\Delta fadD2D1D3$ in all FAs except for $C_{4:0}$ (**Figure 6 C - I**). In SCFAs and MCFAs $\Delta fadD2D1D4$ mutant exhibited shorter lag phase than $\Delta fadD2D1D3D4$ by 10 h to 20 h (**Figure 9 D - F**). Unexpectedly, in $C_{6:0}$ *fadD4* quadruple grew faster than the triple *fadD4* mutant (**Figure 9 C**). Both *fadD4* mutants reached OD similar to $\Delta fadD2D1$ and $\Delta fadD2D1D3$ in $C_{6:0}$ and $C_{8:0}$. Surprisingly, in $C_{10:0}$ both strains grew denser by ~10% compared to $\Delta fadD2D1$, and in $C_{12:0}$ $\Delta fadD2D1D3D4$ reached an OD ~15% higher than $\Delta fadD2D1$, $\Delta fadD2D1D3$ or $\Delta fadD2D1D4$. In LCFA ($C_{14:0}$ - $C_{18:1}{}^{\Delta 9}$) triple and quadruple *fadD4* mutants had the same growth rate and reached final ODs similar to $\Delta fadD2D1$ and $\Delta fadD2D1D3$ at 50 h - 60 h (**Figure 9 G - I**). Presence of *fadD3* mutation alone in the $\Delta fadD2D1$ background did not cause growth deficiencies in any of FAs. However, mutation of *fadD4* in $\Delta fadD2D1$ background resulted in a growth defect in the form of long lag phase in all FAs except $C_{4:0}$. Strain lacking all four *fadD* exhibited an even longer lag phase compared to $\Delta fadD2D1D4$ mutant in $C_{8:0,}$ $C_{10:0}$, and $C_{12:0}$ indicating that *fadD3* also contributes to Fad.

4.2.2 Growth Curve Experiments with *fadD3* and *fadD4* Mutants and *fadD4* Complemented Strains

Some of the results obtained from the first growth curves with the *fadD3* and *fadD4* mutants were surprising i.e. the faster growth of quadruple mutant in $C_{6:0}$, higher final OD of the quadruple and triple mutants in some of the FAs. To confirm the
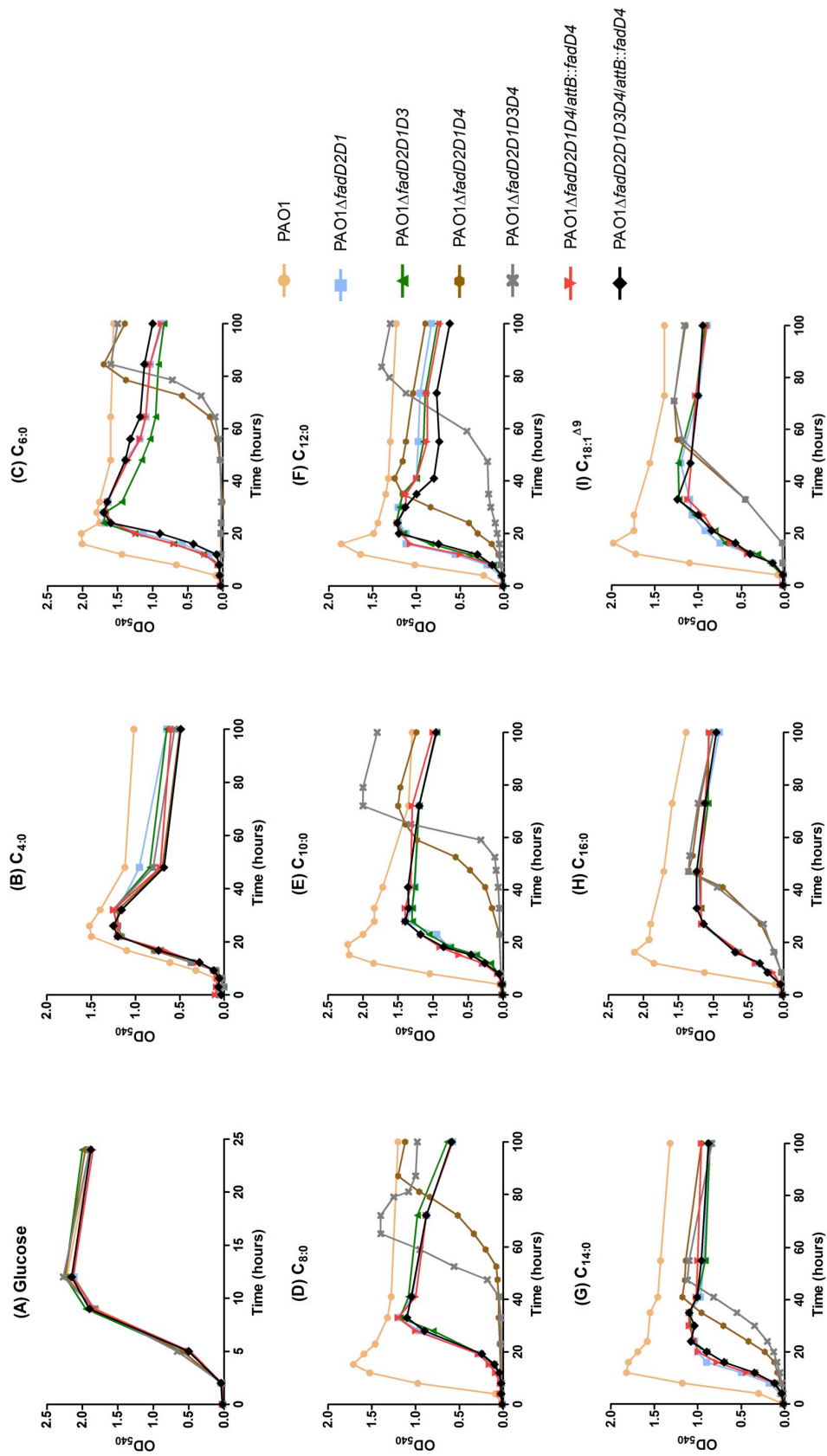
Figure 10. Growth curves of *fadD3* and *fadD4* mutants along with *fadD4* complemented strains in various fatty acids and glucose.

observed mutants' phenotypes the growth curves in all 8 FAs were repeated with the $\Delta fadD2D1$, $\Delta fadD2D1D3$, $\Delta fadD2D1D4$, $\Delta fadD2D1D3D4$, and PAO1 along with new strains: $\Delta fadD2D1D4/attB::fadD4$ and $\Delta fadD2D1D3D4/attB::fadD4$. Previous growth curves (**Figure 6**) showed that growth differences between reference strain, $\Delta fadD2D1$, and triple and quadruple mutants were observable when the $fadD4$ gene was mutated. Thus only $fadD4$ mutants were complemented with $fadD4$.

