Blocking Phosphatidylcholine Utilization in *Pseudomonas aeruginosa*, via Mutagenesis of Fatty Acid, Glycerol and Choline Degradation Pathways, Confirms the Importance of This Nutrient Source *In Vivo*

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**Abstract**

*Pseudomonas aeruginosa* can grow to very high-cell-density (HCD) during infection of the cystic fibrosis (CF) lung. Phosphatidylcholine (PC), the major component of lung surfactant, has been hypothesized to support HCD growth of *P. aeruginosa* *in vivo*. The phosphorylcholine headgroup, a glycerol molecule, and two long-chain fatty acids (FAs) are released by enzymatic cleavage of PC by bacterial phospholipase C and lipases. Three different bacterial pathways, the choline, glycerol, and fatty acid degradation pathways, are then involved in the degradation of these PC components. Here, we identified five potential FA degradation (Fad) related *fadBA*-operons (fadBA1-5, each encoding 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase). Through mutagenesis and growth analyses, we showed that three (fadBA145) of the five *fadBA*-operons are dominant in medium-chain and long-chain Fad. The triple *fadBA145* mutant also showed reduced ability to degrade PC *in vitro*. We have previously shown that by partially blocking Fad, via mutagenesis of *fadBA5* and *fadD*, we could significantly reduce the ability of *P. aeruginosa* to replicate on FA and PC *in vitro*, as well as in the mouse lung. However, no studies have assessed the ability of mutants, defective in choline and/or glycerol degradation in conjunction with Fad, to grow on PC or *in vivo*. Hence, we constructed additional mutants ([ΔfadBA145ΔglpD, ΔfadBA145ΔabetAB, and ΔfadBA145ΔabetABΔglpD]) significantly defective in the ability to degrade FA, choline, and glycerol and, therefore, PC. The analysis of these mutants in the BALB/c mouse lung infection model showed significant inability to utilize PC *in vitro*, resulted in decreased replication fitness and competitiveness *in vivo* compared to the complement strain, although there was little to no variation in typical virulence factor production (e.g., hemolysin, lipase, and protease levels). This further supports the hypothesis that lung surfactant PC serves as an important nutrient for *P. aeruginosa* during CF lung infection.

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**Introduction**

*Pseudomonas aeruginosa* is widespread in nature, inhabiting soil, water, plants and animals. In hospitals, it can be found in sinks, respirators, humidifiers and occasionally on the hands of medical personnel [1,2]. The ubiquitous nature of this bacterium has allowed it to adapt to a broad range of hosts in which it can cause diseases. The role of *P. aeruginosa* as an opportunistic human pathogen is of particular concern, especially because it is a frequent cause of nosocomial infections such as pneumonia, urinary tract infections, and bacteremia [1,3,4]. *P. aeruginosa* infection in the respiratory tract of cystic fibrosis (CF) patients causes a rapid deterioration in lung function and thus patient survival [5,6]. The pathogenicity of *P. aeruginosa* infection in CF patients has been extensively studied in terms of biofilm production [7–9] and quorum sensing (QS) controlled virulence [10–12]. However, little effort has been placed towards the contribution of *P. aeruginosa* nutrient acquisition aids high-cell-density (HCD) replication during lung infection.

Previous studies have shown that *P. aeruginosa* can undergo HCD replication in the lung of CF patients reaching >10^9 CFU/ml [13–15]. HCD replication is highly energy demanding,
requiring efficient nutrient acquisition and metabolism. However, evidence showed that the nutrients in the lung environment are lipids and amino acids derived from proteins or polypeptides [16–18], to allow P. aeruginosa HCD growth and maintenance in vivo. An in vitro study revealed that P. aeruginosa has directional twitching motility toward phosphatidylethanolamine (PE) and phosphatidylincholine (PC) [19]. Mammalian lungs are naturally coated by indispensable lung surfactant, which is composed of approximately 10% protein and 90% lipids, with about 80% of the lung surfactant lipids being phosphatidylcholine (PC) [20–22]. Thus, PC, the most abundant lipid in lung surfactant may provide significant nutrient for HCD bacterial growth in vivo. In accordance with this hypothesis, our initial studies suggest that PC is a major nutrient source of P. aeruginosa during lung infection and supports HCD replication [15,17,18].

