Multiple FadD Acyl-CoA Synthetases Contribute to Differential Fatty Acid Degradation and Virulence in Pseudomonas aeruginosa

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Abstract

A close interconnection between nutrient metabolism and virulence factor expression contributes to the pathophysiology of Pseudomonas aeruginosa as a successful pathogen. P. aeruginosa fatty acid (FA) degradation is complicated with multiple acyl-CoA synthetase homologs (FadDs) expressed in vivo in lung tissue during cystic fibrosis infections. The promoters of two genetically linked P. aeruginosa fadD genes (fadD1 and fadD2) were mapped and northern blot analysis indicated they could exist on two different transcripts. These FadDs contain ATP/AMP signature and FA-binding motifs highly homologous to those of the Escherichia coli FadD. Upon introduction into an E. coli fadD/fadR double mutant, both P. aeruginosa fadDs functionally complemented the E. coli fadD/fadR mutant, allowing degradation of different chain-length FAs. Chromosomal mutagenesis, growth analysis, induction studies, and determination of kinetic parameters suggested that FadD1 has a substrate preference for long-chain FAs while FadD2 prefers shorter-chain FAs. When compared to the wild type strain, the fadD2 mutant exhibited decreased production of lipase, protease, rhamnolipid and phospholipase, and retardation of both swimming and swarming motilities. Interestingly, fadD1 mutant showed only increased swimming motility. Growth analysis of the fadD mutants showed noticeable deficiencies in utilizing FAs and phosphatidylcholine (major components of lung surfactant) as the sole carbon source. This defect translated into decreased in vivo fitness of P. aeruginosa in a BALB/c mouse lung infection model, supporting the role of lipids as a significant nutrient source for this bacterium in vivo.

Introduction

To occupy a diverse range of ecological niches, Pseudomonas aeruginosa must evolve and maintain a wide array of metabolic pathways for nutrient uptake and utilization. This adaptive flexibility allows P. aeruginosa, a ubiquitous Gram-negative saprophyte, to occupy environmental niches in both soil and water and to transition into a potentially pathogenic lifestyle with humans, plants, animals, and other microbes [1–4]. This bacterium has been responsible for a myriad of infections including serious bacteremia and nosocomial pneumonia [5–8], and it has been shown to be the major cause of morbidity and mortality among cystic fibrosis (CF) patients aged 18–24 years [9,10]. P. aeruginosa thrives both environmentally and within a human host because of its extensive repertoire of virulence factors [9,10]. Its capacity to metabolize 70–80 different organic substrates as sole carbon sources, notably different chain-length fatty acids (FA, C14–C18) [11]. Our previous work suggested that P. aeruginosa expresses phospholipases and lipases in vivo that degrades phosphatidylcholine (PC; Fig. 1A) as a nutrient source for bacterial replication in the lungs of CF patients [12]. In support of these results, Miller et al. [13] have shown that P. aeruginosa utilizes type IV pili to twitch towards phospholipids (i.e. phosphatidylethanolamine and PC) and long-chain FA (LCFAs).

Fatty acid degradation (Fad) in the model microbe, Escherichia coli, employs enzymes of the Fad pathway encoded by the fad regulon [14,15]. E. coli possesses a single FadD, a 62-kDa fatty acyl-CoA synthetase (FACS or AMP-forming fatty acid:CoA ligase), encoded by the fadD gene [16,17]. The FadD protein possesses two highly conserved sequence elements corresponding to a proposed ATP/AMP signature motif [17,18], as well as a signature motif involved in FA substrate binding and specificity [19] (Fig. 1C). Following FadL-mediated importation of exogenous FAs through the outer membrane [20–22] and an unknown transportation process through the inner membrane, FadD appears to employ these two motifs to activate FAs in a two-step process [18,19]. In the first step of activation, an acyl bond between the α-phosphoryl group of an ATP and the carboxyl group of a FA is formed creating a fatty acyl-adenylate intermediate and releasing pyrophosphate. In the second step, the release of AMP occurs concomitantly with thioester bond formation.
Figure 1. The proposed FA degradation pathway in P. aeruginosa based on E. coli β-oxidation. (A) Phosphatidylcholine (PC) is the major component of lung surfactant. PC can be cleaved by P. aeruginosa phospholipase C and lipases producing free fatty acids that are degraded via the β-oxidation pathway by this bacterium. (B) FAs are transported through the outer membrane aided by an unidentified P. aeruginosa FadL [13,67]. In E. coli, FA may be transported through the inner membrane via an unknown mechanism coupled to a single peripheral membrane FadD protein [18]. However, P. aeruginosa contains at least two FadDs (FadD1 and FadD2). Although there are over a dozen potential FadE homologues in the P. aeruginosa genome, the specific enzyme(s) that catalyzes this reaction has not been identified. FadB catalyzes the next two steps followed by cleavage of the 3-keto-acyl-CoA by FadA. Two fadBA operons (fadBA1 and fadBA5) have been identified in P. aeruginosa [29,30]. (C) Alignment of the P. aeruginosa FadD1 and FadD2 ATP/AMP-signature and FA-binding motifs with the FadD motifs of E. coli [18,19]. Abbreviation for Fad-proteins:
formation between the fatty acyl group and the sulfhydryl group of coenzyme A in the second step [23]. This FadD-catalyzed reaction produces fatty acyl-CoA, a molecule capable of degradation by the β-oxidation cycle or exerting transcriptional control on the E. coli fad-regulon by interacting with the FadR regulator to derepress fad-genes [24–27]. However, it seems currently that some fad-genes in P. aeruginosa are induced, not by fatty acyl-CoA, but by LCFAs [24–28]. While the biochemistry and physiology of FadD have been well characterized in E. coli, relatively little is known about FadD(s) in P. aeruginosa. The P. aeruginosa β-oxidation cycle in Fad has only been partially characterized with respect to FadBAs (Fig. 1B) [29,30]. Fad enzymes, including the broad substrate specificity of the FACS, have also been characterized in Pseudomonas fragi [31–33]. A study on Pseudomonas putida originally isolated and characterized one FACS with a broad substrate range [34]. Additional work further characterized the role of this P. putida enzyme and identified a second FACS, naming them FadD1 and FadD2, respectively [35,36]. In this dual FadD system, it was shown that FadD1 played a dominant role in FA metabolism while FadD2 was activated only when FadD1 was inactivated [36]. Comparison of the significantly larger size of the Pseudomonas genome relative to that of E. coli, such genetic redundancies are not unexpected. However, the importance of the redundancy and functions of these enzymes in Fad are uncertain.