All 7 strains grew the same in minimal medium with glucose (**Figure 10 A**). In $C_{4:0}$ (**Figure 10 B**) all mutant strains and $fadD4$ complemented strains grew the same but not as dense as PAO1 showing a similar pattern as previously (**Figure 10 B**). In other FAs ($C_{6:0}$ - $C_{18:1}^{\Delta 9}$) growth of $\Delta fadD2D1$ and $\Delta fadD2D1D3$ was the same, with longer lag phase and lower final OD then PAO1 (**Figure 10 C - I**) as observed in growth curves in **Figure 9**. $fadD4$ complemented strains, $\Delta fadD2D1D4/attB::fadD4$ and $\Delta fadD2D1D3D4/attB::fadD4$, had the same growth kinetics as $\Delta fadD2D1$ and $\Delta fadD2D1D3$ in $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ indicating that $fadD4$ mutation was complemented and that no other mutations were causing the growth defect observed in FAs. A similar pattern of growth was observed for the strains having $fadD4$ mutations as in **Figure 9** i.e. much longer lag phase and the same or higher final OD as $\Delta fadD2D1$ and $\Delta fadD2D1D3$. In $C_{6:0}$ $\Delta fadD2D1D4$ had shorter lag than $\Delta fadD2D1D3D4$ (**Figure 10 C**), which was opposite to growth pattern observed previously (**Figure 9 C**). Interestingly, in $C_{8:0}$ $\Delta fadD2D1D3D4$ grew faster than $\Delta fadD2D1D4$ (**Figure 10 D**). A similar growth pattern was observed in previous growth curves in $C_{6:0}$ (**Figure 9 C**). In $C_{8:0}$, $C_{10:0}$, and $C_{12:0}$ $\Delta fadD2D1D3D4$ achieved 20% - 40% higher final OD than $\Delta fadD2D1$, $\Delta fadD2D1D3$, and $\Delta fadD2D1D4$ (**Figure 10 D**, **E**, and **F**). In longer chain fatty acids, $C_{16:0}$ and $C_{18:1}^{\Delta 9}$, triple and

quadruple *fadD4* mutants grew the same (**Figure 10 H** and **I**) as observed previously (**Figure 9 H** and **I**) indicating that *fadD3* does not contribute significantly to degradation of these FAs. Surprising, in $C_{14:0}$ $\Delta fadD2D1D3D4$ had a longer lag phase than $\Delta fadD2D1D4$ (**Figure 10 G**) which was not observed previously (**Figure 9 G**) suggesting that *fadD3* possibly also contributes to degradation of $C_{14:0}$. Based on the growth curves presented in **Figure 9** and **Figure 10** *fadD4* and *fadD3* are involved in degradation of FAs, $C_{6:0}$ - $C_{18:1}^{\Delta 9}$, and $C_{6:0}$ - $C_{12:0}$, respectively. Additionally, the ability of $\Delta fadD2D1D3D4$ mutant to grow in FAs indicates presence of additional *fadD* genes.

4.2.3 Induction studies of *fadD3* and *fadD4*

To further study involvement of *fadD3* and *fadD4* in Fad expression levels of these genes were measured in presence of various FAs. *lacZ* reporter fusions for *fadD3* and *fadD4* were created (**Chapter 2, section 2.7.2**) and fusion strains were grown in minimal media with casamino acids as carbon and energy source in the presence or absence of 0.1% (w/v) of FAs ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:1}^{\Delta 9}$). Both reporter strains had the same growth rates and very similar OD values in all conditions (**Figure 11 A** and **B**) allowing for comparison of gene expression between various FAs and control (CAA alone). Similarly, as observed before [81] death phase between the control media and media with FAs were different. β-galactosidase activity, corresponding to expression of gene of interest, was measured at different stages of growth of *lacZ* fusion strains: early-log (5 h), mid-log (7.5 h), late-log (10 h), early-stationary (12.5 h), and stationary phase (15 h).
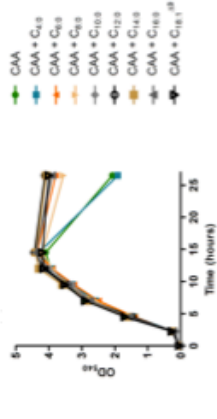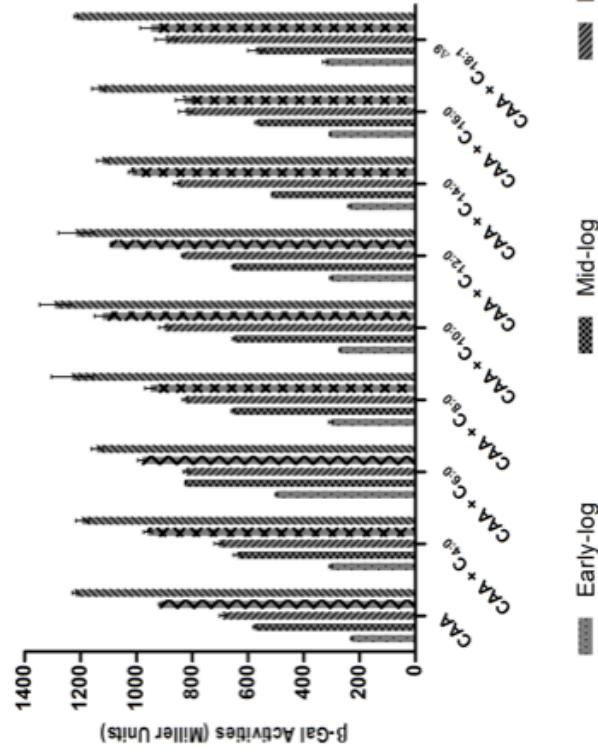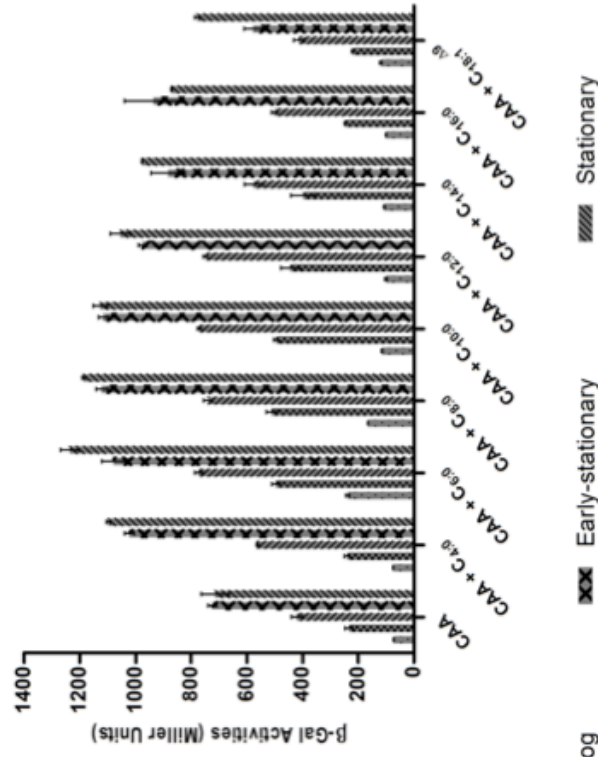
Figure 11. Induction studies of *fadD3* and *fadD4* via *lacZ* gene fusions. (A) and (B) Growth curves of *lacZ* fusion strains of *fadD3* and *fadD4*, respectively, in casamino acids ± different chain length fatty acids. (C) and (D) Induction of *fadD3* and *fadD4*, respectively, by FAs. β-Galactoside activities were determined for both *lacZ* fusion strains for different conditions at different times points in triplicate and are presented as mean ± S.E.M.

4.2.4 Induction of *fadD3* by FAs

fadD3 response to FAs is presented in **Figure 8 C**. *fadD3* is expressed in all conditions (CAA±FAs) and levels of β-galactosidase activity increase from early-log (200 Miller Units) to stationary phase (~1200 Miller Units). Expression of *fadD3* is almost constitutive for most FAs beside $C_{6:0}$. At early-log level of β-galactosidase activity was ~2.3 times higher for $C_{6:0}$ and not much difference was observed between the remaining FAs and CAA. At mid-log *fadD3* expression was ~40% higher in $C_{6:0}$. At late-log expression in $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ was ~20% higher. At early-stationary phase only in $C_{10:0}$ and $C_{12:0}$ levels of β-galactosidase activity was still 20% higher compared to CAA. No significant difference in level of *fadD3* expression between CAA and FAs was observed at stationary phase.

