Elucidating the *Pseudomonas aeruginosa* Fatty Acid Degradation Pathway: Identification of Additional Fatty Acyl-CoA Synthetase Homologues

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Abstract

The fatty acid (FA) degradation pathway of *Pseudomonas aeruginosa*, an opportunistic pathogen, was recently shown to be involved in nutrient acquisition during BALB/c mouse lung infection model. The source of FA in the lung is believed to be phosphatidylcholine, the major component of lung surfactant. Previous research indicated that *P. aeruginosa* has more than two fatty acyl-CoA synthetase genes (*fadD*; PA3299 and PA3300), which are responsible for activation of FAs using ATP and coenzyme A. Through a bioinformatics approach, 11 candidate genes were identified by their homology to the *Escherichia coli* FadD in the present study. Four new homologues of *fadD* (PA1617, PA2893, PA3860, and PA3924) were functionally confirmed by their ability to complement the *E. coli* fadD mutant on FA-containing media. Growth phenotypes of 17 combinatorial *fadD* mutants on different FAs, as sole carbon sources, indicated that the four new *fadD* homologues are involved in FA degradation, bringing the total number of *P. aeruginosa* *fadD* genes to six. Of the four new homologues, *fadD* (PA1617) contributed the most to the degradation of different chain length FAs. Growth patterns of various *fadD* mutants on plant-based perfumery substances, citronellic and geranic acids, as sole carbon and energy sources indicated that *fadD* is also involved in the degradation of these plant-derived compounds. A decrease in fitness of the sextuple *fadD* mutant, relative to the Δ*fadD1D2* mutant, was only observed during BALB/c mouse lung infection at 24 h.

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Introduction

*Pseudomonas aeruginosa* is an important human pathogen [1], [2] responsible for myriad of infections of the human body [3–11]. This ubiquitous bacterium is also a leading cause of mortality and morbidity in patients with cystic fibrosis (CF) [1], [2].

Phosphatidylcholine (PC), the major component of lung surfactant [12], was suggested as a potential nutrient source for pathogenesis during *P. aeruginosa* infection of the CF lung [13]. The major carbon source within the PC molecule comes from the two highly reduced long-chain fatty acids (LCFA). Many fatty acid degradation (β-oxidation) genes are expressed by *P. aeruginosa* during CF lung infection (e.g. *fadD1*: PA3299, *fadD2*: PA3300, *fadA5*: PA3013, and *fadB5*: PA3014) [13] and mutants defective in the fatty acid (FA) degradation pathway were reported to have decreased fitness during mouse lung infection [14]. A link between FA degradation genes and virulence was also observed [14] and *P. aeruginosa* can chemotax towards FA [15]. Furthermore, FA was shown to modulate type three-secretion system expression in this bacterium [16].

Despite the connection between virulence and FA degradation during infections, not all genes involved in *P. aeruginosa* FA degradation are characterized (Fig. 1A). In contrast, genes needed by *Escherichia coli* for aerobic β-oxidation (*fadL, fadD, fadE*, and *fadB* [17–20]), anaerobic FA degradation (*fadK* and *fadF*) [21], and auxiliary genes (*fadH* [22] and *fadM* [23]) are well characterized. For an exogenous FA to be degraded by this pathway, it must first be transported by the membrane transporter (FatD) into the cell [24]. *FA* is then activated with the use of adenosine triphosphate (ATP) and coenzyme A (CoASH) by FatD (fatty acyl-CoA synthetase, FACS) [19], [25]. The activated FA molecule can then proceed through the β-oxidation pathway (Fig. 1A). In *E. coli*, genes encoding enzymes needed for β-oxidation (*fadL, fadD, fadE*, and *fadB* [17–20]) are repressed in the absence of FAs by the transcriptional regulator FadR. Acyl-CoA of chain length ≥ C<sub>12:0</sub> can bind to FadR to induce FA degradation [18], [26], [27] resulting in growth on FA (≥ C<sub>16:0</sub>). Cyclic AMP and receptor protein complex levels [28], presence of oxygen [29], and osmotic pressure [30] also affect expression of FA degradation genes in *E. coli*. However, the existence of a central regulator, such as fadR, is unknown in *P. aeruginosa*, and only a few *fad*-genes have been found to be regulated by a FA sensor, PsaA [31].

*P. aeruginosa* exhibits greater metabolic capabilities for FA degradation than *E. coli* by growing aerobically on short, medium, and long-chain FAs as sole carbon and energy sources [31]. With a...
A genome of 6.3 Mb, *P. aeruginosa* could potentially have more FA degradation genes than *E. coli* [32], suggesting possible redundancies and a higher level of complexity in this pathway. Three potential *fadL* have been investigated thus far in *P. aeruginosa* and their exact role in FA transport still remains unclear [15]. Two *fadBA* operon homologues (*fadAB1* and *fadBA5*) have been studied so far. The *fadAB1* (PA1736–PA1737) operon was shown to be strongly induced by medium-chain fatty acids (MCFA, C10:0 and C12:0) and, to a lesser extent, LCFA (C14:0–C18:1D9) [33]. The *fadBA5* (PA3013–PA3014) operon was determined to be.

Figure 1. *P. aeruginosa* fatty acid degradation pathway (FA degradation). (A) *P. aeruginosa* FA degradation model was based on the *E. coli* β-oxidation pathway. Known *P. aeruginosa* FA degradation enzyme homologues are indicated by numbers: FadD1 (PA3299), FadD2 (PA3300), FadD3 (PA3860), FadD4 (PA1617), FadD5 (PA2893), FadD6 (PA3924), FadAB1 (PA1736–PA1737), and FadBA5 (PA3013–PA3014). Abbreviations: FadA, 3-ketoacyl-CoA thiolase; FadB, cis-Δ3-trans-Δ2-enoyl-CoA isomerase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase, and 3-hydroxyacyl-CoA dehydrogenase; FadD, fatty acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadL, outer membrane long-chain fatty acid translocase; OM, outer membrane; IN, inner membrane. (B) Alignment of FadD homologues motifs with *E. coli* FadD motifs. Amino acids with similar properties are assigned the same colors using CLC Sequence Viewer 6 software (www.clcbio.com).

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involved in LCFA metabolism and to be induced by LCFA, especially olate (C_{18:1}) [31]. We have recently identified two FACS homologues of *P. aeruginosa*, {\textit{fadD1}} (PA3299) and {\textit{fadD2}} (PA3900) [14]. The {\textit{fadD1}} and {\textit{fadD2}} of *P. aeruginosa* were determined to have broad specificity for FA of different chain lengths. {\textit{fadD1}} has preference for LCFA whereas {\textit{fadD2}} has higher activities for shorter chain FAs. {\textit{fadD1}}, {\textit{fadD2}}, and *{\textit{fadD2D1}}* mutants showed growth defects when grown on minimal media with different length FAs as sole carbon sources. {\textit{fadD1}} was determined to be induced by LCFA and to be more important for growth on LCFA while *{\textit{fadD2}}* was important for growth on short-chain fatty acids (SCFA) and was induced by MCFAs. The double mutant *{\textit{fadD2D1}}* displayed an impaired ability to grow on PC as a sole carbon source. This growth defect translated into decreased in vivo fitness during mouse lung infection, indicating that {\textit{fadD1}} and {\textit{fadD2}} may mediate *P. aeruginosa* replication in the CF lung [14]. However, the double mutant *{\textit{fadD2D1}}* was still able to grow on FA, suggesting the involvement of other *{\textit{fadD}}* homologues in FA degradation [14].