Studies on other species have indicated a link between FACS, nutrient metabolism, and the expression of virulence factors [37–43]. In Mycobacterium tuberculosis, 36 fadD homologues were identified [39]. A null mutation in the M. tuberculosis fadD28 gene showed significant replication restriction in mouse lungs, as a result of defects in cell-wall biosynthesis and the production of complex lipids [40]. In addition, fadD33 in the M. tuberculosis H37Ra strain was shown to play a role in supporting growth in mouse livers [42]. Similar to these Mycobacterium studies, the use of random transposon mutagenesis has led to the isolation of a fadD mutant in Salmonella enterica serovar Typhimurium which was shown to reduce the expression of hck (a proposed transcriptional activator of genes in the type III secretion system [38]) and invasion genes three- to five-fold [41]. A Xanthomonas campestris fadD homolog tpb1 mutant has decreased production of protease, endoglucanase, and polygalacturonate lyase due to the inability to generate a diffusible extracellular factor containing a FA moiety [37]. A Tn5 insertion in the fadD gene of Sinorhizobium meliloti displayed an increased swarming phenotype compared to wild-type, resulting in an observed decrease in alfalfa root nodulation [43]. Many of these studies correlated fadD mutations with decreased virulence, but did not confirm or elucidate its enzymatic role in FA metabolism. We have previously shown that P. aeruginosa expresses fadD1 and fadD2 (PA3299 and PA3300) during lung infections in CF patients, suggesting the importance of Fad in lipid nutrient acquisition in vivo [12]. However, the role of fadD on virulence and growth of the bacteria in vivo has not been characterized.

Here, we characterized the FadD1 and FadD2 (PA3299 and PA3300) and the respective genes with relevance to their biochemistry and the effect on P. aeruginosa pathophysiology. The results of genetic analyses and biochemical characterization provided insight into reasons why redundancies in fadD are beneficial to this pathogen. Interestingly, fadD mutants displayed alterations in swimming and swarming motility and the production of lipases, phospholipases, rhamnolipids, and proteases. The fadD mutants with reduced ability to grow on phosphatidylcholine as a sole carbon source showed decreased fitness in a mouse lung infection model. These results provide the initial characterization of P. aeruginosa fadD genes and suggest a pathophysiological link between Fad and virulence.

**Results**

**Comparison of two fadDs in P. aeruginosa**

Our previous work showed that two P. aeruginosa fadDs (fadD1 and fadD2) were expressed in vivo during lung infection in CF patients [12]. FadD1 (PA3299) and FadD2 (PA3300) are 72% similar (54% identical) and 72% similar (53% identical) to the E. coli FadD, respectively, while P. aeruginosa FadD1 and FadD2 are 76% similar (60% identical) to each other. In addition, fadD1 and fadD2 are adjacent genes, separated by 234-bp and a possible Rho-independent transcriptional terminator (Fig. 2C). Convincingly, the ATP/AMP signature and FA-binding motifs described for the E. coli FadD are highly conserved in both P. aeruginosa FadD1 and FadD2 (Fig. 1C). This preliminary analysis suggests that fadD1 and fadD2 are both involved in Fad.

To confirm that fadD1 and fadD2 are important for Fad, we complemented the E. coli fadD1/fadR strain (E2011) with these P. aeruginosa genes. This E. coli strain contains a mutation in the fadR gene (fadR), the main repressor of the fad-regulon in E. coli, allowing the constitutive expression of other fad-genes of the aerobic Fad-pathway [24–27]. Both fadD1 and fadD2 of P. aeruginosa were able to functionally complement the E. coli fadD mutant (Table 1). The E. coli K12 wildtype strain was able to metabolize C12:0 to C18:1 Δ9 as expected, because long-chain acyl-CoA (≥C12:0) binds efficiently to FadR to induce the fad-regulon. Complementation of strain E2011 with the E. coli fadD1 gene (fadD1E) on plasmid pET15b resulted in growth similar to that of E. coli K12 on C12:0 to C18:1 Δ9, as well as on C16:0 because of the deregulated fad-regulon as previously observed [44]. Both P. aeruginosa fadDs were able to complement the E. coli fadD1/fadR strain to a similar level as the fadD1E complement (Table 1), suggesting that both FadD1 and FadD2 could activate LCFA and MCFAs. The various complements did not grow on short-chain FAs (SCFAs), suggesting that other E. coli Fad enzymes may not support growth on SCFAs [45] and not necessarily that the Pseudomonas FadD proteins are incapable of producing short-chain fatty acyl-CoAs. The E2011 control strains, either with or without the pET15b empty vector, showed no growth on any FA (Table 1).

**fadD2 and fadD1 exist on two transcripts and are induced by FA of different lengths**

To gain information on the regulatory regions of the P. aeruginosa fadDs, we mapped their transcriptional start sites to assign putative promoter sequences, and then determined transcription levels of each gene on various carbon sources (Figs. 2 and 3). Promoter mapping experiments indicated that each fadD had an independent transcriptional start site, suggesting that they were independently transcribed; however, northern blot analyses indicated that fadD2 and fadD1 can be co-transcribed on a single larger transcript or as smaller independent transcripts (Fig. 3A and 3B). Both fadD2 and fadD1 can exist as two different transcripts, suggesting some level of regulation by the predicted transcriptional terminator or attenuator sequence within the intergenic region (Fig. 2C). From

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**fadD1** and **fadD2** exist on two transcripts and are induced by FA of different lengths.
Figure 2. SMART mapping of the transcriptional start sites for fadD2 and fadD1. (A) One SMART product was observed after PCR amplification of the cDNA with SMART and fadD2 primers (oligonucleotides #798 and #373). Sequencing of the single band with a nested fadD2 primer (oligonucleotide #374) displayed a reverse-complement sequence chromatogram, showing the fadD2 transcriptional start site (indicated by +1 at the CTTCG sequence) and the underlined SMART primer sequence. (B) Likewise, the downstream fadD1 transcriptional start site was mapped (at the G of the sequence GCCTA) by sequencing a single PCR product. (C) fadD2 and fadD1 coding sequences and the predicted promoter regions are indicated (boxed). The intergenic region between fadD2 and fadD1 contains a potential transcriptional terminator or attenuator sequence (inverted arrows). For each gene, three black arrows indicate primers 1, 2 and 3 (#372/#375, #373/376, and #374/377) used for mapping fadD2 and fadD1. Dashed lines indicate missing protein sequences and dots indicate stop codons.

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Table 1. Complementation of the E. coli fadD mutant with P. aeruginosa fadD homologues.

| Strain | Plasmid       | C4:0 | C6:0 | C8:0 | C10:0 | C12:0 | C14:0 | C16:0 | C18:0 | C18:1 | CAA |
|--------|---------------|------|------|------|-------|-------|-------|-------|-------|-------|-----|
| K12    | none          | -    | -    | -    | -     | +4    | +5    | +4    | +5    | +6    |     |
| E2011  | fadD2-fadR    |      |      |      |       |       |       |       |       |       |     |
| E2011  | fadD2-fadR    |      |      |      |       |       |       |       |       |       |     |
| E2011  | fadD2-fadR    |      |      |      |       |       |       |       |       |       |     |
| E2011  | fadD2-fadR    |      |      |      |       |       |       |       |       |       |     |
| E2011  | fadD2-fadD1   |      |      |      |       |       |       |       |       |       |     |
| E2011  | fadD2-fadD2   |      |      |      |       |       |       |       |       |       |     |

(-) denotes no growth on a patch; (+) denotes growth; (+1) is very little growth and (+6) is heavy growth after 3 days.