Our previous in vivo CF spuata study showed that P. aeruginosa produced phospholipase C (heat-labile hemolysin) and lipases that could cleave exogenous PC into three components: a phosphorylcholine headgroup, glycerol and two long-chain fatty acids (LCFAs) [15] (Fig. 1A). These three components can be further metabolized by the choline (bet), glycerol (glp), and fatty acid degradation (Fad) pathways (Fig. 1B), respectively. Choline and glycerol metabolism by P. aeruginosa are well characterized [23–27]. However, LCFA degradation by P. aeruginosa and the genes involved in this process remain to be fully elucidated. Our previous in vivo CF spuata study also detected the expression of P. aeruginosa genes involved in each pathway for metabolizing all three PC components [15]. The betAB-operon was induced and glpD and glpK genes were constitutively expressed in vivo [15]. Among genes for Fad (Fig. 1B), the expression of fadBA1 was detected when P. aeruginosa was grown on PC in vitro, and fadBA5 and fadA4 were induced and constitutively expressed in CF spuata [15], suggesting the involvement of fadBA1-45 in LCFA degradation. We have also shown the reduced ability of the fadD mutants to utilize FAs as nutrients led to their decreased fitness during mouse lung infection [17,18]. However, further evidence is needed to support the hypothesis that all three components of lung surfactant PC (phosphorylcholine, glycerol and FA) serve as nutrient sources for P. aeruginosa during in vivo lung infection.

In this study, up to five potential fadBA-operns (encoding 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase) were identified, and three of them fadBA1 (PA1737, PA1736), fadBA4 (PA4786, PA4785) and fadBA5 (PA3014, PA3013) were shown to be significantly involved in medium- and long-chain Fad. Coupling of the fadBA145 mutations with mutations in choline and/or glycerol degradation were investigated to determine the importance of these pathways to degrade PC in vitro and in vivo. Competition studies were initiated to analyze the competitive fitness of these mutants relative to strains with intact pathways.

Results and Discussion

P. aeruginosa has five fadBA-operns potentially involved in fatty acid degradation

The well-established aerobic fatty acid degradation (Fad) pathway in E. coli was used as a model to characterize P. aeruginosa Fad. E. coli possesses only a single copy of each fad gene for aerobic Fad [28,29], and the cyclic degradation of fatty acids by two carbons per cycle is primarily catalyzed by an acyl-CoA dehydrogenase coded by fadE, and the products of the fadBA-opern, 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, respectively. Up to five potential fadBA-operns were identified in P. aeruginosa by BLAST analysis of the E.coli fadBA sequence against the P. aeruginosa genome, including fadBA1 (PA1737, PA1736), fadBA2 (PA3590, PA3589), fadBA3 (PA2554, PA2553), fadBA4 (PA4786, PA4785), and fadBA5 (PA3014, PA3013) (Fig. S1). Of these five FadBA, FadBA5 showed the greatest homology to the E.coli FadBA with FadB5 having 72% similar (34% identical) and FadA5 having 76% similar (61% identical) to the E.coli FadBA, respectively [30,31].

Considering the larger size of the P. aeruginosa genome (8.29 Mb) [32] and its wide range of environmental niches and metabolic capabilities, it is not surprising that P. aeruginosa has up to five fadBA-opern homologues. Therefore, it is important next to narrow down which of these five operons are important in Fad, using a mutagenesis approach.

P. aeruginosa fadBA145-operns are important for degrading PC and medium- and long-chain fatty acids

Our previous work showed that the ΔfadBA5 mutant has a reduced ability to utilize LCFA as a sole carbon source, but this ΔfadBA5 mutant can still grow on LCFAs as a carbon source, indicating the existence of other potential fadBA-operns in P. aeruginosa for LCFA degradation [31]. Further supporting this idea, the fadBA1-operon was shown to be induced by medium-chain fatty acids (MCFAs) and to a lesser extent by LCFAs [33]. The fadBA5-operon plays the most significant role in LCFA degradation, because neither the single ΔfadBA1 mutant, nor the single ΔfadBA4 mutant, showed much defects in their ability to utilize MCFA and LCFA as sole carbon sources (Fig. S2). It is possible that the FadBA1 and/or other FadBA(s) might have overlapping functions with FadBA5 in the metabolism of different chain length FAs, but these activities are masked by the more dominant FadBA5. Evidence for the involvement of other FadBA(s) is lacking, and needs to be addressed.