4.2.5 Induction of *fadD4* by FAs

fadD4 was expressed differentially in the presence of $C_{4:0}$ - $C_{16:0}$ (**Figure 8 D**). In general most induction was observed for $C_{6:0}$ and $C_{8:0}$ at all growth phases. *fadD4* was also induced to lesser degrees by $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$ and least by $C_{4:0}$ and $C_{16:0}$. More induction was observed at early growth stages then in latter phases (2 times compare to 1.3 - 1.6 times, respectively). At early-log in $C_{6:0}$ and $C_{8:0}$ levels of β-galactosidase activity were ~3 and ~2 times higher, respectively, compared to CAA and *fadD4* was not responding to other FAs. At mid-log *fadD4* levels were ~2 - 2.3 times greater in $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$. At late-log induction by $C_{6:0}$ - $C_{12:0}$ was ~2 times greater compared to CAA and for $C_{4:0}$ and $C_{14:0}$ it was 20% greater. In early-stationary phase

levels of β-galactosidase were 1.5 times higher for $C_{6:0}$ - $C_{10:0}$ and ~1.3 times greater for $C_{4:0}$, $C_{14:0}$, and $C_{16:0}$. In stationary stage *fadD4* was induced by $C_{4:0}$ - $C_{12:0}$ ~1.6 times and ~1.4 and ~1.2 times by $C_{14:0}$ and $C_{16:0}$, respectively.

Chapter 5. Fatty Acyl-CoA Synthetase Homologues, *fadD5* and *fadD6,* are Involved in Fad of *P. aeruginosa*

5.1 Introduction

Based on *P. aeruginosa* genome analysis there is potentially 6 to 10 times more genes for Fad then in *E. coli* [12]. This *in silico* prediction was showed to have merit when three *fadBA* operons were determined to be responsible for almost all activity of the two last steps of Fad in this bacterium [82]. Previously, two *fadD* genes were determined to be involved in Fad [83]. However, *fadD1* and *fadD2* are not the only fatty acyl-CoA synthetases present in *P. aeruginosa* [83]. *fadD3* and *fadD4* were indentified in this study (**Chapter 3, section 3.2.1**) and determined to also contribute to Fad (**Chapter 4**). Based on growth curve studies with quadruple *fadD* mutants, more fatty acyl-CoA synthetases exist (**Chapter 4**, **section 4.2.1**). Presence of other *fadD* genes makes determining the role in Fad of the newly discovered *fadD* homologues more complicated, especially that of *fadD3*. Additional *fadD* homologues were identified. Candidate genes were tested in conjunction with known *fadD* genes for their contribution to Fad in *P. aeruginosa.*

5.2 Results

5.2.1 Identification of Additional *fadD* Homologues: *fadD5* and *fadD6*

Based on the results of growth curve experiments with Δ*fadD1D2D3D4* (**Chapter 4**, **section 4.2.1** and **4.2.2**) it was certain that more then four *fadD* homologues are present in *P. aeruginosa.* It is possible that some of the genes already tested in **Chapter 3** (**Section 3.2.1**) could actually be *fadD* homologues involved in Fad in *P. aeruginosa.* These genes could have too low activity to be detected in *E. coli fadD*⁻ strain, and/or are not expressed/folded properly in *E. coli.*

Another possibility is that other homologues exist and are involved. Two genes, PA2893 and PA3924, are annotated as putative very-long chain acyl-CoA synthetase (22% identical and 37% similar to *E. coli* FadD) and probable medium-chain acyl-CoA ligase 22% identical and 37% similar to *E. coli* FadD), respectively. Previously, PA2893 was showed to be induced ~10-fold when PAO1 strain was grown in PC [71] and PA3924 was expressed *in vivo* constitutively in lungs of two CF patients [71]. When cloned into high-copy plasmid under control of the *lac* promoter both genes were able to complement the *E. coli fadD*⁻ mutant on $C_{16:0}$ and $C_{18:1}{}^{\Delta 9}$ (Hoang's laboratory unpublished results). However, when both genes were mutated in Δ*fadD2D1* mutant background resulting quadruple mutant grew the same on $C_{4:0}$ - $C_{18:1}{}^{\Delta 9}$ as Δ*fadD2D1* strain (Hoang's laboratory unpublished results). Therefore, PA2893 and PA3924 were considered not to be involved in Fad and the genome of *P. aeruginosa* was searched for other potential homologues resulting in identification of *fadD3* (PA3860) and *fadD4* (PA1617) (**Chapter 3** and **Chapter 4**).
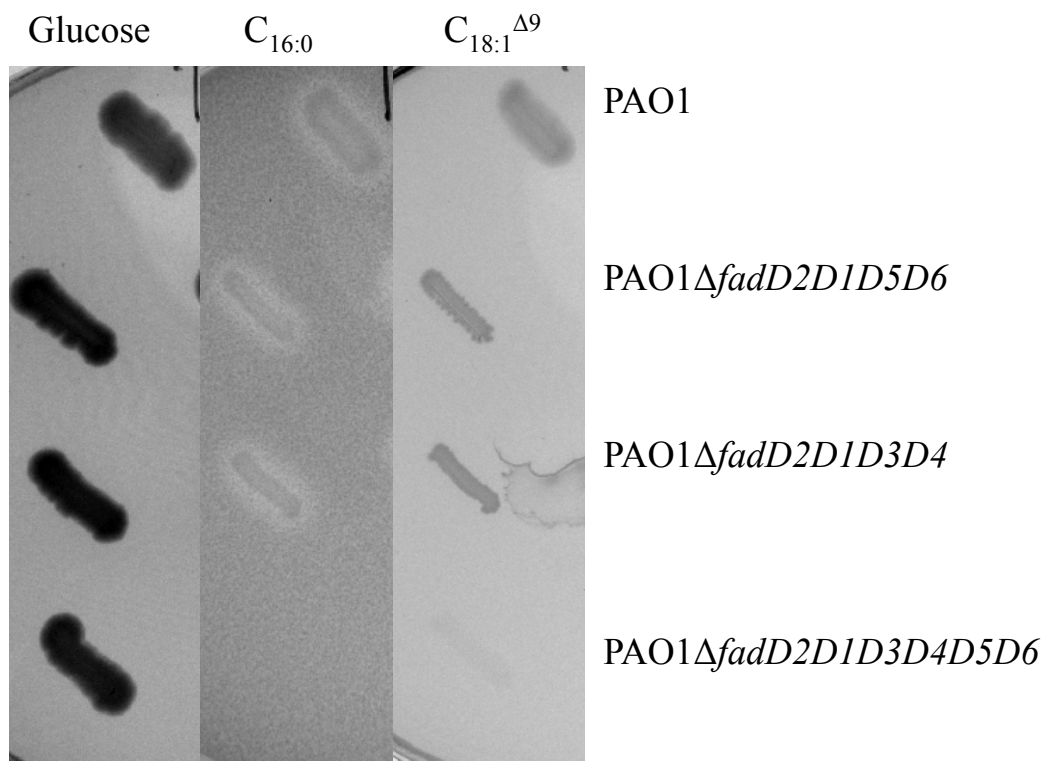
Figure 12. Growth of sextuple *fadD* mutant on FAs. Strains were grown on 1x M9 + 1% Brij-58 medium supplemented with 0.2% (w/v) fatty acids or 20 mM glucose for 5 days at 37°C.