We surveyed the *P. aeruginosa* genome for additional *{\textit{fadD}}* homologues to gain more insight into the degradation of FAs in this bacterium. Four new *{\textit{fadD}}* homologues PA1617, PA2993, PA3860, and PA3924 were identified out of 11 potential candidates. Through genetic analyses, their contribution to FA degradation was assessed. The final four candidates were determined to be FACS homologues, but PA1617 (*{\textit{fadD4}}*) was found to be the major contributor to FA degradation. Involvement of the newly discovered *{\textit{fadD4}}* in catabolism of plant-derived acyclic terpenes suggests that the function of multiple FACS in *P. aeruginosa* is the degradation of compounds closely related to FAs. Growth defect on PC and decreased fitness in mouse lung of the sextuple *{\textit{fadD}}* mutant supports the role of FA as a nutrient in vivo.

### Results

#### Identification of *P. aeruginosa* Fatty acyl-CoA Synthetase Homologues

To identify *{\textit{fadD}}* homologues of *P. aeruginosa*, *E. coli* FadD amino acid sequence was compared to *P. aeruginosa* PAO1 ORFs via BLAST [34]. The amino acid sequence of genes obtained in the search were further analyzed for the presence of ATP/AMP [19], [35–37] and fatty acid binding motifs [38]. Genes that encode eleven proteins containing amino acid sequences with high degree of similarity to the motifs found in *E. coli* FadD (Fig. S1) were chosen for complementation tests. Identity and similarity of the proteins range from 22% to 31% and from 37% to 52%, respectively (Table S1). When cloned into a high copy number pUC19 vector, only genes encoding PA3860, PA1617, PA2993, and PA3924 were found to complement the *E. coli* *{\textit{fadD}}^+/{\textit{fadR}}^+$ (E2011) strain on minimal medium containing olate (C_{18:1}) and decanoate (C_{10:0}) (Table S1) and were designated *{\textit{fadD3}}*, *{\textit{fadD4}}*, *{\textit{fadD5}}*, and *{\textit{fadD6}}*, respectively. Their ATP/AMP and FA binding motifs show high degree of similarity to those of *E. coli* FadD (Fig. 1B).

All four *P. aeruginosa* *{\textit{fadD}}* genes (*{\textit{fadD3}}*, *{\textit{fadD4}}*, *{\textit{fadD5}}*, and *{\textit{fadD6}}*) were tested further for their ability to support growth of *E. coli* *{\textit{fadD}}^+/{\textit{fadR}}^+$ (E2011) on various FAs as a single copy on the *E. coli* chromosome. The *E. coli* *{\textit{fadD}}^+/{\textit{fadR}}^+$ double mutant was used to ensure that FadR does not inhibit expression of other *E. coli* β-oxidation enzymes. Mini-Tn7 based complementation vectors were constructed and integrated into the E2011 chromosome at the *attTn7* site and resulting strains were tested for growth on FAs (Table 1). As expected, wildtype *E. coli* control strain K-12 showed growth on longer FAs (C_{12:0}–C_{18:1}) but not on the MCFAs, C_{10:0}, or SCFAs (C_{4:0}–C_{8:0}). The E2011 and the integrated empty-vector control strain were not able to growth on any of the FAs, E2011 complemented with *E. coli* *{\textit{fadD}}* (*{\textit{fadD6}}*) grew on C_{12:0}–C_{18:1}, and *{\textit{fadD5}}* and *{\textit{fadD6}}* genes individually allowed E2011 to grow on C_{14:0}–C_{18:1} to similar levels as K-12. *{\textit{fadD2}}* and *{\textit{fadD6}}* complemented E2011 to a lesser degree than *{\textit{fadD4}}* and *{\textit{fadD5}}* on C_{17:0}, and four *{\textit{fadD}}* genes supported minimal growth of E2011 on C_{10:0} to the same level as *{\textit{fadD2}}*; E2011 complemented with *{\textit{fadD2}}*, *{\textit{fadD3}}*, *{\textit{fadD4}}*, or *{\textit{fadD6}}* did not grow on C_{10:0}–C_{18:1}, which was in agreement with previous observations that other *E. coli* FA degradation enzymes do not support metabolism of shorter FAs [39].

#### Contribution of *{\textit{fadD3}}*, *{\textit{fadD4}}*, *{\textit{fadD5}}*, and *{\textit{fadD6}}* to FA Degradation

To determine the role of the *{\textit{fadD}}* homologues (*{\textit{fadD3}}*, *{\textit{fadD4}}*, *{\textit{fadD5}}*, and *{\textit{fadD6}}*) in *P. aeruginosa* FA degradation, strains with various combinations of *{\textit{fadD}}* mutations were created. To prevent potential masking of phenotypes by *{\textit{fadD1}}* and *{\textit{fadD2}}*, 15 mutants were constructed in the *P. aeruginosa* PAO1 *{\textit{fadD1D2}}* background. Four triple, seven quadruple, four quintuple mutants and one sextuple mutant (Table 2) were tested for growth on C_{4:0}–C_{18:1} indicating that *{\textit{fadD1}}* and *{\textit{fadD2}}* are most likely providing a majority of FACS activity in *P. aeruginosa* (Tables 3 and 4). No difference in growth was observed between *{\textit{fadD1D2}}* and *{\textit{fadD1D2D3}}*, *{\textit{fadD1D2D5}}*, *{\textit{fadD1D2D6}}*, *{\textit{fadD1D2D5D6}}*, *{\textit{fadD1D2D3D6}}*, or *{\textit{fadD1D2D5D6}}* on C_{4:0}–C_{18:1}. There was significantly less growth for *{\textit{fadD1D2D3}}* on C_{4:0}–C_{18:1} at 24 h in comparison to *{\textit{fadD1D2}}*, suggesting that *{\textit{fadD4}}* is important for degradation of all FAs from C_{4:0} to C_{18:1}. Addition of *{\textit{fadD3}},* *{\textit{fadD5}},* or *{\textit{fadD6}}* mutation to *{\textit{fadD1D2D4}}* strain in a quadruple mutant combination resulted in larger deficiencies in growth on FAs in comparison to the triple *{\textit{fadD1D2D4}}* mutant (Tables 3 and 4), indicating that *{\textit{fadD3}},* *{\textit{fadD5}},* and *{\textit{fadD6}}* also take part in FA degradation and supporting the dominance of FadD4 over these homologues. The *{\textit{fadD1D2D3D4}},* *{\textit{fadD1D2D4D5}},* and *{\textit{fadD1D2D4D6}}* strains showed no growth on C_{4:0} and C_{6:0} even after four days, in contrast to the *{\textit{fadD1D2D4}}* mutant (Table 4), indicating that *{\textit{fadD3}},* *{\textit{fadD5}},* and *{\textit{fadD6}}* are involved in the degradation of these FAs.

All quintuple mutants exhibited some level of growth on several FAs after 96 h (Table 4), whereas no growth was present for the sextuple mutant combination (*{\textit{fadD1D2D3D4D5D6}}*), indicating that all four new *{\textit{fadD}}* homologues contribute to FA degradation and that only six aerobic FACS genes are likely present in *P. aeruginosa*. Quintuple mutants with both *{\textit{fadD4}}* and *{\textit{fadD5}}* mutations (*{\textit{fadD1D2D3D4D5}}* and *{\textit{fadD1D2D4D5D6}}*) were most deficient in FA degradation (Table 3). Growth patterns of the four quintuple mutants after 96 h (Table 4) suggest that *{\textit{fadD4}},* besides *{\textit{fadD7}},* and *{\textit{fadD2}},* is much more important for FA degradation than *{\textit{fadD3}},* *{\textit{fadD5}},* and *{\textit{fadD6}}* combined, and *{\textit{fadD5}}* contributes to FA degradation more than *{\textit{fadD3}}* and *{\textit{fadD6}}*. Furthermore, by comparing the phenotypes of double, triple, and quadruple mutants at two time points (Tables 3 and 4) a hierarchy of contributions of *{\textit{fadD}}* homologues to the degradation of different
C6:0 and C18:1 mutant had almost identical growth in comparison to PAO1 in D of growth, and no increase in turbidity was observed for P. aeruginosa fatty acids (FA) degradation. In comparison to PAO1 on FAs (Fig. 2B–2E). The phenotype of calculated from growth curves in Fig. 2 are presented in Table 3 and 4; iii) FadD3 degrades C6:0–C12:0 (fadD1D2D4D5D6 in Tables 3 and 4); iv) FadD6 degrades C6:0–C18:1 (fadD1D2D4D5D6 versus fadD1D2D4). Growth on different carbon sources Growth on acyclic terpenes possibly suggesting the involvement of other homologue(s) [40]. To determine the role of fadD5 and other fadD homologues in degradation of acyclic terpenes as plant-derived nutrient sources, we grew PAO1 along with 17 combinatorial fadD mutants in 1x M9 minimal media +1% (w/v) Brij-58 and 0.1% (w/v) of CA or GA (300 nM IPTG for three days at 37°C). – indicates no growth on a patch and + denotes growth.