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Figure 3. Transcriptional profile of fadD1 and fadD2 in various FAs. For a short-(C₈:0), medium-(C₁₀:0), and long-chain FA (C₁₈:1Δ⁹), northern blot analysis indicated two possible transcripts for both fadD genes when probed with either fadD1 (A) or fadD2 (B). Gene-fusion studies of strains P518 (PfadD1-lacZ) and P520 (PfadD2-lacZ), grown to mid-log phase, showed differential expression of fadD1 and fadD2 in the presence of different FAs (C and D). (C) fadD1 was up-regulated in the presence of the unsaturated LCFA (C₁₈:1Δ⁹), while fadD2 expression was significantly increased in the presence of shorter chain FAs (C₈:0, C₁₀:0) (D). For (C) and (D), all cultures had identical growth-rates and overall cell densities (data not shown). doi:10.1371/journal.pone.0013557.g003

fadD mutants showed reduced ability to grow on various FAs

To further confirm the involvement of each fadD in Fad, we generated single and double mutants for growth analysis on various FAs as sole carbon sources (Fig. 4). As previously observed for fadA mutants [30], growth defects were exemplified by slower growth and lower overall final cell densities in various FA media, presumably, due to reduced rates of Fad and growth inhibiting intermediates. Both fadD1 and fadD2 single mutants had various levels of defects when grown on all FAs (Fig. 4). However, the fadD1 mutant displayed a greater growth defect on all FAs than the single fadD2 mutant, with the exception of C₈:0 and C₁₀:0 where fadD2 seems to be equally as important as fadD1 (Fig. 4D and 4E). The fadD1ΔfadD2 mutant showed more dramatic growth defects on all FAs than the individual single mutants, indicating that both proteins were involved in the metabolism of all chain-length FAs tested. The lack of a complete defect in Fad of this double mutant suggests that other fadDs exist in P. aeruginosa. The complemented single and double mutants fully restored growth on all FAs (Fig. 4), while empty vector miniCTX2 controls did not complement growth on the FAs (data not shown). No apparent defects were observed for any mutant grown with casamino acids (CAA) as a sole carbon source (Fig. 4A). Based on the physiological data (Fig. 3 and 4), fadD1 was found to be important for the metabolism of all FAs, particularly the unsaturated LCFA oleate, while fadD2 was also important in Fad but more so for MCFA (C₉:0 and C₁₀:0) degradation.
Kinetic properties of purified FadDs support differential FA chain-length preferences

To determine the substrate specificities of each FadD and further clarify the reason why *P. aeruginosa* possesses multiple *fadD* homologues, both FadD proteins were purified to near homogeneity from an *E. coli fadD*- strain to ensure that all acyl-CoA synthetase activities were derived only from purified recombinant FadD1 or FadD2 (Fig. 5A). FadD1 of *P. aeruginosa* coupled CoASH to LCFA better than to SCFA or MCFA, as exemplified by larger $V_{\text{max}}$ and lower $K_m$ values for C18:1 D9 and C16:0 than FAs of other chain-lengths (Fig. 5B and Table 2). The reverse was true for FadD2, where this enzyme had higher $V_{\text{max}}$ and lower $K_m$ for SCFA and MCFA than LCFA (Fig. 5C and Table 2). The catalytic efficiency ($k_{cat}/K_m$) of FadD1 was significantly higher for LCFA (C18:1 D9, C16:0, and C14:0) than MCFA (C12:0 to C8:0) or SCFA (C6:0 and C4:0), while the catalytic efficiency of FadD2 was higher for MCFA and SCFA than LCFA (Table 2). The kinetic parameters for ATP and catalytic efficiency of both enzymes were comparable when ATP was limited in the reaction, with FadD1 being a slightly better catalytic enzyme for ATP than FadD2 (Table 2). Clearly, multiple FadDs in *P. aeruginosa*, with broad substrate conversion capabilities and overlapping chain-length preferences, afford this bacterium the ability to optimally metabolize FAs of various chain-lengths.

*fadD* mutants influence virulence behavior of *P. aeruginosa*

Based on work in other bacteria that showed an interconnection between *fadD* genes and expression of virulence factors [37–43], we sought to determine if a similar connection existed in *P. aeruginosa*. Increased swarming motility of *S. meliloti*, leading to altered virulence, was previously attributed to hyperflagellation observed by transmission electron microscopy (TEM) [43]. While no apparent differences in structure or numbers of flagella were observed for the *fadD* mutants compared to wildtype PAO1 using TEM in the current study (data not shown), we showed that *fadD* mutations could still significantly influence swarming and swimming motility in *P. aeruginosa* (Fig. 6). The *fadD2* mutant was severely defective in swimming and swarming motility relative to the wildtype PAO1 strain (Fig. 6). Although the *fadD1* single mutant showed no apparent difference in swimming motility, it displayed increased swimming migration compared to PAO1. Swarming was most pronounced in the ΔfadD2D1 mutant. In the ΔfadD2D1 mutant, it was very interesting to observe that the *fadD1*...
mutation suppressed the swarming and swimming defects of the fadD2 mutation. Each complemented strain showed that swimming and swarming motility could be restored to wildtype levels, indicating no unforeseen secondary or polar mutations affected these behaviors.

The production of other virulence factors was also monitored for the fadD mutants. Interestingly, the fadD2 mutant showed significantly decreased production of total hemolysins, proteases, lipases, and rhamnolipids (Fig. 7). No other mutant or complement showed noticeable decreases in the production of these virulence factors compared to the wildtype PAO1 strain. The suppression of the fadD2 mutation by the fadD1 mutation, in the ΔfadD2ΔD mutant, reversed the reduction in virulence factor expression seen in the single fadD1 mutation alone (Fig. 7). Similar suppression was observed in swimming and swarming motilities (Fig. 6). The altered virulence behaviors and suppression were not due to differences in growth-rates or overall final cell densities, as all seven strains (i.e. wildtype, mutants, and complements) grew identically in LB media prior to testing for these virulence traits (Fig. 7E). Mechanisms governing these differences remain to be elucidated.

Compromised ability of fadD mutants to degrade FA and PC leads to reduced P. aeruginosa fitness in mice

Since fadD1 and fadD2 are expressed in vivo during CF lung infections [12] and are potentially important for PC degradation (Fig. 1A), it was necessary to determine whether these mutants are deficient in growth on PC. Growth analysis on PC showed only slight decreases in the maximum cell density of the individual single fadD mutants, while the fadD2 mutant exhibited a delayed log phase (Fig. 8A). The ΔfadD2ΔD mutant exhibited the greatest growth defect, while the single and double fadD complements restored growth to wildtype levels. Since PC is the major component (70%) of the essential lung surfactant [46] and is a potential nutrient source in vivo [12], it was important to assess whether the growth defects of these mutants on PC would result in decreased fitness in vivo.