Because it is too overwhelming to test all possible double, triple, and quadruple fadBA-muant combinations and the complicated dominance of fadBA5, we demonstrated the involvement of each fadBA-operon by testing different triple mutant combinations. In this study, we generated several triple mutants (ΔfadBA125, ΔfadBA135, ΔfadBA145, ΔfadBA225, ΔfadBA245, and ΔfadBA345) and a quintuple mutant ΔfadBA1-5 (Table 1) for growth analysis on MCFAs and LCFAs as sole carbon sources, to further characterize the function of these fadBA-operns. The growth defects were previously defined by the slower growth rate and lower overall final cell densities compared to wildtype strain PAO1, which suggest a reduced ability to metabolize these FAs, presumably due to the accumulation of growth inhibiting intermediates [17,31]. Significant growth defects were observed for any combinations of triple mutants in which both the fadBA5 and fadBA1-operns were deleted (i.e., ΔfadBA125, ΔfadBA135 and ΔfadBA145 mutants), revealing the importance of FadBA1 and fadBA5 contributing to growth on MCFAs and LCFAs (Fig. 2A–D). Although the level of defects is different for each type of FA used, the trend is consistent for these mutants in all FAs (Fig. 2A–D). Only the ΔfadBA145 triple mutant showed the same growth defect as the ΔfadBA1-5 quintuple mutant on all fatty acids tested, suggesting the importance of all three fadBA1, fadBA4, and fadBA5 operons and the minor role of fadBA2 and fadBA3-operns in metabolizing MCFAs and LCFAs. The importance of fadBA4 was further confirmed by the fact that the ΔfadBA245 and ΔfadBA345 triple mutants showed an additional growth defect on all FAs compared to the ΔfadBA245 mutant (Fig. 2A–D). However, the fadBA4-operon displays less of an involvement in metabolizing all FAs tested compared to both fadBA1 and fadBA5-operns (i.e., comparing ΔfadBA4 to ΔfadBA125 or ΔfadBA345 to ΔfadBA1-5). Knowing that only fadBA1, fadBA4, and fadBA5 are important for degrading...
Figure 1. Phosphatidylycholine (PC) degradation pathways in *Pseudomonas aeruginosa*. (A) PC is the main component of lung surfactant and can be cleaved by phospholipase C and lipases, producing free fatty acids, glycerol, and phosphorylcholine. Three different pathways then further metabolize each component: the bet pathway for choline head group metabolism, the glp pathway for glycerol metabolism, and the β-
oxidation pathway for the degradation of the FAs. (B) The proposed P. aeruginosa FA β-oxidation pathway. 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, out membrane; IN, inner membrane.

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MCFA and LCFA of PC, we continued with further experiments from this point forward in our study by using the ΔfadBA145 mutant background, rather than the ΔfadBA145-5 quintuple mutant.

We next tested the ΔfadBA145 mutant for its ability to grow on PC. The ΔfadBA145 triple mutant displayed a reduced growth rate and lower final cell density as compared to wildtype PAO1 when grown with PC as a sole carbon source (Fig. 3A). There was no growth defect observed for this mutant when grown in control LB medium (Fig. 3B). The ΔfadBA145 mutant strain is competitively less fit than the complement in the in vitro competition study when grown in PC or FA (C\textsubscript{18:1} n\textsubscript{9}) (Fig. 4A panel i). No competitive defect was observed when the ΔfadBA145 mutant was grown on LB, glucose, casamino acids (CAA), glycerol or choline controls (Fig. 4A panel i). We concluded that the reduced growth of the ΔfadBA145 mutant strain on PC observed in figure 3A is due to a decreased ability to degrade LCFA of PC and not glycerol or choline. All the evidence we have here strongly suggests the involvement of the three fadBA1,4,5-operons in Fad and PC degradation. We complemented ΔfadBA145 mutant strain by integrating minCTX2-fadBA45 (i.e., the dominant fadBA operon as explained above) as a single copy into the ΔfadBA145 mutant background (Table 1). The complemented strain was fully restored to wildtype growth on PC (Fig. 3A) and FAs (not shown). Hence, all complementation experiments in this study for any ΔfadBA145 mutant background were performed with only the dominant fadBA45-operand.

Mutants blocked in FA, glycerol, and choline degradation displayed dramatically reduced ability to utilize PC in vitro

The enzymatic activity of phospholipase C on PC releases the phosphorylcholine headgroup and the diacylglycerol (DAG) molecule (Fig. 1A). The phosphorylcholine headgroup is first transported across the cell membrane and dephosphorylated by a phosphatase [23,34,35] to yield choline, which has previously been shown to be sufficient for P. aeruginosa to grow on as a sole carbon, nitrogen, and energy source [36]. P. aeruginosa BetAB (encoding choline dehydrogenase and a glycine betaine aldehyde dehydrogenase) catalyzes the conversion of choline to glycine betaine [23]. Glycine betaine is successively demethylated to form dimethylglycine (DMG), sarcosine (monomethylglycine), and finally glycine [24,37]. The DAG molecule is cleaved by the P. aeruginosa lipase, liberating a glycerol molecule and two LCFA. Glycerol metabolism has been well characterized in P. aeruginosa. The operon primarily consists of glpD (a sn-glycerol-3-phosphate dehydrogenase [38]), glpF (a membrane-associated glycerol diffusion facilitator [27,39]), glpK (a glycerol kinase [27,39]), glpM (a membrane protein affecting alginic synthesis [26]), and glpR (a regulator of the glp operon [25]).