Contribution of *fadD3* to Fad could be only verified when *fadD1*, *fadD2* and *fadD4* were mutated as showed in **Chapter 4**, **section 4.2.1** and **4.2.2**. It was possible that in Δ*fadD1D2D4* or Δ*fadD1D2D3D4* backgrounds roles of PA2893 and PA3924 in Fad also could be determined. A sextuple mutant strain with *fadD1*, *fadD2*, *fadD3*, *fadD4*, PA2893 (*fadD5*), and PA3924 (*fadD6*) genes inactivated was constructed (as described in **Chapter 2**, **section 2.7.3**) and tested for growth on $C_{16:0}$ and $C_{18:1}^{\Delta 9}$. No growth was observed on both FAs after five days (**Figure 12**) indicating that either one of the genes or both is/are the last *fadD* homologue(s) involved in Fad awaiting discovery.

5.2.2 Growth Study of Various *P. aeruginosa fadD*s Mutants on Different Fatty Acids

To determine level of involvement of *fadD3*, *fadD4*, *fadD5*, and *fadD6* in Fad, mutant strains with various combinations of *fadD*s were constructed (as described in **section 2.7.3** of **Chapter 2**). Seven quadruple mutants, four quintuple mutants and one sextuple mutant were tested for growth on $C_{4:0}$ - $C_{18:1}^{\Delta 9}$ along with PAO1 and other control mutant strains (as described in **Chapter 2**, **section 2.15**). Data for this growth study at 24 h, 48 h, 72 h, and 96 h are presented in **Table 8**, **9**, **10**, and **11**, respectively.

5.2.3 Growth of Various *P. aeruginosa fadD*s Mutants on Different FAs at 24 h

After 24 h, as expected, all 15 mutant strains grew the same as PAO1 on glucose (**Table 8**). Growth of all strains on $C_{4:0}$ was the same indicating none of the *fadD* homologues contribute to degradation of this FA or the differences were to small (as can be seen in **Figure 9** and **10** in **Chapter 4** between PAO1 and Δ*fadD2D1*) to be detected

Table 8. Growth of various PAO1 *fadD*s mutants on FAs after 24 h.

Strain	Growth on different carbon sources*								
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:1}{}^{\Delta 9}$	**Glu**
PAO1	+2	+3	+4	+4	+3	+3	+3	+3	+4
Δ*fadD2D1*	+2	+2	+3	+3	+2	+3	+2	+3	+4
Δ*fadD2D1D3*	+2	+2	+3	+3	+2	+3	+2	+3	+4
Δ*fadD2D1D4*	+2	-	-	+1	+1	+1	+1	+1	+4
Δ*fadD2D1D3D4*	+2	-	-	-	-	+1	+1	+1	+4
Δ*fadD2D1D3D5*	+2	+2	+3	+3	+2	+3	+2	+3	+4
Δ*fadD2D1D3D6*	+2	+2	+3	+3	+2	+3	+2	+3	+4
Δ*fadD2D1D4D5*	+2	-	-	-	-	-	-	+1	+4
Δ*fadD2D1D4D6*	+2	-	-	-	-	+1	+1	+1	+4
Δ*fadD2D1D5D6*	+2	+2	+3	+3	+2	+3	+2	+3	+4
Δ*fadD3D4D5D6*	+2	+3	+4	+4	+3	+3	+3	+3	+4
Δ*fadD2D1D3D4D5*	+2	-	-	-	-	-	-	-	+4
Δ*fadD2D1D3D4D6*	+2	-	-	-	-	+1	+1	+1	+4
Δ*fadD2D1D3D5D6*	+2	+2	+1	+3	+1	+3	+1	+3	+4
Δ*fadD2D1D4D5D6*	+2	-	-	-	-	-	-	-	+4
Δ*fadD2D1D3D4D5D6*	+2	-	-	-	-	-	-	-	+4

Strains were grown on 1x M9 +1% (w/v) Brij-58 medium supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu). * - indicates no growth on a patch; + denotes growth: +1 is very little growth whereas +6 is very heavy growth.

-	+1	+2	+3	+4	+5	+6

via plate base growth assay. $\Delta fadD3D4D5D6$ strain had the same growth as PAO1 on $C_{4:0}$ - $C_{18:1}^{\Delta 9}$ indicating that FadD1 and FadD2 are providing majority of fatty acyl-CoA synthetase activity in *P. aeruginosa*. No difference in growth was observed between $\Delta fadD2D1D3$, $\Delta fadD2D1D5D6$, $\Delta fadD2D1D3D5$, $\Delta fadD2D1D3D6$ and $\Delta fadD2D1$ on $C_{6:0}$ - $C_{18:1}^{\Delta 9}$. No growth was observed on $C_{6:0}$ and $C_{8:0}$ for $\Delta fadD2D1D4$ and there was less growth on $C_{10:0}$ - $C_{18:1}^{\Delta 9}$ in comparison to $\Delta fadD2D1$ suggesting that *fadD4* is important for degradation of all FAs tested. All quadruple mutant strains with *fadD4* mutations were more deficient in growth on FAs then the triple *fadD4* mutant. $\Delta fadD2D1D3D4$ and $\Delta fadD2D1D4D6$ mutants had the same amount of growth on $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ as $\Delta fadD2D1D4$ but did no grow on $C_{8:0}$ and $C_{10:0}$ suggesting similar involvement in degradation of both FAs. $\Delta fadD2D1D4D5$ strain only showed growth on $C_{18:1}^{\Delta 9}$ (similar to $\Delta fadD2D1D4$) and lack of growth on $C_{10:0}$ - $C_{16:0}$ indicates involvement of *fadD5* in degradation of these FAs. No growth was present for $\Delta fadD2D1D3D4D5D6$ and quintuple mutants with both *fadD4* and *fadD5* mutations ($\Delta fadD2D1D3D4D5$ and $\Delta fadD2D1D3D4D5$) on $C_{6:0}$ - $C_{18:1}^{\Delta 9}$. $\Delta fadD2D1D3D4D6$ grew the same as $\Delta fadD2D1D3D4$ and $\Delta fadD2D1D4D6$. Strain $\Delta fadD2D1D3D5D6$ grew the same on $C_{6:0}$, $C_{10:0}$, $C_{14:0}$, and $C_{18:1}^{\Delta 9}$ as $\Delta fadD2D1$. Growth patterns at 24 h of these four quintuple mutants suggest that *fadD4* is much more important for Fad than *fadD3*, *fadD5*, and *fadD6* combined and that *fadD5* contributes to Fad more than *fadD3* and *fadD6*.

Table 9. Growth of various PAO1 *fadD*s mutants on FAs after 48 h.

Strain	Growth on different carbon sources*								
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:1}^{\Delta9}$	Glu
PAO1	+3	+5	+5	+5	+5	+5	+4	+5	+6
Δ*fadD2D1*	+3	+4	+4	+4	+4	+4	+3	+4	+6
Δ*fadD2D1D3*	+3	+4	+4	+4	+4	+4	+3	+4	+6
Δ*fadD2D1D4*	+3	-	-	+3	+4	+4	+3	+4	+6
Δ*fadD2D1D3D4*	+3	-	-	+1	-	+4	+3	+4	+6
Δ*fadD2D1D3D5*	+3	+4	+4	+4	+4	+4	+3	+4	+6
Δ*fadD2D1D3D6*	+3	+4	+4	+4	+4	+4	+3	+4	+6
Δ*fadD2D1D4D5*	+3	-	-	-	-	-	-	+2	+6
Δ*fadD2D1D4D6*	+3	-	-	+3	+4	+4	+3	+4	+6
Δ*fadD2D1D5D6*	+3	+4	+4	+4	+4	+4	+3	+4	+6
Δ*fadD3D4D5D6*	+3	+5	+5	+5	+5	+5	+4	+5	+6
Δ*fadD2D1D3D4D5*	+3	-	-	-	-	-	-	-	+6
Δ*fadD2D1D3D4D6*	+3	-	-	-	-	+4	+3	+2	+6
Δ*fadD2D1D3D5D6*	+3	+4	+4	+4	+4	+4	+3	+4	+6
Δ*fadD2D1D4D5D6*	+3	-	-	-	-	-	-	-	+6
Δ*fadD2D1D3D4D5D6*	+3	-	-	-	-	-	-	-	+6

Legend: | - | +1 | +2 | +3 | +4 | +5 | +6 |

Strains were grown on 1x M9 +1% (w/v) Brij-58 medium supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu). * - indicates no growth on a patch; + denotes growth: +1 is very little growth whereas +6 is very heavy growth.