Table 2. Kinetic properties of FadD1 and FadD2 with various substrates.

| Substrate varied | FadD1 Kinetic Parameter | FadD2 Kinetic Parameter |
|------------------|-------------------------|-------------------------|
|                  | Vmax \(^b\)             | kcat \(^c\)             | Km \(^d\)             | kcat/Km \(^e\)         | Vmax \(^b\)             | kcat \(^c\)             | Km \(^d\)             | kcat/Km \(^e\)         |
| ATP              | 213                     | 0.219                   | 10.6                  | 20.7                     | 182                     | 0.187                   | 10.9                  | 17.2                     |
| C4:0             | 137                     | 0.141                   | 27.4                  | 5.1                      | 167                     | 0.172                   | 33.3                  | 5.2                      |
| C6:0             | 133                     | 0.137                   | 26.7                  | 5.1                      | 159                     | 0.164                   | 31.8                  | 5.2                      |
| C8:0             | 125                     | 0.128                   | 25.0                  | 5.1                      | 204                     | 0.210                   | 20.4                  | 10.3                     |
| C10:0            | 116                     | 0.119                   | 23.3                  | 5.1                      | 182                     | 0.187                   | 36.4                  | 5.1                      |
| C12:0            | 130                     | 0.134                   | 26.0                  | 5.1                      | 137                     | 0.141                   | 41.1                  | 3.4                      |
| C14:0            | 130                     | 0.134                   | 13.0                  | 10.3                     | 109                     | 0.112                   | 43.5                  | 2.6                      |
| C16:0            | 154                     | 0.158                   | 15.4                  | 10.3                     | 99                      | 0.102                   | 49.5                  | 2.1                      |
| C18:1 \(^a\)    | 217                     | 0.223                   | 21.7                  | 10.3                     | 101                     | 0.104                   | 50.5                  | 2.1                      |

\(^a\)Kinetic constants (Vmax and Km) determined using Hanes-Woolf plot.
\(^b\)nmol of acyl-CoA formed/min/mg of protein.
\(^c\)s\(^-1\); determined using MW of FadD1 (61,655) and FadD2 (61,373).
\(^d\)mM of ATP or FA.
\(^e\)mM\(^{-1}\) s\(^{-1}\); represents enzyme catalytic efficiency.
In vitro competition between the various \( fadD \) mutants and their complements were first tested to determine if the defect reduced their ability to utilize various sole carbon sources. Each mutant and complement pair was inoculated into seven growth media with different sole carbon sources including LB, CAA, glucose, PC, \( \text{C}_{18:1} \) \text{D} \text{9}, choline, and glycerol, and bacterial CFU were determined after 24–48 h growth (Fig. 8B). As expected, all three single or double \( fadD \) mutants were less competitive than their corresponding complements but only in media containing PC or \( \text{C}_{18:1} \) \text{D} \text{9} as sole carbon sources. Next, to evaluate the fitness of the \( fadD \) mutants within the lung environment, in vivo competition between the mutants and their complements was analyzed. Following intratracheal inoculation of equal ratios of each mutant and its complement pair into BALB/c mice (6\( \times \)10\(^6\) CFU/animal), bacterial CFU recovered from the lungs were determined 24 h and 48 h postinfection and the competitive index (CI) was calculated (Fig. 8C and 8D). The CI is defined as the ratio of mutant CFU relative to CFU of the respective complement [47]. In all of these competition experiments, with the exception of the \( fadD2 \) mutant after 48 h, the average total CFU/mouse recovered was greater than the initial inoculum showing that these strains maintained the ability to replicate within the mouse lung. Although the \( fadD1 \) single mutant showed decreased competitive fitness within the lung compared to the complement, the CI of the \( fadD1 \) mutant remained relatively unchanged between the two time points analyzed. At 24 h postinfection, all of the mutants exhibited decreased competition levels relative to their respective complements and the \( fadD2 \) and \( \Delta fadD2D1 \) mutant strains showed greater reduced fitness than the \( fadD1 \) mutant. By allowing the infections to persist for 48 h, the reduced CI of the \( fadD2 \) mutant showed a significantly higher defect in competitive fitness. By 48 h, the CI of the \( \Delta fadD2D1 \) mutant was half of that observed at 24 h. Clearly, the \( \Delta fadD2D1 \) mutant with significantly reduced ability to degrade PC (Fig. 8A), while showing no altered virulence factor secretion (Fig. 7), had its competitive fitness reduced by three-fold. This strongly suggests that the ability to degrade PC as a nutrient source allows \( P. \text{aeruginosa} \) to replicate within the lung environment.

Figure 6. Altered swimming and swarming motility of \( P. \text{aeruginosa} fadD \) mutants. (A) Swimming motility of \( fadD \) mutants and their complements. (B) Swarming migration of \( fadD \) mutants and their complements. These figures are representative of multiple experiments. Strain designation is the same as shown in Table 3: wildtype PAO1, P007; \( fadD1 \), P175; \( fadD2 \), P547; \( \Delta fadD2 \), P177; \( fadD1 \) complement, P541; \( fadD2 \) complement, P549; and \( \Delta fadD2 \) complement, P543.

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Discussion

This study focused on characterizing two *P. aeruginosa* acyl-CoA synthetases (FadD1 and FadD2), which are expressed during lung infection in CF patients suggesting their importance in lipid degradation for bacterial replication [12]. The transcriptional profile and substrate preferences of FadD1 and FadD2 were determined to initially shed light onto why *P. aeruginosa* has this genetic redundancy. The fadD1 and fadD2 of *P. aeruginosa* were differentially regulated in response to the type of available FA (Fig. 3), suggesting that each FadD has a different FA substrate preference. While both fadD1 and fadD2 were controlled by their common and independent promoters, the expression of fadD1 downstream could be partially attenuated by some FAs due to a putative intergenic Rho-independent transcriptional-terminator (Fig. 2). Based on the growth defects, both fadD genes are individually important for the degradation of all chain-length FAs tested. These results are further supported by the observation that the ΔfadD2Δ1 mutant had the greatest growth defect on all chain-length FAs (Fig. 4). These growth defects may be due to a bottleneck in the conversion of exogenous FA to acyl-CoA created by the inactivation of two genes that facilitate this process. The complementation study in *E. coli* was initially inconclusive with respect to metabolism of SCFA and some MCFAs by *P. aeruginosa* FadDs (Table 1), as other *E. coli* Fad-proteins (e.g. FadE, FadA and FadB) do not allow the metabolism of shorter-chain FAs [45]. However, the kinetic parameters, especially the catalytic efficiencies (Table 2), provided more precise biochemical evidence for the differences in substrate preferences. By comparing the kinetic measurements of these enzymes, it appears that FadD1 preferred LCFA for degradation, while FadD2 was more suited to the degradation of SCFAs and MCFAs (Fig. 5 and Table 2). Together, the enzyme kinetics, gene-fusion, and growth analyses data all support the importance of FadD2 for degradation of SCFA and MCFA and FadD1 is more suited for LCFA degradation for bacterial replication [12]. The transcriptional profile and substrate preferences of FadD1 and FadD2 were determined to initially shed light onto why *P. aeruginosa* has this genetic redundancy. The fadD1 and fadD2 of *P. aeruginosa* were differentially regulated in response to the type of available FA (Fig. 3), suggesting that each FadD has a different FA substrate preference. While both fadD1 and fadD2 were controlled by their common and independent promoters, the expression of fadD1 downstream could be partially attenuated by some FAs due to a putative intergenic Rho-independent transcriptional-terminator (Fig. 2). Based on the growth defects, both fadD genes are individually important for the degradation of all chain-length FAs tested. These results are further supported by the observation that the ΔfadD2Δ1 mutant had the greatest growth defect on all chain-length FAs (Fig. 4). These growth defects may be due to a bottleneck in the conversion of exogenous FA to acyl-CoA created by the inactivation of two genes that facilitate this process. The complementation study in *E. coli* was initially inconclusive with respect to metabolism of SCFA and some MCFAs by *P. aeruginosa* FadDs (Table 1), as other *E. coli* Fad-proteins (e.g. FadE, FadA and FadB) do not allow the metabolism of shorter-chain FAs [45]. However, the kinetic parameters, especially the catalytic efficiencies (Table 2), provided more precise biochemical evidence for the differences in substrate preferences. By comparing the kinetic measurements of these enzymes, it appears that FadD1 preferred LCFA for degradation, while FadD2 was more suited to the degradation of SCFAs and MCFAs (Fig. 5 and Table 2). Together, the enzyme kinetics, gene-fusion, and growth analyses data all support the importance of FadD2 for degradation of SCFA and MCFA and FadD1 is more suited for LCFA degradation (Fig. 3, 4 and 5). Therefore, we can conclude that these proteins have different substrate preferences and are not functionally equivalent.