Table 1. Single copy complementation of the E. coli fadD mutant with P. aeruginosa fadD homologues.

| Strain                  | C6:0 | C8:0 | C10:0 | C12:0 | C14:0 | C16:0 | C18:1 | Glu   |
|------------------------|------|------|-------|-------|-------|-------|-------|-------|
| K12                    | –    | –    | –     | +4    | +5    | +5    | +5    | +6    |
| E2011 (fadD“fadR”)     | –    | –    | –     | –     | –     | –     | –     | +6    |
| E2011/attTn7:miniTn7-Gm' | –    | –    | –     | +1    | +5    | +5    | +5    | +6    |
| E2011/attTn7:fasD2     | –    | –    | –     | +1    | +3    | +5    | +5    | +6    |
| E2011/attTn7:fasD3     | –    | –    | –     | +1    | +5    | +5    | +5    | +6    |
| E2011/attTn7:fasD4     | –    | –    | –     | +1    | +2    | +5    | +6    |
| E2011/attTn7:fasD5     | –    | –    | –     | +1    | +5    | +5    | +6    |
| E2011/attTn7:fasD6     | –    | –    | –     | +1    | +5    | +5    | +6    |

Strains were grown on 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu) +0.25 mM IPTG for three days at 37°C. +1 is very little growth whereas +6 is very heavy growth comparable to K12 on glucose at day 3.

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fadD1 and fadD2 in Comparison to fadD3, fadD4, fadD5, and fadD6

The growth phenotypes of various combinatorial mutants on FAs indicated that out of the newly discovered FACS genes (fadD3, fadD4, fadD5, and fadD6) fadD1 is most important for FA degradation (Tables 3 and 4), in addition to fadD1 and fadD2 [14]. To investigate further the contribution of fadD4 to FA degradation in comparison to fadD1 and fadD2, growth curve experiments were performed on SCFA, MCFA, and LCFA's with ΔfadD1D2D4, ΔfadD3D4D5D6, ΔfadD1D2D3D5D6, and ΔfadD1D2D3D4D5D6 mutants along with PAO1 and ΔfadD1D2D4 strains (Fig. 2). The growth experiments on FAs were conducted up to 30 h, which was sufficient to distinguish differences in growth patterns between various strains. The growth rates calculated from growth curves in Fig. 2 are presented in Table S3. The ΔfadD1D2D4 mutant strain had impaired growth in comparison to PAO1 on FAs (Fig. 2B–2E). The phenotype of ΔfadD1D2D3D4D5D6 in C6:0–C18:1 (Fig. 2B–2E) was characterized by lower final optical density (OD) and/or longer lag phase than ΔfadD1D2, indicating that fadD3, fadD5, and fadD6 also contribute to FA degradation. In comparison to ΔfadD1D2 and ΔfadD1D2D3D5D6, ΔfadD1D2D4 exhibited very small amounts of growth, and no increase in turbidity was observed for ΔfadD1D2D3D4D5D6 on FAs (Fig. 2B–2E). The ΔfadD3D4D5D6 mutant had almost identical growth in comparison to PAO1 in C6:0 and C18:1 (Fig. 2B and 2E). In C10:0 and C14:0 ΔfadD3D4D5D6 showed a similar final OD as PAO1 but longer lag phase (Fig. 2C and 2D). These data indicate that, although the activity of FadD4 is masked by the dominance of FadD1 and FadD2, the FadD4 plays a significant role in the degradation of FAs in P. aeruginosa.

Role of fadD Homologues in the Utilization of Plant-derived Acyclic Terpenes

One of the P. aeruginosa fadD homologues, fadD5 (PA2893; atanH), was proposed to be part of the acyclic terpenes utilization (ATU) pathway and to contribute to degradation of citronellol and geraniol (perfumery compounds found in plants) by activating citronellolic acid (CA) and geranic acid (GA) through addition of CoASH [40]. However, mutation of PA2893 alone did not abolish growth on acyclic terpenes possibly suggesting the involvement of other homologue(s) [40]. To determine the role of fadD5 and other fadD homologues in degradation of acyclic terpenes as plant-derived nutrient sources, we grew PAO1 along with 17 combinatorial fadD mutants in 1x M9 minimal media +1% (w/v) Brij-58 with 0.1% (w/v) of CA or GA (Fig. 3). All strains had similar OD measurements after one day of growth on glucose (Fig. 3A). After 24 h, all nine strains with the fadD4 mutation (triple, quadruple, quintuple, and sextuple combinations) had significantly lower OD for both compounds in comparison to PAO1 (20% or less) (Fig. 3C and 3E). All other mutants had comparable growth on PA1 in CA and GA (82%–96% and 88%–115%, respectively). None of the strains with fadD4 mutations had higher OD in CA or GA at day six, than at day one, and the remainder of the mutants grew the same as PAO1 (Fig. S2). Since only strains with fadD4 mutations exhibited growth defects in CA and GA, involvement of FadD4 in degradation of these compounds was further investigated using the single fadD4 mutant (Fig. 3D and 3F). Single copy complementation returned growth of the ΔfadD4 mutant to PAO1 levels indicating that fadD4 is responsible for the majority of CA and GA degradation.

fadD3, fadD4, fadD5, and fadD6 and Virulence in P. aeruginosa

A link between fadD and production of virulence factors was previously observed in P. aeruginosa [14]. To determine if newly discovered homologues modulate virulence, single unmarked mutants ΔfadD3, ΔfadD4, ΔfadD5, ΔfadD6, along with ΔfadD1D2D3D4D5D6 strain and its complement were tested for production of proteases, lipases, phospholipases, and rhamnolipids. No difference in production of these virulence determinates was observed between PAO1 and all strains tested (data not shown).
Involvement of New fadD Homologues in PC Degradation and in vivo Growth

Our previous study indicated that the ΔfadD1D2 mutant had a decreased ability to degrade PC and was less fit in BALB/c mice lungs [14]. We hypothesized that the sextuple fadD mutant, which does not grow on FAs, would exhibit impaired growth on PC and have significantly decreased in vivo fitness. We first investigated the role of the four newly discovered FACS genes in PC degradation (Fig. 4A). Before death phase, ΔfadD1D2 exhibited slower growth rate and lower final turbidity than PAO1. ΔfadD1D2D4 had a longer lag phase in comparison to ΔfadD1D2 before reaching a similar OD, implying that fadD4 contributes to degradation of PC. The ΔfadD1D2D3D4D5D6 mutant further exhibited a significant growth defect on PC. The large differences in growth rate and final OD between the sextuple mutant and ΔfadD1D2D4 suggest that not only fadD4 but also fadD3, fadD5, and fadD6 are required for growth on PC, which contains a mixture of FA chain lengths.