5.2.4 Growth of Various *P. aeruginosa fadD*s Mutants on Different FAs at 48 h

In general at 48 h (**Table 9**) more growth was observed than at 24 h (**Table 8**) for most of the strains. There was no difference between growth of 16 strains on glucose as well as on $C_{4:0}$. Growth of PAO1 and $\Delta fadD3D4D5D6$ on $C_{6:0}$ - $C_{18:1}{}^{\Delta9}$ was the same and more copious than for other strains. Similar growth was observed on $C_{6:0}$ - $C_{18:1}{}^{\Delta9}$ between $\Delta fadD2D1$, $\Delta fadD2D1D3$, $\Delta fadD2D1D3D5$, $\Delta fadD2D1D3D6$, $\Delta fadD2D1D5D6$, and $\Delta fadD1D2D3D5D6$ indicating that *fadD4* is important for degradation of these FAs. $\Delta fadD2D1D4$ and $\Delta fadD2D1D4D6$ exhibited the same level of growth as $\Delta fadD2D1$ on $C_{12:0}$ - $C_{18:1}{}^{\Delta9}$ and only slightly less on $C_{10:0}$. Amount of growth for $\Delta fadD2D1D3D4$ mutant was similar to that of $\Delta fadD2D1D4$ and $\Delta fadD2D1D4D6$ strains on $C_{6:0}$, $C_{8:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:1}{}^{\Delta9}$. $\Delta fadD2D1D3D4$ grew only slightly on $C_{10:0}$ and did not grow on $C_{12:0}$ suggesting involvement of *fadD3* in degradation of both FAs. For $\Delta fadD2D1D4D5$ strain some growth was only present on $C_{18:1}{}^{\Delta9}$ indicating that *fadD5* contributes to degradation of $C_{10:0}$ - $C_{16:0}$ whereas *fadD3* and/or *fadD6* could contribute to degradation of $C_{18:1}{}^{\Delta9}$. Quintuple mutant, $\Delta fadD2D1D3D4D6$, exhibited similar growth on $C_{14:0}$ and $C_{16:0}$ as $\Delta fadD2D1$ and similar growth on $C_{18:1}{}^{\Delta9}$ as $\Delta fadD2D1D4D5$. No growth was observed for $\Delta fadD2D1D3D4D5$, $\Delta fadD2D1D4D5D6$, and $\Delta fadD2D1D3D4D5D6$ mutants on $C_{6:0}$ - $C_{18:1}{}^{\Delta9}$ suggesting that possibly both *fadD3* and *fadD6* are required for Fad.

5.2.5 Growth of Various *P. aeruginosa fadD* Mutants on Different FAs at 72 h

At 72 h (**Table 10**) growth for most of the strains showed similar pattern as at 48 h (**Table 9**). The same amount of growth was observed for all strains on glucose and $C_{4:0}$

Table 10. Growth of various PAO1 *fadD*s mutants on FAs after 72 h.

Growth on different carbon sources*

Strain	C$_{4:0}$	C$_{6:0}$	C$_{8:0}$	C$_{10:0}$	C$_{12:0}$	C$_{14:0}$	C$_{16:0}$	C$_{18:1}^{\Delta 9}$	Glu
PAO1	+4	+5	+6	+6	+6	+6	+6	+6	+6
Δ*fadD2D1*	+4	+4	+4	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D3*	+4	+4	+4	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D4*	+4	-	-	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D3D4*	+4	-	-	+2	+1	+4	+4	+4	+6
Δ*fadD2D1D3D5*	+4	+4	+4	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D3D6*	+4	+4	+4	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D4D5*	+4	-	-	+1	+3	+2	+3	+3	+6
Δ*fadD2D1D4D6*	+4	-	-	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D5D6*	+4	+4	+4	+4	+4	+4	+4	+4	+6
Δ*fadD3D4D5D6*	+4	+5	+6	+6	+6	+6	+6	+6	+6
Δ*fadD2D1D3D4D5*	+4	-	-	+2	+1	+4	-	+2	+6
Δ*fadD2D1D3D4D6*	+4	-	-	+2	+1	+4	+4	+3	+6
Δ*fadD2D1D3D5D6*	+4	+4	+4	+4	+4	+4	+4	+4	+6
Δ*fadD2D1D4D5D6*	+4	-	-	+1	+3	-	-	-	+6
Δ*fadD2D1D3D4D5D6*	+4	-	-	-	-	-	-	-	+6

-	+1	+2	+3	+4	+5	+6

Strains were grown on 1x M9 +1% (w/v) Brij-58 medium supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu). * - indicates no growth on a patch; + denotes growth: +1 is very little growth whereas +6 is very heavy growth.

(less than on glucose). On $C_{6:0}$ - $C_{18:1}{}^{\Delta 9}$ PAO1 and $\Delta fadD3D4D5D6$ exhibited more growth than rest of strains and there was no growth for the sextuple mutant, $\Delta fadD2D1D3D4D5D6$. $\Delta fadD2D1$, $\Delta fadD2D1D3$, $\Delta fadD2D1D3D5$, $\Delta fadD2D1D3D6$, $\Delta fadD2D1D5D6$, and $\Delta fadD1D2D3D5D6$ strains had the same growth on $C_{6:0}$ - $C_{18:1}{}^{\Delta 9}$. Growth of $\Delta fadD2D1D4$ and $\Delta fadD2D1D4D6$ was comparable to $\Delta fadD2D1$ on $C_{10:0}$ - $C_{18:1}{}^{\Delta 9}$. Both strains, as well as $\Delta fadD2D1D3D4$ and $\Delta fadD2D1D4D5$, still showed no growth on $C_{6:0}$ and $C_{8:0}$ suggesting that *fadD4* is important for degradation of these FAs. $\Delta fadD2D1D3D4$ had the same amount of growth as $\Delta fadD12$ on $C_{14:0}$ - $C_{18:1}{}^{\Delta 9}$. Less growth was observed on $C_{10:0}$ and $C_{12:0}$ than on other FAs indicating that *fadD3* is involved in degradation of $C_{10:0}$ and $C_{12:0}$. $\Delta fadD2D1D4D5$ had more growth present on $C_{18:1}{}^{\Delta 9}$ in comparison to 48 h (**Table 9**) and growth on $C_{10:0}$ - $C_{16:0}$ increased from none to minimal or moderate indicating that *fadD3* and *fadD6* contribute to degradation of $C_{10:0}$ - $C_{18:1}{}^{\Delta 9}$. $\Delta fadD2D1D3D4D6$ mutant had the same or almost the same amount of growth on $C_{14:0}$ - $C_{18:1}{}^{\Delta 9}$ as *fadD2D1* and similar growth as $\Delta fadD2D1D3D4$ strain on $C_{10:0}$ and $C_{12:0}$. $\Delta fadD2D1D3D4D5$ exhibited small level of growth on $C_{18:1}{}^{\Delta 9}$ (not present at 48 h, **Table 9)** and no growth was present on $C_{6:0}$ - $C_{16:0}$. Growth pattern of these two strains on FAs suggest that *fadD5* is involved in degradation of $C_{10:0}$ - $C_{16:0}$ and *fadD6* in $C_{18:1}{}^{\Delta 9}$. Presence of small to moderate levels of growth on $C_{10:0}$ and $C_{12:0}$ for $\Delta fadD2D1D4D5D6$ mutant suggests that *fadD3* contributes to degradation of these FAs.