We showed here that mutations in fadD genes, important for FA β-oxidation in *P. aeruginosa*, also influenced two modes of motility and virulence factor expression. Our fadD2 mutant had reduced swimming and swarming motilities and decreased virulence factor expression (proteases, lipases, rhamnolipids, and lipopolysaccharides), while the fadD1 mutant and the ΔfadD2Δ1 double mutant only showed an increased swimming phenotype. In the double mutant background, the presence of the fadD1 mutation suppresses the phenotype of the fadD2 mutation (Figs. 6 and 7). It will be interesting to determine the exact cause of the phenotypic suppression in future investigations. At this point, we speculate that the reduced swimming phenotype of the fadD2 mutant is due to decreased production of rhamnolipids (Fig. 6 and 7), as rhamnolipids were previously shown to be necessary for *P. aeruginosa* swarming motility [48]. These phenotypic differences in virulence factor expression further support the observation that FadD1 and FadD2 are not functionally equivalent. Although we did not exhaust the large list of virulence determinants, nor were able to show the exact method by which FadD2 influences their expression, this characterization of several virulence factors links Fad and virulence factor expression in *P. aeruginosa*.

Additionally, the expression of genes in vivo that encode proteins with β-oxidative activity, along with several other PC degradation genes, strongly support the hypothesis that lipids within the lung may be important nutrient sources for *P. aeruginosa* [12] and serve as signals to control virulence factor expression [13]. Phospholipase- and lipase-derived components of PC (LCFA, glycerol, and phospholylcholine) could individually serve as sole carbon sources (Fig. 1A) and provide nitrogen and phosphorous contributing to virulence [49,50]. Of these three PC components, the two LCFAs from each PC molecule yield the most carbon and energy. Therefore, the determination that both FadD1 and FadD2 were important for LCFA degradation was pivotal as PC, the major component of lung surfactant, is primarily composed of LCFA (C16:0, 50–60%; C18:0, C16:1, C18:1, and C18:2 each at 10–20%) [46]. To that end, we analyzed the growth of these mutants on PC as a sole carbon source. The delayed log-phase of the fadD2 double mutant is likely due to the decreased expression of lipase and phospholipase, thereby reducing the cleavage rate of exogenous PC into its usable components and thus slowing growth. Since it was shown that the ΔfadD1Δ2 mutant had no apparent deficiencies in lipase or phospholipase expression, yet exhibited the greatest decrease in growth on all FAs tested, we believe that its reduced growth on PC is attributed to a reduced ability to degrade FAs, as this double mutant degrades phospholylcholine and glycerol as well as the complement. Since the fadD mutants fully retained the ability to degrade choline and glycerol and only had reduced levels of FA degradation, it was not surprising that the ΔfadD1Δ2 double mutant could still degrade PC.

Because PC is a major lung surfactant common in all mammals, including mice [51], a mouse lung infection model [52] was utilized to analyze the competitive growth in vivo between the *P. aeruginosa* fadD mutants and their complements. Replication of the fadD2 mutant was observed after 24 h, while all other strains showed an increase after 48 h, indicating all strains were able to replicate in vivo. This could be explained by the fact that our ΔfadD1Δ2 double mutant still had significant ability to degrade PC and its components, and that *P. aeruginosa* expresses genes in the lung for both amino acid and PC degradation [12] and possibly DNA [53]. After 24 h and 48 h, all mutants showed lower in vivo fitness than their complements, which means that lipids are significant nutrient sources in the mouse lung. The CIs of the fadD2 mutant were consistently lower than those of the fadD1 mutant after both 24 h and 48 h, attributed to the fadD2 mutant's decreased production of virulence factors. Although the ΔfadD2Δ1 mutant only showed partial defect in PC degradation and no effect in virulence factor expression or the ability to grow with amino acids, this partial defect in PC degradation translated into significantly reduced in vivo lung fitness. This is further supported by the in vitro competition results where all single and double fadD mutants exhibited competition defect only on PC and C16:1. Therefore, the significantly lowered CI after 48 h for the double ΔfadD1Δ2 mutant can only be due to a reduced ability to degrade PC as a nutrient source for replication in the mouse lung.

In this study, a pathophysiological link between the acquisition of lipid nutrients and virulence in vivo was established by i) characterizing these two *P. aeruginosa* fadD genes expressed during lung infection in CF patients, ii) determining that there may be some connection between fadD-genes and the expression of certain virulence traits, and iii) showing that mutations in these genes
correspond to a deficiency in the ability to replicate in mouse lungs. These data support results from a previous in vivo gene expression study showing that _P. aeruginosa_ expresses _gfp_, _bet_, and especially, _fad_-genes to degrade PC as one of the nutrient sources in the lungs [12]. We predict that _P. aeruginosa_ mutants, completely blocked in PC utilization, will have significantly lower fitness, further supporting PC as a significant nutrient source for this important pathogen in mammalian lungs.

Materials and Methods

Ethics statement

All animal experiments were conducted in compliance with the NIH (National Institutes of Health) Guide for the Care and Use of Laboratory Animals and were approved by the University of Hawaii Institutional Animal Care and Use Committee (protocol No. 06-023-04).

Bacterial strains and growth media

Strains and plasmids used in this study are shown in Tables 3 and 4. _E. coli_ EPMMax10B was routinely used as a strain for cloning and was cultured in Luria-Bertani (LB) medium (Difco). _P. aeruginosa_ strain PAO1 and derivatives were cultured on _Pseudomonas_ Isolation Agar or Broth (PIA or PIB; Difco) or LB medium. _P. aeruginosa_ fadD1::FRT, _P. aeruginosa_ fadD2::FRT, _P. aeruginosa_ fadD1::::FRT, and their complements were cultured in 1x M9 minimal medium +1% Brij-58 (Sigma) +1% casamino acids (CAA) or 0.2% (w/v) of the individual FA, C14:0 to C16:0, or C18:1 Δ9 (Sigma; Fig. 4), and 1x M9 minimal medium +1% Brij-58 +0.2% phosphatidylcholine (PC, Sigma; Fig. 8A) for growth analyses. The fusion strains _P. aeruginosa_ fadD1::::FRT-lacZ/attB::miniCTX2-fadD1 and _P. aeruginosa_ fadD2::::FRT-lacZ/attB::miniCTX2-fadD1 were cultured in 1x M9 +1% Brij-58 +1% CAA±0.1% (w/v) C16:0 or C16:1, or C18:1 Δ9 for induction studies (Fig. 3C and 3D). For _in vivo_ competition studies (Fig. 3B), each mutant/complement mixture of equal cell density was grown in seven different media, including LB, 1x M9 +20 mM glucose, 1x M9 +1% CAA, 1x M9 +1% Brij-58 +0.2% PC, 1x M9 +1% Brij-58 +0.2% C18:1 Δ9, 1x M9 +40 mM glycerol, and 1x M9 +30 mM choline. Stock solutions of all FAs were made as previously described [30]. Unless indicated otherwise, all liquid cultures were grown at 37°C with a shaking speed of 200 r.p.m.