When in vitro competition studies were conducted on the sextuple fadD mutant and its competitor the complemented sextuple fadD mutant, mutation of all six FACS genes did not affect fitness when the bacteria were grown in rich Luria Bertani (LB) medium, and minimal medium supplemented with casamino acids, glucose, glycerol, and choline (Fig. 4B). In contrast, the in vitro competitive index (CI) in oleate (C18:1) and PC were low (~0.15 and ~0.3, respectively) indicating that ΔfadD1D2D3D4D5D6 has a growth disadvantage on these carbon sources.

Table 2. Strains utilized in this study.

| Strain                  | Lab ID | Relevant Properties                      | Source/reference |
|-------------------------|--------|------------------------------------------|------------------|
| E. coli                 |        |                                          |                  |
| K-12                    | E0577  | Prototroph                               | ATCC #23740      |
| E. coli fadD'fadR        | E2011  | Km', fadD' (oldDB8) fadR; Km'           |                  |
| 2011/attTn7:miniTn7-Gm' | E2665  | Gm', Km'; 2011 with miniTn7-Gm' vector inserted at attTn7 site | This study       |
| 2011/attTn7:fadD3        | E2666  | Gm', Km'; 2011 with fadD3 inserted at attTn7 site | This study       |
| 2011/attTn7:fadD4        | E2667  | Gm', Km'; 2011 with fadD4 inserted at attTn7 site | This study       |
| 2011/attTn7:fadD5        | E2799  | Gm', Km'; 2011 with fadD5 inserted at attTn7 site | This study       |
| 2011/attTn7:fadD6        | E2798  | Gm', Km'; 2011 with fadD6 inserted at attTn7 site | This study       |
| 2011/attTn7:fadD7        | E2385  | Gm', Km'; 2011 with fadD7 inserted at attTn7 site | This study       |
| P. aeruginosa            |        |                                          |                  |
| PA01                    | P007   | Prototroph                               | [59]             |
| ΔfadD4                  | P691   | PA01-fadD4::FRT                          | This study       |
| ΔfadD4/attB:fadD4        | P1041  | Tc; PA01-fadD4::FRT/attB::miniCTX2:fadD4 | This study       |
| ΔfadD1D2                | P177   | PA01-ΔfadD1D2::FRT                       | [14]             |
| ΔfadD1D2D3               | P678   | PA01-ΔfadD1D2::FRT/ΔfadD3::FRT           | This study       |
| ΔfadD1D2D4               | P696   | PA01-ΔfadD1D2::FRT/ΔfadD4::mFRT          | This study       |
| ΔfadD1D2D5               | P246   | PA01-ΔfadD1D2::FRT/ΔfadD5::FRT           | This study       |
| ΔfadD1D2D6               | P969   | PA01-ΔfadD1D2::FRT/ΔfadD6::FRT           | This study       |
| ΔfadD1D2D3D4             | P698   | PA01-ΔfadD1D2D4::FRT/ΔfadD3::FRT/ΔfadD4::mFRT | This study       |
| ΔfadD1D2D3D5             | P768   | PA01-ΔfadD1D2D5::FRT/ΔfadD3::FRT/ΔfadD4::mFRT | This study       |
| ΔfadD1D2D3D6             | P769   | PA01-ΔfadD1D2D6::FRT/ΔfadD3::FRT/ΔfadD4::mFRT | This study       |
| ΔfadD1D2D4D5             | P770   | PA01-ΔfadD1D2D4::FRT/ΔfadD3::FRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D4D6             | P771   | PA01-ΔfadD1D2D6::FRT/ΔfadD3::FRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D5D6             | P722   | PA01-ΔfadD1D2D5::FRT/ΔfadD3::FRT/ΔfadD6::FRT | This study       |
| ΔfadD3D4D5D6             | P781   | PA01-ΔfadD3D4D5D6::mFRT/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D3D4D5            | P772   | PA01-ΔfadD1D2D3D4D5::mFRT/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D3D4D6            | P773   | PA01-ΔfadD1D2D3D4D6::mFRT/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D4D5D6            | P726   | PA01-ΔfadD1D2D4D5D6::mFRT/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D3D4D5D6          | P766   | PA01-ΔfadD1D2D3D4D5D6::mFRT/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D3D4D5D6/mucA−   | P973   | Cb'; P767/mucA−/pUC18                    | This study       |
| ΔfadD1D2D3D4D5D6/complement/ mucA− | P1021 | Gm', Tc'; P767/attB::miniCTX2::fadD1D2D4/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT | This study       |
| ΔfadD1D2D3D4D5D6/complement/ mucA− | P1028 | Cb', Gm', Tc'; P767/attB::miniCTX2::fadD1D2D4/ΔfadD3::FRT/ΔfadD4::mFRT/ΔfadD5::FRT/mucA−/pUC18 | This study       |

Abbreviations:
Cb'; carbencillin resistance; Ec, E. coli; fadD, gene encoding fatty acyl-CoA synthetase; Flp, Saccharomyces cerevisiae recombinase; FRT, Flp recognition target; Gm', gentamicin resistance; Km', kanamycin resistance; mucA, anti-sigma factor, repressor of alginate biosynthesis in P. aeruginosa; Po, P. aeruginosa; pheS, gene encoding a mutated ≥ subunit of phenylalanyl tRNA synthase; Tc', tetracycline resistance.

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Table 3. Growth of various P. aeruginosa fadD mutants on FAs after 24 h.

| Strain | C4:0 | C6:0 | C8:0 | C10:0 | C12:0 | C14:0 | C16:0 | C18:1 \(16:1^b\) | Glu |
|--------|------|------|------|-------|-------|-------|-------|----------------|-----|
| PAO1   | +2   | +3   | +4   | +4    | +3    | +3    | +3    | +3            | +4  |
| ΔfadD1D2 | +2   | +2   | +3   | +3    | +3    | +2    | +3    | +3            | +4  |
| ΔfadD1D2D3 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD1D2D4 | +2   | –    | –    | +1    | +1    | +1    | +1    | +1            | +4  |
| ΔfadD1D2D5 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD1D2D6 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD1D2D3D4 | +2   | –    | –    | –     | –     | –     | –     | +1            | +4  |
| ΔfadD1D2D3D5 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD1D2D3D6 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD2D3D4D5 | +2   | –    | –    | –     | –     | –     | –     | –             | +4  |
| ΔfadD2D3D5D6 | +2   | –    | +3   | +4    | +4    | +3    | +3    | +3            | +4  |
| ΔfadD2D3D4D5D6 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD2D3D4D5D6D6 | +2   | +2   | +3   | +3    | +2    | +3    | +2    | +3            | +4  |
| ΔfadD3D4D5D6 | +2   | –    | –    | –     | –     | –     | –     | –             | +4  |
| ΔfadD1D2D3D4D6 | +2   | +2   | +1   | +3    | +1    | +3    | +1    | +3            | +4  |
| ΔfadD1D2D3D5D6 | +2   | +2   | +1   | +3    | +1    | +3    | +1    | +3            | +4  |
| ΔfadD1D2D3D4D5D6 | +2   | –    | –    | –     | –     | –     | –     | –             | +4  |

Strains were grown on 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu).
- indicates no growth on a patch and + denotes growth:
+1 is very little growth.
+4 is a heavy growth comparable to PAO1 on glucose at 96 h.

- indicates no growth on a patch and + denotes growth:
+1 is very little growth.
+4 is a heavy growth comparable to PAO1 on glucose at 24 h.
+6 is a very heavy growth comparable to PAO1 on glucose at 96 h.
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sources. The in vivo competition study showed that the sextuple fadD mutant was out numbered by its complement (Fig. 4C). An almost 10 fold increase in CFU per lung above inoculum (6 x 10^6) was observed for both time points indicating bacterial replication in vivo. At 24 h, the amount of the sextuple fadD mutant was half of its complement, which is lower than the reported CI for the ΔfadD1D2 mutant at 24 h [14]. Even at 48 h the CI was significantly lower than 1, indicating that deletion of fadD genes decreases in vivo fitness of sextuple fadD mutant.