5.2.6 Growth of Various *P. aeruginosa fadD*s Mutants on Different FAs at 96 h

At day four (96 h, **Table 11**) growth of all 16 strains on $C_{4:0}$ was identical and all strains had the same amount of growth on glucose. Similarly to results for 72 h time point

Table 11. Growth of various PAO1 *fadD*s mutants on FAs after 96 h.

Strain	Growth on different carbon sources*								
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:1}^{\Delta 9}$	Glu
PAO1	+4	+6	+6	+6	+6	+6	+6	+6	+6
ΔfadD2D1	+4	+4	+4	+4	+4	+4	+4	+4	+6
ΔfadD2D1D3	+4	+4	+4	+4	+4	+4	+4	+4	+6
ΔfadD2D1D4	+4	+2	+1	+4	+4	+4	+4	+4	+6
ΔfadD2D1D3D4	+4	-	-	+2	+2	+4	+4	+4	+6
ΔfadD2D1D3D5	+4	+4	+4	+4	+4	+4	+4	+4	+6
ΔfadD2D1D3D6	+4	+4	+4	+4	+4	+4	+4	+4	+6
ΔfadD2D1D4D5	+4	-	-	+1	+3	+3	+4	+4	+6
ΔfadD2D1D4D6	+4	-	-	+4	+4	+4	+4	+4	+6
ΔfadD2D1D5D6	+4	+4	+4	+4	+4	+4	+4	+4	+6
ΔfadD3D4D5D6	+4	+6	+6	+6	+6	+6	+6	+6	+6
ΔfadD2D1D3D4D5	+4	-	-	-	-	-	+3	+3	+6
ΔfadD2D1D3D4D6	+4	-	-	+2	+2	+4	+4	+4	+6
ΔfadD2D1D3D5D6	+4	+4	+4	+4	+4	+4	+4	+4	+6
ΔfadD2D1D4D5D6	+4	-	-	+1	+3	+1	-	+1	+6
ΔfadD2D1D3D4D5D6	+4	-	-	-	-	-	-	-	+6

Legend: - | +1 | +2 | +3 | +4 | +5 | +6

Strains were grown on 1x M9 +1% (w/v) Brij-58 medium supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu). * - indicates no growth on a patch; + denotes growth: +1 is very little growth whereas +6 is very heavy growth.

(**Table 10**) PAO1 and $\Delta fadD3D4D5D6$ showed most growth on $C_{6:0}$ - $C_{18:1}^{\Delta9}$ than the rest of strains. $\Delta fadD2D1$, $\Delta fadD2D1D3$, $\Delta fadD2D1D3D5$, $\Delta fadD2D1D3D6$, $\Delta fadD2D1D5D6$, and $\Delta fadD1D2D3D5D6$ had the same amount of growth on $C_{6:0}$ - $C_{18:1}^{\Delta9}$. Other mutants exhibited different levels of deficiencies in growth on different FAs. $\Delta fadD2D1D4$ and $\Delta fadD2D1D4D6$ had the same amount of growth on $C_{10:0}$ - $C_{18:1}^{\Delta9}$ as $\Delta fadD2D1$. On $C_{6:0}$ and $C_{8:0}$ minimal amount of growth was present for $\Delta fadD2D1D4$ but not for $\Delta fadD2D1D3D4$, $\Delta fadD2D1D4D6$, and $\Delta fadD2D1D4D5$ indicating that $fadD3$, $fadD5$, and $fadD6$ could contribute to degradation of $C_{6:0}$ and $C_{8:0}$. $\Delta fadD2D1D3D4$ and $\Delta fadD2D1D4D5$ had similar growth on $C_{14:0}$ - $C_{18:1}^{\Delta9}$ as $\Delta fadD2D1$. On $C_{10:0}$ and $C_{12:0}$ $\Delta fadD2D1D3D4$ exhibited small levels of growth. In comparison, $\Delta fadD2D1D4D5$ grew less on $C_{10:0}$ and more on $C_{12:0}$. Interestingly at this time point $\Delta fadD2D1D3D4D6$ showed the same growth pattern on $C_{6:0}$ - $C_{18:1}^{\Delta9}$ as $\Delta fadD2D1D3D4$. The quintuple mutant, $\Delta fadD2D1D3D4D5$, had more growth present on $C_{16:0}$ and $C_{18:1}^{\Delta9}$ then at 72 h (**Table 10**) indicating that $fadD6$ contributes to degradation of these FAs. For strain $\Delta fadD2D1D4D5D6$ similar growth as at 72 h was observed on $C_{10:0}$ and $C_{12:0}$. Very little growth was also present on $C_{14:0}$ and $C_{18:1}^{\Delta9}$ suggesting that $fadD3$ contributes to degradation of $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, and $C_{18:1}^{\Delta9}$.

Chapter Six: Discussion

The purpose of this study was to characterize the role of fatty acyl-CoA synthetase homologue *fadD3* (PA3860) in fatty acid degradation (Fad) of *P. aeruginosa*. The Fad pathway was showed to be involved in degradation of major lung surfactant PC, in nutrient acquisition by *P. aeruginosa in vivo* during CF lung infection, and replication in mice lung [71, 83]. Despite its importance in lung infection, the Fad pathway in this bacterium has not yet been fully characterized. In contrary to the model organism for Fad, *E. coli*, *P. aeruginosa* has more than one copy of each gene involved in Fad [12]. Before FA can be degraded via β-oxidation pathway it has to be activated to fatty acyl-CoA by fatty acyl-CoA synthetase (FadD). In *P. aeruginosa* two *fadD* genes, *fadD1* and *fadD2*, were characterized and determined not to be the only homologues involved in Fad. *fadD2* gene was also showed to be connected to expression of virulence factors [83].

In the initial search for other *fadD* homologues of *P. aeruginosa* only ORF PA3860 was determined to encode fatty acyl-CoA synthetase (**Table 5**). PA3860 (FadD3) contains two regions similar to FadD motifs (ATP/AMP binding and FACS) (**Figure 4**) but most importantly it complements *E. coli fadD*⁻ mutant growth on FAs even at single copy level (**Table 6**). Based on the complementation test in *E.coli* FadD3 can activate FAs of different chain length from $C_{10:0}$ to $C_{18:1}^{\Delta 9}$. Since other *E. coli* Fad enzymes do not support catabolism of shorter chain FAs ($C_{4:0}$ - $C_{8:0}$) [128] no insights into FadD3 activity towards those FAs could be obtained through this experiment (even tough *fadR* mutation which allows growth on FAs shorter then $C_{12:0}$, such as $C_{10:0}$, was present [80]). *fadD3* was able to support growth of *E. coli fadD*⁻/*fadR*⁻ mutant to very similar level as *E. coli fadD* gene suggesting comparable biochemical properties of

encoded proteins. However, complementation with FadD1 and FadD2 also gave the same results as *E. coli* FadD even though both enzymes have different substrate preferences [83]. Similar complementation on $C_{10:0}$ to $C_{18:1}{}^{\Delta 9}$ was also observed at single copy level for another *fadD* homologue identified PA1617 (*fadD4*) (**Table 6**).