General molecular methods

All molecular methods and their components were employed as previously described [34]. Oligonucleotides utilized in this study were ordered through Integrated DNA Technology (IDT, Table 5).

Complementation of _E. coli_ fadD mutant

The _E. coli_ fadD fadD::Km mutant (E2011) was engineered by transferring the fadD::Km mutation from JWC285 into the K27 (fadD) strain via Pl transduction. The resulting double mutant strain was then used for the complementation study. To construct the _E. coli_ complementation vectors, coding regions of _P. aeruginosa_ fadD1 and fadD2 were amplified from PAO1 chromosomal DNA using oligos #341 ++#342 and #339 + #340, respectively. PCR products were then digested with NdeI + BamHI and ligated individually into PET15b, digested with the same enzymes, yielding PET15b-fadD1 and PET15b-fadD2. As a control for the complementation study, the _E. coli_ fadD gene (fadD_Ec) was also amplified from strain K12 chromosomal DNA using oligos #1092 + #1093. The 1.8-kb PCR product was digested with AseI + BamHI, and ligated with PET15b digested with NdeI + BamHI, yielding PET15b-fadD_Ec. These three vectors, PET15b-fadD1, PET15b-fadD2, and PET15b-fadD_Ec, were introduced into _E. coli_ strain E2011 for complementation. E2011, harboring each complementation vector, was patched on 1x M9 minimal medium +1% Brij-58 +1% CAA or 0.2% various fatty acids, and growth was determined after 3 days incubation at 37°C (Table 1).

Construction of PAO1 fadD mutant and complementation strains

Three fadD mutant strains (PAO1-fadD1::FRT, PAO1-fadD2::FRT, and PAO1-fadD1::::FRT) were engineered, respectively, using three allelic-replacement plasmids (pEX18T-fadD1::Gm-FRT, pEX18T-fadD2::Gm-FRT, and pEX18T-fadD1::::Gm-FRT) as previously described [55]. These gene-replacement vectors were constructed by inserting the SmaI Gm’-FRT cassette at the EcoRV site to inactivate fadD1 gene, the SmaI site to inactivate fadD2 gene, or at the deleted fadD2D1 SmaI-EcoRV locus. These PAO1 fadD mutants were confirmed by PCR (data not shown).

These newly engineered mutant strains, PAO1-fadD1::FRT, PAO1-fadD2::FRT, and PAO1-fadD1::::FRT, were complemented using the relevant gene(s) on the miniCTX2 single copy integration vector as described previously [56]. The resulting strains, PAO1-fadD1::FRT/attB::miniCTX2-fadD1, PAO1-fadD2::FRT/attB::miniCTX2-fadD2, and PAO1-fadD1::::FRT/attB::miniCTX2-fadD1, were used in the growth curve experiments (Fig. 4 and 8A). Controls were also performed with the empty miniCTX2 integrated into each mutant strain.

Growth characterization of fadD mutant and complemented strains

The fadD mutants, their corresponding complemented strains, and the PAO1 wildtype strain were initially grown overnight in PIB medium. The overnight cultures were centrifuged and the cell pellets were washed twice with 1x M9 minimal media and resuspended with equal volumes of the same 1x M9 media. The cell resuspensions were then diluted 100-fold in 1x M9 +1% Brij-58 +1% CAA or 0.2% of the individual FAs (C16:0 to C16:1, or C18:1 Δ9; Fig. 4) or 0.2% PC (Fig. 8A), and growth was then initiated. At each time point, aliquots of individual cultures were diluted 4-fold in 4% Brij-58 (pre-incubated at 42°C) to clarify any insoluble FA and OD540 measurements were taken.

Figure 8. Growth analysis on phosphatidylcholine and competition studies. (A) The _A. fadD2D1_ mutant exhibited a growth defect when grown on PC as a sole carbon source, while the _fadD2_ mutant had a delayed log phase compared to the wildtype PAO1 strain. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wildtype PAO1 strain. (B) _In vitro_ competition studies of the _fadD_ mutants and their complemented strains in different growth media (n = the number of independent _in vitro_ competition experiments performed with each carbon source). _In vivo_ lung competition of the various _fadD_ mutants and their complemented strains after 24 h (C) and 48 h (D). n equals the number of mice in each group that were inoculated with a total of 6 x 10⁶ CFU/mouse. The solid red line indicates the geometric mean of the competitive indices (CI) in each competition group. CI < 1 indicates the _fadD_ mutant was less competitive than its complemented strain in various growth media (B) or within the lungs (C and D) (* P < 0.05 based on one sample _t_ test) [47]. Numbers above the red line represent the average total recovered CFU/mouse for each competition group.

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### Table 3. Bacterial strains used in this study*.

| Strains           | Lab ID* | Relevant properties                                                                 | Reference |
|-------------------|---------|-------------------------------------------------------------------------------------|-----------|
| **E. coli**       |         |                                                                                     |           |
| EPMax108          | E1231   | F: λ; recA Δ(mrr-hsdR51-mcrBC) pRS415 ΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL nupG | BioRad    |
| K12               | E0577   | Prototroph                                                                          | ATCC #2374|
| K27               | E0410   | fadD' (oldD88) mutant                                                                | [16]      |
| K27-T7            | E1063   | Gm'; K27 with T7 expression system                                                  | Lab strain|
| JW285             | E1560   | Km'; fadD::T7(ara,leu)7697::Kmr                                                     | [44]      |
| K27-fadD1         | E2011   | Km'; fadD'::lacZ (transduced from strain JWC285 P1-lysate into strain K27)         | This study |
| **P. aeruginosa** |         |                                                                                     |           |
| PAO1              | P007    | prototroph                                                                          | [68]      |
| PAO1-fadD1::FRT   | P175    | PAO1 with fadD1 insertion mutation                                                 | This study |
| PAO1-fadD2::FRT   | P547    | PAO1 with fadD2 insertion mutation                                                  | This study |
| PAO1-ΔfadD2D1::FRT| P177    | PAO1 with fadD2 deletion mutation                                                   | This study |
| PAO1-fadD1::FRT/attB::miniCTX2-fadD1| P541| Tet'; PAO1-fadD1::FRT complemented with miniCTX2-fadD1 | This study |
| PAO1-fadD2::FRT/attB::miniCTX2-fadD2| P549| Tet'; PAO1-fadD2::FRT complemented with miniCTX2-fadD2 | This study |
| PAO1-ΔfadD2D1::FRT/attB::miniCTX2-fadD2| P543| Tet'; PAO1-ΔfadD2D1::FRT complemented with miniCTX2-fadD2 | This study |
| PAO1-fadD1::FRT-lacZ/attB::miniCTX2-fadD1| P518| Tet', Gm'; fadD1 complement strain with fadD1::FRT-lacZ fusion | This study |
| PAO1-fadD2::FRT-lacZ/attB::miniCTX2-fadD2| P520| Tet', Gm'; fadD2 complement strain with fadD2::FRT-lacZ fusion | This study |
| PAO1-fadD1::FRT/ mucA::pUC18 | P663 | Cb'; fadD1 mutant with pUC18 inserted in mucA gene                                | This study |
| PAO1-fadD2::FRT/ mucA::pUC18 | P667 | Cb'; fadD2 mutant with pUC18 inserted in mucA gene                                | This study |
| PAO1-ΔfadD2D1::FRT/ mucA::pUC18 | P665 | Cb'; fadD2D1 mutant with pUC18 inserted in mucA gene                              | This study |
| PAO1-fadD1::FRT/attB::miniCTX2-fadD1/ mucA::pUC18 | P657 | Cb'; Tet', fadD1 mutant with pUC18 inserted in mucA gene                           | This study |
| PAO1-fadD2::FRT/attB::miniCTX2-fadD2/ mucA::pUC18 | P659 | Cb'; Tet', fadD2 mutant with pUC18 inserted in mucA gene                          | This study |
| PAO1-ΔfadD2D1::FRT/attB::miniCTX2-fadD2/ mucA::pUC18 | P661 | Cb'; Tet', fadD2D1 mutant with pUC18 inserted in mucA gene                        | This study |