Discussion

Previous research on fadD1 and fadD2 indicated that more than two FACS genes are present in P. aeruginosa [14]. In this study, we focused on identification of additional fadD homologues. Four genes, fadD3, fadD4, fadD5, and fadD6 (PA3860, PA1617, PA2893, and PA3924, respectively) were found to encode FACS (Tables S1 and 1). Each of these genes contributes at a varying degree to FA degradation (Tables 3 and 4). Surprisingly, none of the new fadDs were involved in degradation of butyrate (C_{1:0}; Table 3). It is possible that other unidentified genes with acyl-CoA synthetase functions are responsible for growth on C_{1:0}. Butyrate could also be processed through the acetocetate degradation pathway (ato), an alternative pathway for degradation of SCFA [41]. This could be possible since two homologues of both of E. coli acetocetyl-CoA transferase complex proteins, AtoA and AtoD, are present in P. aeruginosa. PA2000 (identity 45% and similarity 62%), PA0227 (identity 28% and similarity 62%), PA1999 (identity 40% and similarity 64%), and PA5445 (identity 33% and similarity 55%), respectively.

Growth studies with various mutants using FAs as sole carbon and energy sources indicated that FACS homologues are not of equal physiological significance and that there are disparities in importance and FA preference between them. fadD1 and fadD2, along with fadD4, are responsible for almost all FA degradation and dominate over other homologues. When fadD1 and fadD2 are inactivated, the majority of growth on SCFAs, MCFAs and LCFA is due to fadD4 (Tables 3 and 4, Fig. 2). In comparison, fadD3, fadD5, and fadD6 have small contributions to overall growth on FAs and their individual involvement can be only observed when fadD1, fadD2 and fadD4 are absent (Table 4). This is not unprecedented, since Pseudomonas putida FadD2 is only active when FadD1 is not present [42]. It could be possible that gene(s) ruled out by screening in E. coli for growth on LCFA (Table S1), might be involved in SCFA and/or MCFA degradation. However, lack of growth for the sextuple fadD mutant on C_{6:0}–C_{10:1} \(16:1^b\) (Table 4) strongly indicates that P. aeruginosa has a total of six aerobic FACS genes.

P. aeruginosa is commonly found in soil, water, and on plant surfaces [43–45] and it is known to degrade over 70 different organic substances such as aromatic compounds, organic acids (e.g. isovalerate), alcohols, and acyclic terpenes (e.g. citronellol and geraniol) [44]. Sources of nutrients for pseudomonads on plant surfaces have not been determined. Citronellol and geraniol (perfumery compounds and possible bacterial nutrient sources found in plants) are degraded through the acyclic terpene utilization (ATU) pathway, β-oxidation pathway, and leucine/isovalerate utilization pathway [40,46]. The fadD5 (PA2893 or atuH) was proposed to be part of ATU and to be involved in activation of the CA and GA intermediates of the
Table 4. Growth of various P. aeruginosa fadD mutants on FAs after 96 h.

| Strain          | C4:0 | C6:0 | C8:0 | C10:0 | C12:0 | C14:0 | C16:0 | C18:1 \(\alpha\) | Glu |
|-----------------|------|------|------|-------|-------|-------|-------|-----------------|-----|
| PAO1            | +4   | +6   | +6   | +6    | +6    | +6    | +6    | +6              | +6  |
| ΔfadD1D2        | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D3      | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D4      | +4   | +2   | +1   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D5      | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D6      | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D3D4    | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D3D5    | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D3D6    | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D5D6    | +4   | +4   | +4   | +4    | +4    | +4    | +4    | +4              | +6  |
| ΔfadD1D2D4D5D6  | +4   | +6   | +6   | +6    | +6    | +6    | +6    | +6              | +6  |
| ΔfadD1D2D3D4D5  | +4   | –    | –    | –     | –     | –     | +3    | +3              | +6  |
| ΔfadD1D2D3D5D6  | –    | –    | –    | –     | –     | –     | –     | –               | –   |
| ΔfadD1D2D4D5D6  | –    | –    | –    | –     | –     | –     | –     | –               | –   |
| ΔfadD1D2D3D4D5D6| –    | –    | –    | –     | –     | –     | –     | –               | –   |

Strains were grown on 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu).

- indicates no growth on a patch and + denotes growth.
+1 is very little growth.
+4 is a heavy growth comparable to PAO1 on glucose at 24 h.
+6 is a very heavy growth comparable to PAO1 on glucose at 96 h.

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pathway. However, fadD5 was confirmed experimentally not to be part of ATU, and other homologues were thought to be also involved and to ’mask’ the phenotype [40]. We investigated the possible role of fadD homologues in the degradation of acyclic terpenes, and we reasoned that combination of various fadD mutations would allow involvement of FACS homologues in ATU to be assessed. Surprisingly, fadD5 along with fadD1, fadD2, fadD3, and fadD6 had minimal if any contributions to the degradation of CA and GA (Fig. 3). Interestingly, fadD5 is located right next to genes known to be involved in ATU and seems to be the last gene in atuABCDEFGH cluster [40]. On the other hand, fadD4 is not only involved in ATU but it is almost solely responsible for degradation of these compounds as can be observed from growth phenotypes of the single fadD4 mutant and its complement (Fig. 3D and 3F). Notably, homologues of fadD4 with high similarity are present in Pseudomonas fluorescens (e.g., PFL1_4205 in P0-1, 72% identity and 84% similarity), Pseudomonas protegens (e.g., PFL1_1744 in strain Pf-5, 71% identity and 82% similarity), and Pseudomonas mendocina (e.g., MDS_2302 in strain NK-01, 75% identity and 87% similarity) and some strains of these pseudomonads are known to degrade acyclic terpenes [40], [47].

The ability of P. aeruginosa to degrade lipids and FAs, especially the main component of lung surfactant PC, has been linked to replication of this opportunistic pathogen during infection of CF patients’ lungs [13]. Previously, we determined that ΔfadD1, ΔfadD2, and double ΔfadD1D2 mutants have decreased fitness in BALB/c mice due to their deficiencies in degradation of FAs and PC [14]. We hypothesized that P. aeruginosa strains with greater defects in utilization of FAs and PC in vitro will have larger disadvantages during in vivo growth. ΔfadD1D2D3D4D5D6 mutant exhibited the most significant growth defect in FAs and PC (Fig. 2, 4A and 4B), and similar level of virulence factors (i.e. proteases, hemolysins, lipases) production was observed between sextuple fadD mutant, its complement, and PAO1 (data not shown). The ΔfadD1D2D3D4D5D6 mutant had some decrease of in vivo fitness in comparison to the ΔfadD1D2 at 24 h (Fig. 4C and [14]); but at 48 h, ΔfadD1D2D3D4D5D6 mutant was not less fit in mice lungs than ΔfadD1D2 mutant. This latter result was surprising, as the impaired ability to utilize PC did not result in a more dramatic phenotype in vivo at 48 h (Fig. 4C). There are several possibilities, which could account for this unexpected phenotype. The sextuple mutant could utilize in vivo other constituents of PC such as choline and glycerol later in the infection. Additionally, pulmonary surfactants are composed of 10% proteins [48] and amino acids were suggested to be used by P. aeruginosa during lung infection [49] and could serve as an alternative nutrient source for sextuple fadD mutant. Other FACS genes (i.e. anaerobic which we could not identify because of limitations of our aerobic in vitro screening method) could be important for in vivo growth.