To further characterize FadD3, FadD3 was over expressed in *E. coli*, purified, and its activity was assayed. Since the *E. coli fadD* expression strain was utilized for purification all activity measured resulted from expressed FadD3 even though protein preparation was not 100% homogeneous. Activity towards all tested FAs was observed indicating that this enzyme is fatty acyl-CoA synthetase with broad substrate spectrum (**Figure 6**) similar to FadD1 and FadD2 [83]. FadD3 has preference for long and medium chain length FAs and is most efficient at activating $C_{10:0}$ - $C_{16:0}$ and five times less efficient for $C_{4:0}$ and $C_{6:0}$ (**Table 7**). Its overall catalytic efficiency for various FAs is as follow: $C_{12:0} > C_{14:0} > C_{10:0} > C_{16:0} > C_{18:1}{}^{\Delta 9} > C_{8:0} > C_{6:0} > C_{4:0}$, **Table 7**. Based on its kinetic parameters (**Table 7**) FadD3 is not as active an enzyme as FadD1 or FadD2 [83]. Affinities (K_m) values of FadD3, FadD1 and FadD2 are in similar ranges. However, the lowest activities of FadD1 and FadD2 are two fold greater than FadD3 highest ones, and the highest V_{max} of FadD1 and FadD2 are four fold larger than those of FadD3. FadD3 has 2 to 5 fold less catalytic efficiencies for most of FAs in comparison to FadD1or FadD2. With FadD1 having substrate preference for long chain FAs and FadD2 for medium and short chain FAs [83], FadD3 does not seem to have any obvious activity niche. Because of low enzymatic activity, FadD3 could possibly not contribute significantly to Fad in *P. aeruginosa*.

Contribution of enzyme to *in vivo* process such as for instance Fad cannot be only determined from biochemical properties of enzyme especially when several homologues are present. The role of *fadD3* gene in growth of *P. aeruginosa* on FAs was studied utilizing *fadD3* mutants. Initially *fadD3* did not seem to be involved in degradation of any FAs because no growth difference was presence not only between single *fadD3* mutant and PAO1 (data not shown) but also between Δ*fadD2D1* mutant and Δ*fadD2D1D3* (**Figure 9** and **10**) strain supporting the enzyme assay results. Previously it was shown that contribution of *fadBA1* and *fadBA4* operons to Fad of *P. aeruginosa* could be only detected in Δ*fadBA5* and Δ*fadBA1BA5* backgrounds, respectively [82]. It was possible that a similar pattern exists in respect to *fadD* homologues and that the role of *fadD3* in Fad could only determine when other homologues, possibly more important for Fad, are absent.

Gene PA1617 was identified to be another *fadD* homologue, *fadD4*, (**Table 5** and **Table 6**) and strain having combination of *fadD3* and *fadD4* mutations in Δ*fadD2D1* background were tested on FAs. *fadD4* gene is important for Fad as Δ*fadD2D1D4* did not grow as well as Δ*fadD2D1* or Δ*fadD2D1D3* on $C_{6:0}$ - $C_{18:1}^{\Delta9}$ (**Figure 9 C - I**). *fadD3* is also involved in degradation of $C_{6:0}$ - $C_{12:0}$ since growth of Δ*fadD2D1D4* and Δ*fadD2D1D3D4* on these FAs was not the same. Surprisingly, presence of *fadD3* mutations did not necessarily result in a greater overall growth defect for the quadruple mutant. Shorter lag phase (in $C_{6:0}$) and greater final OD (in $C_{12:0}$) were observed in comparison to Δ*fadD2D1D4* (**Figure 9 C** and **F**, respectively). When the growth study was repeated similar outcome resulted (**Figure 10**). As expected Δ*fadD2D1D4* exhibited growth defects in comparison to Δ*fadD2D1*, Δ*fadD2D1D3*, and complemented *fadD4*

mutant strains on $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ (**Figure 10 C - I**). $\Delta fadD2D1D3D4$ mutant had different

growth than $\Delta fadD2D1D4$ not only on $C_{6:0}$ - $C_{12:0}$ but also on $C_{14:0}$. The growth pattern of

$\Delta fadD2D1D3D4$ was not exactly the same as previously observed in the same FAs

(**Figure 6**). In general shorter lag phase, longer lag phase or longer lag phase with higher

final OD than $\Delta fadD2D1D4$ were observed for quadruple mutant (**Figure 10 C - G**).

Based on growth of $\Delta fadD2D1$, $\Delta fadD2D1D3$, $\Delta fadD2D1D4$ and $\Delta fadD2D1D3D4$

strains, the *fadD4* gene is more important for Fad than *fadD3*. Furthermore, *fadD1*,

fadD2, *fadD3*, and *fadD4* are not the only *fadD* homologues in *P. aeruginosa*. Absence of

growth difference for triple and quadruple *fadD4* mutants and $\Delta fadD2D1D4$ in $C_{4:0}$

indicates that *fadD3* and *fadD4* genes are not important in degradation of this FA. This

suggests that other genes yet to be identified are involved in activation of this short FA.

The unexpected phenotypes of $\Delta fadD2D1D3D4$ mutant could be result of expression or

over expression of other *fadD* homologues in the absence of more important FadD3 and

FadD4. Such scenario was reported in *P. putida*, which has two *fadD* genes and *fadD2* is

only active when dominant *fadD1* gene is absent [107]. On the other hand, the long lag

phase and different outcomes of growth exhibited by $\Delta fadD2D1D3D4$ suggest that

mutation directly or indirectly affecting other *fadD* homologues could be responsible for

growth of the quadruple mutant. Those possibilities require further investigation.

More insight into roles of *fadD3* as well as *fadD4* in Fad of *P. aeruginosa* was

obtained when expression profiles of both genes were obtained (**Figure 11**). *fadD3* was

not induced significantly by FAs beside $C_{6:0}$ (at early-log phase and mid-log phase, ~2.3

and ~1.4 fold, respectively) (**Figure 11 C**). Expression of *fadD3* is almost constitutive

through out all growth stages. Such a pattern could indicate that *fadD3* is not important

for degradation of any specific FA beside $C_{6:0}$. This would be in agreement with results of the enzyme assay and growth curves, which indicated that *fadD3* does not have a significant role in Fad. On the other hand, *fadD4* is expressed differentially in the presence of FAs (**Figure 11 D**). Induction by $C_{4:0}$ - $C_{16:0}$ was observed at different growth stages. Most significant expression, ~ 2 fold increase, of *fadD4* was in the presence of $C_{6:0}$ and $C_{8:0}$ (from early-log to stationary phase) and also $C_{10:0}$ and $C_{12:0}$. These high expression levels would suggest that *fadD4* contributes to degradation of these FAs. Growth curve studies indicated involvement of *fadD4* in degradation of $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ and greatest growth defect were observed in $C_{6:0}$ - $C_{12:0}$ (**Figure 9 C - F** and **Figure 10 C - F**). In comparison, higher levels of *fadD1* and *fadD2* transcript were only observed in presence of $C_{18:1}^{\Delta 9}$ and $C_{8:0}$ and $C_{10:0}$, respectively, even though *fadD1* and *fadD2* are important for degradation of all FAs. Furthermore, the higher expression levels observed for *fadD3* and *fadD4* (~ 2 fold) are comparable to those observed for *fadD1* and *fadD2* [83].

Based on enzyme assay, mutant growth curve results, and induction studies *fadD3* gene has a small role in Fad. With the puzzling growth phenotypes of Δ*fadD2D1D3D4* mutant resulting from the existence of other *fadD* homologues exact contribution of this gene to Fad is difficult to ascertain. It was reasoned that with knowledge of four fatty acyl-CoA synthetase genes it could be possible to identify remaining homologues. Genes PA2893 and PA3924, previously thought to be *fadD* homologues [71], were confirmed to be involved in Fad (**Table 8 - 11**). PA2893 (*fadD5*) and PA3924 (*fadD6*) are the last ORFs of *P. aeruginosa* encoding fatty acyl-CoA synthetase required for degradation of $C_{6:0}$ - $C_{18:1}^{\Delta 9}$. No growth was present only for sextuple *fadD* mutant whereas quintuple

mutants exhibited some growth on $C_{6:0}$ - $C_{18:1}^{\Delta9}$ (**Table 10** and **11**). Growth on $C_{4:0}$ was identical between PAO1, Δ*fadD2D1*, and even Δ*fadD2D1D3D4D5D6* indicating that plate assay is not sensitive enough to detect small growth differences (as seen for PAO1 and Δ*fadD2D1* in **Figure 9 A** and **10 A**) and that other unidentified homologues are responsible for the degradation of this short FA.