*For strains constructed in this study, please see text for further details.

Please use lab ID for requesting strains.

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**Construction of fadD1-lacZ and fadD2-lacZ fusion strains and induction by FAs**

To take advantage of the native fadD1-promoter and create a transcriptional fusion of Pseudomonas FadD and Virulence

The resulting fusion strain, PAO1-fadD1::FRT-lacZ was PCR confirmed using oligos #341 + #713, which are specific for the fadD1 and lacZ genes, respectively. Similarly, PAO1-fadD2::FRT-lacZ was constructed and PCR confirmed using oligos #377 + #501 (data not shown). Complementation vectors miniCTX2-fadD1 and miniCTX2-fadD2 were then integrated into these newly developed lacZ fusion strains, to yield two complemented fusion strains, PAO1-fadD1::FRT-lacZ/attB::miniCTX2-fadD1 and PAO1-fadD2::FRT-lacZ/attB::miniCTX2-fadD2, respectively.

β-Galactosidase activities were measured for these two complemented fusion strains under various growth conditions. Cells were first grown overnight in PIB medium, washed twice with one volume of 1x M9, and resuspended in an equal volume of the same medium. Cell resuspensions were then diluted 100-fold into fresh 1x M9 + 1% Brij-58 + 1% CAA + 0.1% of the individual FAs (C4:0 to C16:0, or C18:1 Δ9, and growth curve experiments were performed. Cell cultures were taken at mid-log phase (OD600)}
~2.0) and β-galactosidase assays were performed in triplicate and Miller Units (mean ± s.e.m.) were determined [57] (Fig. 3).

**FadD1 and FadD2 purification**

Histidine-tagged FadD1 and FadD2 were expressed on the pET15b vector and purified using a Ni²⁺-NTA column (Qiagen, Valencia, CA) as described elsewhere [58]. The *E. coli* K27-T7 (fadD) strain was used for protein expression to prevent any FadD contamination in protein preparations (Fig. 5A).

**Measurement of fatty acyl-CoA synthetase (FadD1 and FadD2) activity**

Fatty acyl-CoA synthetase activity was monitored using Ellman’s reagent, as previously described in several studies, to detect the amount of free thiol (i.e., CoASH used in the reaction) [23,59–61]. Reactions (450 µL total) were prepared with 20 µg of purified FadD1 (or FadD2) in a reaction buffer containing final concentrations of 150 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 2 mM EDTA, 0.1% Triton X-100, 5 mM ATP, 0.5 mM coenzyme A (CoASH), and an individual FA (30 to 300 µM) in thin-walled glass tubes. Briefly, to perform the reaction, each mixture was assembled containing all components above (excluding CoASH) and the 450 µL mixture was pre-incubated at 37°C for 3 min, quickly mixed, and incubated at 37°C during the course of the reaction. Immediately after mixing, a time zero point was taken by removing 75 µl from the 450 µl reaction mix and adding it to 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 A₄₁₂ was measured. Subsequent 75 µl aliquots of the reaction were taken at 20-sec intervals and mixed with DTNB under these conditions. Reactions with FadD enzymes were performed exactly as above to show no change in absorbance at 412 nm and verify the stability of CoASH and DTNB under these conditions. Reactions with FadD were repeated to obtain triplicate data for each FA at each concentration. For each FA substrate, decreases in A₄₁₂ values for the reaction were taken at 20-sec intervals and mixed with DTNB for additional measurements. Additionally, control experiments without FadD enzymes were performed exactly as above to show no change in absorbance at 412 nm and verify the stability of CoASH and DTNB under these conditions. Reactions with FadD were repeated to obtain triplicate data for each FA at each concentration. For each FA substrate, decreases in A₄₁₂ values for the reaction were taken at 20-sec intervals and mixed with DTNB for additional measurements. Additionally, control experiments without FadD enzymes were performed exactly as above to show no change in absorbance at 412 nm and verify the stability of CoASH and DTNB under these conditions.

**Motility assays**

Strains for swarming and swimming were grown overnight in LB medium. Cell pellets of 500 µl culture aliquots were washed twice with equivalent volumes of 1x M9 medium and resuspended...
Table 5. Primers used in this study.

| Number and Name | Sequence* |
|-----------------|-----------|
| **fadD cloning** |           |
| 302; fadD-up-Hind | 5′-ATCGAGAATCTCCGCGGTGTCGGCGCGAT-3′ |
| 303; fadD-down | 5′-TCTGAGAATCTCCGCGGTGTCGGCGCGAT-3′ |
| 339; fadD2-Ddel | 5′-CAGAACTATGAACACTGTTGAAACG-3′ |
| 340; fadD2-BamHI | 5′-CGGCCAAGATCCGGTACGAGCGTTGATG-3′ |
| 341; fadD1-Ddel | 5′-TGGGCATGACGAACTGTTGAAACG-3′ |
| 342; fadD1-BamHI | 5′-GGGCGGCGATGACGAGCGTTGATG-3′ |
| 501; Gm-up-reverse | 5′-CATAGCGACTTCCGATACAG-3′ |
| 713; lacZa | 5′-TTGGGGAAGGCGGATC-3′ |
| 1092; EcfadD-up-Axel | 5′-AAGGATATAAAAGGAAGGGTTGCTTAAC-3′ |
| 1093; EcfadD-down-BamHI | 5′-AACGCGGATCCGAGGGTTGATG-3′ |
| **Promoter mapping** |           |
| 372 – fadD2-race3b | 5′-GAGGCGCTGCAACCTGCGA-3′ |
| 373 – fadD2-race2 | 5′-GAGGCGCTGCAACCTGCGA-3′ |
| 374 – fadD2-race1 | 5′-TGCTCCAGAATTAGCGTTCG-3′ |
| 375 – fadD1-race3b | 5′-AGGCGATGTCGCTGGCTCGA-3′ |
| 376 – fadD1-race2 | 5′-CGGGATGTCGCTGGCTCGA-3′ |
| 377 – fadD1-race1 | 5′-GTATCTGTCTCTTCCATTACGT-3′ |
| 797 – SMART-IId | 5′-AAGCGGTGTTATCAGCGAGATCGGCCGGG-3′ |
| 798 – SMART-IIIb | 5′-AAGCGGTGTTATCAGCGAGATCGGCCGGG-3′ |
| **mucA cloning** |           |
| 937 – mucA-up | 5′-GAAGCGGATGTAACCTCGAG-3′ |
| 938 – mucA-down | 5′-AGTACGACGCGGCTAG-3′ |

*Restriction enzyme sites utilized in this study are underlined.