In summary, we have identified four additional FACS homologues of P. aeruginosa and determined their involvement in degradation of different FAs. The dual catabolic function of fadD4 (PA1617) for FAs and acyclic terpenes exemplifies the interconnection of metabolic pathways and multiple roles that FACS homologues play in this ubiquitous bacterium. Our in vivo data show that nutrient acquisition during lung infection is a complicated process, involving alternative pathways that require
further investigation. Knowledge of all fadD genes needed for FA degradation significantly increases our understanding of the FA degradation pathway and its importance for in vivo replication of P. aeruginosa.

Figure 2. fadD mutants and growth on FAs. Various strains were grown on glucose (A), C6:0 (B), C10:0 (C), C14:0 (D), and C18:1 Δ9 (E) to investigate further the role of fadD4 in FA degradation in comparison to rest of homologues. These growth curves demonstrate the hierarchical dominance of fadD1, fadD2 and fadD4 over other fadDs. Growth experiments were performed twice and representative curves are shown.

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Materials and Methods

Ethics Statement

All animal experiments were approved by University of Hawaii at Manoa Institutional Animal Care and Use Committee (protocol no. 06-023-6) and were conducted in compliance with the NIH
Bacterial Strains and Growth Media

Strains and plasmids utilized in this study are listed in Tables 2, S2, and 5, respectively. All *P. aeruginosa* mutants constructed and utilized in this study are derived from strain PAO1. *E. coli* E1869 strain (Table S2) was routinely used for cloning and *E. coli Δasd* or Δ*fadD4* strains (E464, E1353, and E2072, Table S2) were used for mobilization of plasmids as described previously [50]. *E. coli* and *P. aeruginosa* strains were cultured in rich and minimal media as

Figure 3. Growth phenotypes of various *fadD* homologues mutants on acyclic terpenes. Strains were grown in liquid 1x M9 medium +1% (w/v) Brij-58 supplemented with 20 mM glucose, 0.1% (w/v) of citronelllic acid, or 0.1% (w/v) geranic acid at 30°C. Optical densities (ODs) of cultures were measured and compared to PAO1 at day one (A, C, and E). Growth of Δf*adD4* mutant and Δf*adD4/attB-fadD4* complement strain in different carbon source were compared to PAO1 and ODs from day six are presented (B, D, and F). Results shown are from representative experiments that were performed twice by measuring triplicate cultures.
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described by Kang et al. [14] unless indicated otherwise. Fatty acids stocks were prepared as previously described [31].

General Molecular Techniques

Molecular techniques were performed as previously described [50]. Oligonucleotides (Table 6) were synthesized through Integrated DNA Technologies.

Identification of P. aeruginosa Fatty acyl-CoA Synthetase Homologues

Potential P. aeruginosa fadD homologues were identified through BLAST [34] utilizing E. coli FadD sequence and alignment of E. coli FadD ATP/AMP [19], [35–37] and fatty acid binding motifs [38] with the FadD motifs of P. aeruginosa fadD homologues. Prediction of function of genes was obtained from Pseudomonas Genome Database (www.pseudomonas.com) [51]. PA2557, PA3860, and PA4198 were PCR amplified and cloned into pUC19 as BamHI fragments. The fadD homologues PA1617, PA1997, PA2555, PA3568, PA2893, and PA3924, were PCR amplified and cloned into pUC19 as HindIII/EcoRI, BamHI/SmaI, HindIII/KpnI, HindIII/SalI, and XbaI/BamHI fragments, respectively. For functional complementation testing, pUC19 vectors containing PAO1 fadD homologues were transformed into E. coli fadD<sup>-/-</sup>/fadR<sup>-/-</sup> strain (E2011) and the resulting transformants were patched onto 1x M9+1% (w/v) Brij-58+ampicillin 100 μg/ml supplemented with 20 mM glucose, 0.2% (w/v) oleate (C<sub>18:1</sub>Δ<sub>9</sub>) or decanoate (C<sub>10:0</sub>).

Single Copy Complementation of the E. coli fadD<sup>-/-</sup>/fadR<sup>-/-</sup> Mutant

To construct fadD<sub>3</sub>, fadD<sub>5</sub>, and fadD<sub>6</sub> single copy complementation vectors, first fadD<sub>3</sub>, fadD<sub>5</sub>, and fadD<sub>6</sub> PCR product were cloned into pET15b as NdeI/BamHI fragments. Next, the pUC19 vectors containing PAO1 fadD homologues were transformed into E. coli fadD<sup>-/-</sup>/fadR<sup>-/-</sup> strain (E2011) and the resulting transformants were patched onto 1x M9+1% (w/v) Brij-58+ampicillin 100 μg/ml supplemented with 20 mM glucose, 0.2% (w/v) oleate (C<sub>18:1</sub>Δ<sub>9</sub>) or decanoate (C<sub>10:0</sub>).
Table 5. Plasmids used in this study.

| Plasmid                  | Lab ID   | Relevant Properties                                      | Source/reference |
|-------------------------|----------|---------------------------------------------------------|------------------|
| miniCTX2                | E0076    | Tc<sup>+</sup>; P. aeruginosa site specific integration vector | [58]             |
| miniCTX2-fadD2D1        | E2143    | Tc<sup>+</sup>; fadD2D1 cloned into miniCTX2           | [14]             |
| miniCTX2-fadD2D1D4      | E2811    | Tc<sup>+</sup>; fadD4 gene cloned into miniCTX2-fadD2D1 |                 |
| miniCTX2-fadD4          | E2589    | Tc<sup>+</sup>; fadD4 gene cloned into miniCTX2         | This study       |
| miniTn7-Gm<sup>+</sup>  | E2643    | Ap<sup>+</sup>, Gm<sup>+</sup>; pUC18K6mini-Tn7 [52] with FRT8-Gm<sup>+</sup> cassette and lac promoter cloned | Laboratory collection |
| miniTn7-fadD3           | E2645    | Ap<sup>+</sup>, Gm<sup>+</sup>; fadD3 cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-fadD4           | E2647    | Ap<sup>+</sup>, Gm<sup>+</sup>; fadD4 cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-fadD5           | E2793    | Ap<sup>+</sup>, Gm<sup>+</sup>; fadD5 cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-fadD6           | E2794    | Ap<sup>+</sup>, Gm<sup>+</sup>; fadD6 cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-fadD6<sub>a</sub> | E2378   | Ap<sup>+</sup>, Gm<sup>+</sup>; E. coli fad cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-PA3860          | E2377    | Ap<sup>+</sup>, Gm<sup>+</sup>; fadD3 with native rbs cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-PA3924          | E2854    | Ap<sup>+</sup>, Gm<sup>+</sup>; fadD6 with native rbs cloned into miniTn7-Gm<sup>+</sup> | This study       |
| miniTn7-fadD5-fadD6     | E2860    | Ap<sup>+</sup>, Gm<sup>+</sup>, fadD5, fadD6, and fadD6 with native rbs cloned into miniTn7-Gm<sup>+</sup> | This study       |
| pCD135K-flp oriT        | E0783    | Sp<sup>+</sup>; suicidal Flp-expressing plasmid          | [33]             |
| pET15b                  | E0047    | Ap<sup>+</sup>; T7 expression vector                    | Novagen          |
| pET15b-fadD3            | E2658    | Ap<sup>+</sup>; pET15b with fadD3 gene                  | This study       |
| pET15b-fadD5            | E1127    | Ap<sup>+</sup>; pET15b with fadD5 gene                  | This study       |
| pET15b-fadD6            | E2790    | Ap<sup>+</sup>; pET15b with fadD6 gene                  | This study       |
| pET28a                  | E0158    | Km<sup>+</sup>; T7 expression vector                    | Novagen          |
| pET28a-fadD4            | E2644    | Km<sup>+</sup>; pET28a with fadD4 gene                  | This study       |
| pEX18T                  | E0055    | Ap<sup>+</sup>; gene replacement vector                 | [53]             |
| pEX18T-fadD3-Gm<sup>+</sup>-pheS<sub>594</sub> | E2438   | Ap<sup>+</sup>, Gm<sup>+</sup>, Gm<sup>+</sup>-pheS<sub>594</sub>-FRT cassette inserted into fadD3 | This study       |
| pEX18T-fadD4-Gm<sup>+</sup>-pheS<sub>594</sub> | E2506   | Ap<sup>+</sup>, Gm<sup>+</sup>, Gm<sup>+</sup>-pheS<sub>594</sub>-mFRT cassette inserted into fadD4 | This study       |
| pEX18T-fadD5-Gm<sup>+</sup> | E0828   | Ap<sup>+</sup>, Gm<sup>+</sup>, Gm<sup>+</sup>-mFRT cassette inserted into fadD5 | This study       |
| pEX18T-fadD6-Gm<sup>+</sup> | E1476   | Ap<sup>+</sup>, Gm<sup>+</sup>, Gm<sup>+</sup>-FRT cassette inserted into fadD6 | This study       |
| pFLP<sup>+</sup>FRT-Gm<sup>+</sup>-pheS<sub>594</sub> | E0067   | Ap<sup>+</sup>; broad-host range FLP expressing plasmid | Laboratory collection |
| pPS56<sup>a</sup>       | E0050    | Ap<sup>+</sup>, Gm<sup>+</sup>; plasmid with Gm<sup>+</sup>-pheS<sub>594</sub>-mFRT cassette | [53]             |
| pTN52<sup>a</sup>       | E1189    | Ap<sup>+</sup>; helper plasmid for Tn7 transposition system | [52]             |
| pUC18-‘mucA’            | E1907    | Ap<sup>+</sup>; pUC18 with internal fragment of mucA cloned | [14]             |
| pUC19                   | E0014    | Ap<sup>+</sup>; cloning vector                          | [60]             |
| pUC19-PA1617            | E2472    | Ap<sup>+</sup>; PAO1 PA1617 gene cloned into pUC19      | This study       |
| pUC19-PA3860            | E2356    | Ap<sup>+</sup>; PAO1 PA3860 gene cloned into pUC19      | This study       |
| pwFRT-Gm<sup>+</sup>-pheS<sub>594</sub> | E2380   | Ap<sup>+</sup>, Gm<sup>+</sup>; plasmid with Gm<sup>+</sup>-pheS<sub>594</sub>-FRT cassette | Laboratory collection |