Growth patterns of seven quadruple mutants, four quintuple mutants along with control strains allowed determining the role of each *fadD* gene in Fad. Based on the growth of Δ*fadD2D1* strain on FAs it was thought that other *fadD* homologues contribute to half of the catabolic ability for FAs. However, from the growth of Δ*fadD3D4D5D6*, it is obvious that *fadD1* and *fadD2* are most important fatty acyl-CoA synthetases, and can support growth of *P. aeruginosa* on FAs to wildtype level even when *fadD3*, *fadD4*, *fadD5*, and *fadD6* are absent (**Table 8 - 11**). *fadD4* contribute significantly more to Fad than *fadD3*, *fadD5*, and *fadD6* combined. Mutation of *fadD4* in Δ*fadD2D1* background is sufficient to cause a growth defect, which is not the case with other *fadD* genes. Furthermore, presence of only *fadD4* allows Δ*fadD2D1D3D5D6* mutant to have a similar growth as Δ*fadD2D1* at 48 h (**Table 9**). *fadD5* contributes more to Fad than *fadD3* and *fadD6* since greater growth defects are present for Δ*fadD2D1D4D5* than other for quadruple mutants with *fadD4* mutations. Also, quintuple mutant with *fadD5* intact grows better on FAs than quintuple mutants with *fadD3* and *fadD6* genes present (**Table 8 - 11**). Mutation of *fadD3* gene resulted in greater defects in comparison to *fadD6* mutation as was seen from growth of Δ*fadD2D1D4*, Δ*fadD2D1D3D4*, and Δ*fadD2D1D4D6* (**Table 8 - 11**). Taken together the growth results of 15 various *fadD*

mutants indicate that overall importance of each *fadD* homologue in Fad to be as follow:

fadD1 and *fadD2* >> *fadD4* >> *fadD5* > *fadD3* > *fadD6*.

Levels of growth of tested *fadD* mutants at different times (24 h - 96 h, **Table 8** - **11**) also indicated contribution of *fadD3*, *fadD4*, *fadD5*, and *fadD6* to degradation of individual FAs. *fadD3* is most important for degradation of $C_{12:0}$ and $C_{10:0}$ which is in agreement with its biochemical properties (catalytic efficiency is highest for $C_{12:0}$, **Table 7**). *fadD3* also contributes to lesser degree to Fad of $C_{14:0}$, and $C_{18:1}^{\Delta 9}$ as can be seen from growth of Δ*fadD2D1D4D5*, Δ*fadD2D1D3D4D5*, and Δ*fadD2D1D4D5D6* (**Table 8** - **Table 11**). *fadD4* is involved in degradation of $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ with largest contribution to catabolism of short and medium chain length FAs ($C_{6:0}$ - $C_{12:0}$) which correlates strongest with induction of this gene in the presence of these FAs (**Figure 11 D**). *fadD5* gene has the greatest impact on degradation of $C_{14:0}$, $C_{16:0}$, and $C_{18:1}^{\Delta 9}$ and some on $C_{10:0}$ and $C_{12:0}$ as observed from growth abilities of Δ*fadD2D1D4D5* and Δ*fadD2D1D3D4D6* (**Table 8** - **Table 11**). *fadD6* is important in catabolism of $C_{18:1}^{\Delta 9}$ and also $C_{16:0}$ as indicated by growth of Δ*fadD2D1D3D4D5* (**Table 10** and **Table 11**). *fadD3*, *fadD5*, *fadD6* are also involved in degradation of $C_{6:0}$ and $C_{8:0}$ as can be seen from growth differences between Δ*fadD2D1D4* and the corresponding quadruples mutants (**Table 9** - **11**). It is possible, based on the amount of growth present for Δ*fadD2D1D4,* that if the duration of plate growth assay was extended beyond 96 h these quadruple mutants would exhibit growth on $C_{6:0}$ and $C_{8:0}$ to levels observed for Δ*fadD2D1D3D4* during growth curves studies (**Figures 9 C**, **D** and **10 C**, **D**).

The reason for presence of six homologues of fatty acyl-CoA synthetase in *P. aeruginosa* is unclear. *fadD1* and *fadD2* are obviously the most importance genes for Fad

and *fadD4* seems to have a more auxiliary function. Ability of *fadD1* and *fadD2* to support growth on FAs to wildtype level, in the absence of other *fadD* homologues, suggests that primary function of FadD3, FadD5, FadD6, and possibly FadD4 is activation of other organic substances, not FAs. *P. aeruginosa* can degrade many substance such as aromatic compounds, organic acids (e.g. isovalerate), alcohols, and acylic terpenes (e.g. citronellol and geraniol) [7]. Citronellol is a methyl branched aroma compound found in plants which catabolism was investigated in *P. aeruginosa*. This compound is thought to be degraded through acyclic terpene utilization (Atu) pathway, β-oxidation pathway, and leucine/isovalerate utilization (Liu) pathway to acetate, three molecules of acetyl-CoA and acetoacetate [129, 130]. Citronellol is oxidized by Atu pathway enzymes to citronellate, which then is activated by AtuH via addition of CoASH. Function of atuH was assigned to the last gene in *atuADCDEFGH* cluster, PA2893 [130] identified in this study as *fadD5* (**Chapter 5**). Most of the genes in Atu cluster were confirmed experimentally to be involved in citronellol degradation beside *atuH* [130]. *atuH* mutant was able to grow on acyclic terpenes and this phenotype was attributed to expression of homologues [130]. Since *fadD5* is involved in Fad and most likely also in Atu it is possible that *fadD3*, *fadD4*, and *fadD6* could also contribute to Atu. FadD3, FadD4, FadD5, and FadD6 could be broad substrate range enzymes that contribute to utilization of other organic compounds beside FAs. It can be predicted that the *P. aeruginosa* sextuple *fadD* mutant, or even Δ*fadD3D4D5D6*, mutant would be defective in degradation acyclic terpenes and possibly many other organic compounds.

In this study, the role of FadD3 (PA3860) in Fad of *P. aeruginosa* was successfully determined through growth curve studies, gene induction studies, and

biochemical characterization. In the process of studying *fadD3* three more *fadD* homologues needed for degradation of $C_{6:0}$ - $C_{18:1}^{\Delta 9}$ were discovered and their contribution to degradation of FAs was also determined. *fadD3* gene was shown to be constitutively express and to encode, a fatty acyl-CoA synthetase of moderate activity with broad substrate spectrum and preference for medium and long chain FAs. Contribution of *fadD3* to Fad is not very significant and greatest impact for degradation was observed for $C_{10:0}$ and $C_{12:0}$. Two other homologues *fadD4* and *fadD5* are more important for degradation of FAs. It is possible that the main function of *fadD3* and other homologues in *P. aeruginosa* is for activating other organic compounds that contain possibly branched acyl tails or even ring structures. Identifying all *fadD* genes involved in fatty acid catabolism and determining the level of their contribution significantly increased our knowledge of Fad pathway, which is important in pathogenesis, virulence and *in vivo* replication of *P. aeruginosa*.

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