*Primers synthesized Rnase free and HPLC purified.

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in equal volumes of the same medium. Swarming motility was assayed by spotting 5 µl of the resuspended cultures onto BM2-glucose swarm agar plates, made as described previously [63]. Swimming motility was assayed by pin-stabbing 0.3% LB agar plates with the overnight liquid cultures grown in LB. All inoculated plates were allowed to dry at room temperature for 10 min, incubated at 37°C for 16 to 18 h, and motility zones were compared (Fig. 6).

Protease, phospholipase, lipase, and rhamnolipid detection

Strains were grown in LB medium and cultures were used for OD₆₀₀ measurements at various time points (Fig. 7E). For protease, phospholipase, and lipase assays, clarified supernatants were obtained at 24 h from 1 ml culture aliquots centrifuged (16,000 × g) for 2 min at 4°C and filtered through 0.2 Î¼m hydrophilic PVDF filters (Fisher Scientific). To quantify protease and phospholipase activities, 4-mm diameter holes were punched into 2% skim milk NB agar protease plates or blood agar phospholipase plates (PML Microbiological) and filled twice with 50 µl of each cell-free supernatant, respectively. Both the skim milk and blood agar plates were incubated at 37°C for 18 h before analyzing. Similarly, 50 µl of the same cell-free supernatants were applied five times into 4-mm holes in rhodamine B agar plates [64], and the plates were imaged using a UV transilluminator after incubation at 37°C for 3 days to visualize lipase activity. These plate-based assays were conducted in triplicate and the clearance zone diameters for skim milk and blood agar plates or the fluorescent halo diameters for the rhodamine B plates were measured and compared by percentage conversion relative to the wildtype PAO1 value and were expressed as an average ± s.e.m (Fig. 7A, 7B and 7C). Rhamnolipid production was assessed using a previously published methylene blue complexation assay [65]. All strains were grown for 24 h in LB medium and 1.5 ml of clarified supernatant for rhamnolipid extraction was obtained from each culture by room temperature centrifugation (16,000 × g). This assay was conducted in triplicate and average absorbance was compared by percentage conversion relative to the wildtype PAO1 value and was expressed as an average ± s.e.m (Fig. 7D).

Promoter mapping

The transcriptional start sites of the fadD1 and fadD2 genes were determined as previously described [50]. Briefly, PAO1 was grown in 1x M9 minimal media supplemented with 0.2% C₁₆₁₀ to mid-log phase. This FA was chosen prior to the gene induction studies and, in retrospect, it was as appropriate as any other FA to map the fadD promoters. Cells were harvested and total RNA was isolated to perform cDNA synthesis using a SMART-IIA primer (#797, Table 5) and the first gene-specific primer (#375 for fadD1 and #372 for fadD2, Table 5). The cDNA was subsequently used as the template in PCR, using oligos #798 + #376 and #798 + #373 for fadD1 and fadD2, respectively. Finally, the PCR product was sequenced using a second nested oligo #377 for fadD1, or #374 for fadD2 (Fig. 2).

Northern blot analysis

Wildtype strain, PAO1, was grown in 1x M9 minimal medium supplemented with 0.2% C₁₆₁₀, C₁₄₁₀, or C₁₈₁₀ as sole carbon sources. After reaching mid-log phase (OD₆₀₀=1.0), cells were harvested at 4°C and total RNA was isolated. Thirty µg of each RNA sample was used for northern analysis as described previously [66]. The fadD1 and fadD2 genes were PCR amplified from pET15b-fadD1 and pET15b-fadD2 using oligos #341 + #342, and #339 + #340, respectively, and used individually as probes (Fig. 3A and 3B).

In vitro and in vivo competition studies

Various fadD mutant strains (fadD1, fadD2 and fadD2D1 mutants) and their corresponding complemented strains were used for the in vitro and in vivo competition studies (Table 3). A mucA insertional mutation was introduced into all strains to overproduce alginate, as we used a mouse model to allow these mucoid strains to survive and replicate in the lung as described previously [52]. Briefly, a 450-bp internal region of the mucA gene was PCR amplified from PAO1 chromosomal DNA using oligos #973 and #974 and cloned into the PsvuI site of pUC18. The resultant vector pUC18-muca was electroporated into the various fadD1 mutant/complemented strains and the mucoid transformants were selected on PIA plates supplemented with 500 µg/ml carbencillin (Cb500). One mucoid colony of each mutant/compplemented strain was then inoculated separately in 3 ml of PIB + Cb500. After 24 h of incubation in a shaking incubator at 37°C, these cultures were diluted 100 times into 5 ml of fresh PIB + Cb500 and grown overnight. Three ml of each overnight culture was centrifuged (20,000 × g) for 10 min at 4°C and clarified supernatants were collected. The cell density of each culture was calculated by plating 10-fold serial dilutions on LB plates. Each culture was then adjusted to 2×10⁶ CFU/ml in its own clarified supernatant, obtained above. At this point, each diluted fadD mutant strain (fadD1, fadD2 and fadD2D1 mutants) and its
corresponding complemented strain were mixed at a 1:1 CFU ratio and the resulting mixtures (fadD1/complement, fadD2/complement, and fadD2D1/complement) were used for inoculation into various growth media (in vitro competition) or mouse lungs (in vivo competition). For in vitro competition, each mutant/complement mixture of equal cell density was diluted 100x into various growth media with LB, glucose, CAA, PC, C\(_{18.1}\), glycerol, or choline as sole carbon sources. All cultures were grown at 37°C with shaking for 1–2 days until the total cell densities reached 3–7×10\(^8\) CFU/ml. Bacteria were then quantified by plating dilutions onto LB plates with and without tetracycline to determine the total number of bacteria (growth with no tetracycline) and the number of complemented bacteria (growth with tetracycline). These numbers were used to determine the in vivo CI (CFU\(_{\text{mutant}}$/CFU\(_{\text{complement}}\) when grown in mouse lungs) [47]. A control condition was included using PAO1-mucA\(_{p}\)/pUC101/PAO1-mucA\(_{p}\)/pUC10-miniCTX2 to show that no competitive advantage or disadvantage was conferred by the presence of the Tet’ marker during in vivo growth (data not shown). Statistical analysis was performed using GraphPad Prism 5.0 software (Fig. 8C and 8D).

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Author Contributions

Conceived and designed the experiments: YK CBW TTH Performed the experiments: YK JZS MHN. Analyzed the data: YK TTH. Contributed reagents/materials/analysis tools: JZS CBW MHN. Wrote the paper: YK TTH.

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