Abbreviations:
- Ap<sup>+</sup>, ampicillin resistance; lac, E. coli lactose operon; rbs, ribosomal binding site; Sp<sup>+</sup>, streptomycin resistance.

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with XbaI. To construct miniTn7-fadD<sub>6</sub>, the fadD<sub>6</sub> PCR product was cloned as BamHI/blunt-end fragment into miniTn7-Gm<sup>+</sup> digested with XbaI, blunt ended and digested BamHI.

Various miniTn7 vectors were integrated into E2011 using pTNS2 [52]. For the complementation study, two colonies of K-12, E2011, E2011/attTn7::miniTn7-Gm<sup>+</sup>, E2011/attTn7::fadD<sub>6</sub>, E2011/attTn7::fadD<sub>3</sub>, E2011/attTn7::fadD<sub>4</sub>, E2011/attTn7::fadD<sub>5</sub>, and E2011/attTn7::fadD<sub>6</sub> were patched onto 1x M9 medium +1% (w/v) Brij-58+0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) supplemented with 0.2% (w/v) FAs or 20 mM glucose. Plates were incubated for three 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 comparable to K12 on glucose at day three was marked as +6.

Construction of Mutant Strains of PAO1

The fadD3, fadD4, fadD5, and fadD6 gene replacement vectors were obtained as follows. pEX18T-fadD3-Gm<sup>+</sup>-pheS<sub>594</sub> was constructed by digesting pUC19-PA3860 with MscI and SgrAI, blunt-ending, and ligation it with Gm<sup>+</sup>-pheS<sub>594</sub>-FRT cassette that was Smal excised from pwFRT-Gm<sup>+</sup>-pheS<sub>594</sub>. The PA3860-Gm<sup>+</sup>-pheS<sub>594</sub> fragment was excised from the resulting vector using BamHI and cloned into pEX18T. Similarly, pEX18T-fadD4-Gm<sup>+</sup>-pheS<sub>594</sub> was obtained by first sub-cloning fadD4 gene as a HindIII/EcoRI fragment from pUC19-PA1617 into pEX18T, and fadD4 was deactivated at the XhoI site by inserting the Gm<sup>+</sup>-pheS<sub>594</sub>-mFRT cassette SalI excised from pmFRT-Gm<sup>+</sup>-pheS<sub>594</sub>. pEX18T-fadD5-Gm<sup>+</sup> was constructed by cloning fadD5 PCR product (oligos #437 and #438) as BamHI/blunt-end fragment into pEX18T that was
digested with BamHI and SmaI, and *fadD5* was deactivated at the blunt-ended XhoI site by inserting the Gm r-*fadD5* cassette, to obtain unmarked mutant strains P685, P239, P416, P677, P772, P773, P969, and P972. Strain Δ*fadD3D4D5D6* (P781) was constructed in the PAO1-*fadD3D4D5D6* background by subsequent transfer of mutation from strains P685, P239, and P146 followed by Flp mediated excision of Gm r-*fadD3D4D5D6* cassette. Presence or absence of mutations of *fadD2D1, fadD5, fadD4, fadD5*, and *fadD6* in all mutant strains were confirmed by PCR (data not shown).

### Growth Phenotypes of Multiple *fadD* Mutants on 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 purified on LB. After 24 h incubation at 37°C, two colonies of each strain were patched onto 1x M9 solid medium +0.2% (w/v) FAs or 20 mM glucose. Plates were incubated at 37°C for four days. Growth of each strain was scored from 0 to 6 (very heavy growth comparable to PAO1 on glucose growth) to +6 (very heavy growth comparable to PAO1 on glucose at 96 h).

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**Table 6. Oligonucleotides primers utilized in this study.**

| Primer number and name | Sequence* |
|------------------------|-----------|
| 438; PA2893-BamHI<sup>c</sup> | 5'-CAGTAGGATCCCAAGGTGATCAAGAGCGGT-3' |
| 512; PA3924-BamHI<sup>c</sup> | 5'-TGCTTGAGACCCGCGTTCGGCGGTGA-3' |
| 1093; EcfadD-down-BamHI<sup>b</sup> | 5'-AACCGGATCCAGCTTTTATGTCC-3' |
| 1109; PA3924-Ndel<sup>b</sup> | 5'-GTGACCGCATATGCTGTACCCACC-3' |
| 1151; PA1221 BamHI-up<sup>a</sup> | 5'-AAGCCTGGATCATCTACGTGGTTCTC-3' |
| 1152; PA1221 BamHI-down<sup>a</sup> | 5'-AGCGCGCTTTTCGCGGAAAGGGGTCC-3' |
| 1153; PA2557 BamHI-up<sup>a</sup> | 5'-TGGGCCGATCCGCGTTCGTGTTAC-3' |
| 1154; PA2557 BamHI-down<sup>a</sup> | 5'-GAAGGCCAGTCCGCTCTTCGAGGCCGGA-3' |
| 1155; PA3860 BamHI-up<sup>d</sup> | 5'-GAACCGGATCCAGCTGAAGCACTTGCTCC-3' |
| 1156; PA3860 BamHI-down<sup>d</sup> | 5'-CTGAGGAAATCCCAACAGATCGGGTTCTGCT-3' |
| 1157; PA4198 BamHI-up<sup>a</sup> | 5'-CCAGGAGTCCAGCGGTTCGTGCTGCTC-3' |
| 1158; PA4198 BamHI-down<sup>a</sup> | 5'-CGAAGCAGCTGTGGAGGAGGGATCCGAC-3' |
| 1218; fadDE-Flp-oriT<sup>b</sup> | 5'-CTCATGATGCTTGGGTTGCGATGAC-3' |
| 1251; fadD3-Ndel<sup>b</sup> | 5'-AACCATATGAGACCTCCGATCG-3' |
| 1252; PA3568-Up-HindII<sup>b</sup> | 5'-AATCTAGATGACGTACCTACCTACTCCATC-3' |
| 1253; PA3568-Down-Sall<sup>c</sup> | 5'-AGCGCGCTTTTCGCGGAAAGGGGTCC-3' |
| 1254; PA1997-Up-BamHI<sup>b</sup> | 5'-AAGCCTGGATCATCTACGTGGTTCTC-3' |
| 1255; PA1997-Down-Sall<sup>c</sup> | 5'-TGAAAGAATGCATTGCTG-3' |
| 1256; PA0996-Up-BamHI<sup>b</sup> | 5'-GCTTCTGCTTGGTGCAC-3' |
| 1257; PA0996-Down-BamHI<sup>d</sup> | 5'-CCAGGAGTCCAGCGGTTCGTGCTGCTC-3' |
| 1258; PA2555-Up-HindII<sup>d</sup> | 5'-GCGTGAGCTCCGCGTTCGTGCTGCTC-3' |
| 1259; PA2555-Down-Sall<sup>d</sup> | 5'-CGAAGCAGCTGTGGAGGAGGGATCCGAC-3' |
| 1260; PA1617-Up-HindII<sup>c</sup> | 5'-CCATAGGTTCGCTCAGGACTCTAC-3' |
| 1261; PA1617-Down-EcoRI<sup>b</sup> | 5'-CGCTATGACAGAGCAGCAAC-3' |
| 1441; PA1617-Ndel<sup>c</sup> | 5'-CTGATCTATGACAGAGCAGCAAC-3' |
| 1444; PA1617-Ndel<sup>b</sup> | 5'-ATGCCATATGATGTCACTCGAAATCTG-3' |
| 2109; PA2893-up<sup>c</sup> | 5'-GGCTATGACAGAGCAGCAAC-3' |
| 2110; PA3924-up<sup>d</sup> | 5'-GGATCCGTACATGACAGAGCAGCAAC-3' |

*Restriction enzyme sequences are underlined.
*Single copy complementation in *E. coli.*
*Single copy complementation in *P. aeruginosa.*

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Growth Curves Experiments

To further characterize various fadD mutants of P. aeruginosa, growth curve studies were performed using FAs as sole carbon source as described previously [14]. Doubling time of various strains in log-phase (Table S3) was calculated as follows: doubling time = \[ \frac{0.301(t_2-t_1)}{\log OD_2 - \log OD_1} \] [57].

Growth of fadD Mutants on Acyclic Terpenes

The \( \Delta \)fadD1 and attB::fadD4 strain was constructed using a single copy complementation vector miniCTX2/fadD4, which was obtained by cloning the fadD4 PCR product (oligos \#1443 and \#1261) as HindIII and EcoRI fragment into miniCTX2 and integrated into \( \Delta \)fadD4 mutant chromosome as described previously [50]. Stocks of citronellic (Sigma) and geranic acid (Sigma) (3% (w/v)) were prepared by neutralizing the compounds with equal molar sodium hydroxide and dissolving in 1% (w/v) Brij-58. PAO1 and various fadD mutants were grown overnight (14–16 h), starter culture were prepared as described by Kang et al. [14] and inoculated at 200-fold dilution into 1x M9 minimal medium +1% (w/v) Brij-58 supplemented with 0.1% (w/v) of citronellic acid, 0.1% (w/v) geranic acid or 20 mM glucose. Triplicate cultures were shaken at 30°C and optical densities were measured at day one and day six.

Virulence Factors Production

Lipase, protease, phospholipase, and rhamnolipid productions by fadD mutants were tested as previously described [14].

In vitro and in vivo Competition Studies

For \( \text{in vitro} \) and \( \text{in vivo} \) in competition studies, the \( \Delta \)fadD1D2D3D4D5D6 strain was complemented with fadD2D1 and fadD4 cloned into miniCTX2 and fadD3, fadD5, and fadD6 cloned into miniTn7-Gm\(^{\prime}\). MiniCTX2-fadD2D1D4 complementation vector, was constructed by cloning fadD4 gene PCR product (oligos \#1443 and \#1261) as HindIII/blunt-end fragment into miniCTX2-fadD2D1 digested with XhoI, blunt-ended and digested with HindIII. To construct miniTn7-fadD3/fadD5/fadD6 vector, first fadD3 was sub-cloned as BamHI fragment from pUC19-PA3990 into miniTn7-Gm\(^{\prime}\), resulting in miniTn7-PA3860. The fadD6 was amplified with oligos \#512 and \#2210 and cloned as a BamHI/XbaI fragment into miniTn7-Gm\(^{\prime}\), resulting in miniTn7-PA3924. The fadD5 was amplified with oligos \#438 and \#2109 and digested with BamHI, blunt-ended and digested with XbaI. To construct the final vector, the miniTn7-PA3924 was digested with XbaI, blunt-ended and digested with NdeI and the 2.5 kb fragment (containing fadD6) was cloned simultaneously along with fadD5 fragment into miniTn7-PA3860 digested with NdeI and SpeI. Integration of these plasmids into the P. aeruginosa chromosomes was performed as described previously [58] and [52].

The \( \text{in vitro} \) competition between \( \Delta \)fadD1D2D3D4D5D6 and its complement (strain P1021) on LB, or casamino acids (CAA), choline, glucose, glycerol, oleate (C\(_{18:1}\)\(^\Delta8\)) or PC was performed as described previously [14].

The \( \text{in vivo} \) competition study was performed as previously described [14]. Briefly, mucA was inactivated in the PAO1-\( \Delta \)fadD1D2D3D4D5D6 and its complement strains utilizing pUC119-mucA\(^{\prime}\). Equal amounts of alginate overproducing sextuple mutant and its complement were resuspended in their own supernatants and mixed. Fourteen BALB/c mice were inoculated intratracheally with 6 x 10\(^7\) colony forming units (CFU) of mixture of mutant (strain P973) and complement (strain P1020) as described previously [14]. At each time point (24 h and 48 h) seven mice were manually euthanized, lungs were homogenized in 0.85% (w/v) saline and serial dilutions were plated on LB and LB+tetracycline 100 \(\mu\)g/ml to determine the total CFU and the complemented strain CFU. The competitive index (CI) was calculated as described [14].

Supporting Information

Figure S1 Alignment of motifs of potential fatty acyl-CoA synthetase homologues. Amino acids with similar properties are assigned the same colors using CLC Sequence Viewer 6 software (www.clcbio.com).

Figure S2 Growth phenotypes of various fadD homologues mutants on acyclic terpenes at day six. Strains were grown in liquid 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.1% (w/v) of citronellic acid or 0.1% (w/v) geranic acid at 30°C. Optical densities (ODs) of cultures were measured and compared to PAO1.

Table S1 Potential FadD homologues of P. aeruginosa identified through BLAST and tested for complementation in E. coli fadD \(^{\text{fadR}}\) (E2011).

Table S2 Additional strains utilized in this study.

Table S3 Doubling time in minutes (min) of various strains in log-phase were calculated from growth curves in Fig. 2.

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Author Contributions

Conceived and designed the experiments: JZS MHN YK TTH. Performed the experiments: JZS MHN. Analyzed the data: JZS MHN YK TTH. Contributed reagents/materials/analysis tools: JZS MHN YK ZS APB IM. Wrote the paper: JZS TTH. Edited manuscript: JZS MHN YK ZS APB IM.

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