Since our previous data showed that betAB and glpD were expressed in vivo [15], they may potentially be involved in PC degradation during lung infection. However, before we could address the in vivo aspect of PC degradation, further experiments are needed to characterize P. aeruginosa PC degradation in vitro. We engineered double pathway mutants ΔfadBA145ΔbetAB (blocked in FA and choline degradation) and ΔfadBA145ΔglpD (blocked in FA and glycerol degradation) and a triple pathway mutant ΔfadBA145ΔbetABΔglpD (blocked in FA, choline, and glycerol degradation) (Table 1), to further determine whether these mutants are deficient in growth on PC. As expected, all three mutants experienced various growth defects with decreased cell density and delayed log-phase when grown on PC (Fig. 3A). The triple pathway mutant ΔfadBA145ΔbetABΔglpD exhibited the most significant reduced ability to utilize PC, reaching to only about one-third of the wildtype final cell density. We complemented these mutants (i.e., ΔfadBA145ΔbetAB, ΔfadBA145ΔglpD, and ΔfadBA145ΔbetABΔglpD) by integrating the respective miniCTX2-fadBA5/betAB, miniCTX2-fadBA5/glpD or miniCTX2-fadBA5/betAB/glpD as a single copy on the P. aeruginosa chromosome and the complemented strains fully recovered these mutants’ ability to grow on PC as compared to wildtype PAO1 (Fig. 3A). No mutants or complement showed any growth defects on control LB medium (Fig. 3B).

We performed an in vitro competition study between pathway mutants (ΔfadBA145ΔbetAB, ΔfadBA145ΔglpD, ΔfadBA145ΔbetABΔglpD) and their complements to examine whether the mutation reduced their ability to metabolize various carbon sources. As expected, all the pathway mutants were less competitive than their respective complements in media containing PC and other sole carbon sources involved in the respective pathways (Fig. 4A panels ii-iv). For example, the ΔfadBA145ΔbetAB mutant was less competitive than its complement when grown using PC, FA, or choline, as sole carbon source (Fig. 4A panel ii), which is not the case in other control media (e.g., LB, glucose, CAA or glycerol). Likewise, the ΔfadBA145ΔglpD mutant was less competitive than its complement only if PC, FA, or glycerol was used as sole carbon source (Fig. 4A panel iii). The triple pathway mutant was almost completely outcompeted by its complemented strain with the competitive indices (CI) dramatically reduced to ~0.1 when growing in the media containing PC, choline, glycerol, or oleate FA (Fig. 4A panel iv). Overall, these in vitro data showed that we have three mutants (ΔfadBA145ΔbetAB, ΔfadBA145ΔglpD, ΔfadBA145ΔbetABΔglpD) and their complement that could be used to assess the utilization of PC in vivo, through competitive index experiments within the mouse lung.

Blocking FA, glycerol, and choline degradation significantly reduces replication fitness of P. aeruginosa in vivo

Since PC is the major component of lung surfactant in mammals, including mice [21], a mouse lung infection model [40] was utilized for our in vivo competition study to evaluate the fitness of the PC degradation pathway mutants within the lung environment. The mucoid, exopolysaccharide alginate-overproducing phenotype is a distinguishing feature of P. aeruginosa isolated from CF patients [40,41]. An alginate-overproducing strain carrying a mucA insertional mutation, which allows the mucoid strain to survive and replicate in the lung, has been successfully used in BALB/c mouse model to establish the connection between nutrient acquisition and in vivo lung fitness for P. aeruginosa [17]. Therefore, we constructed various mucA\textsuperscript{−} alginate-overproducing strains, such as ΔfadBA145-mucA\textsuperscript{−}, ΔfadBA145ΔglpD-mucA\textsuperscript{−}, ΔfadBA145ΔbetAB-mucA\textsuperscript{−}, and their complemented strains for
this study. Prior to the animal study, the phenotypes of all mucA strains were confirmed by patching on minimal media plates with FA, choline, or glycerol as sole carbon sources along with all appropriate controls (all mucA wild-type strains and complemented strains). As expected, the mucA mutation did not affect the metabolism of any of these carbon sources (Fig. S3). As previously described [17,40], BALB/c mice were inoculated via intratracheal intubation with equal ratios of each mutant and its complemented pair (6 × 10⁶ CFU/animal). At 24 h post-infection, bacterial CFU recovered from the lungs were determined, followed by CI calculations. For all the strains, the average total CFU per mouse lung recovered at 24 hours post-infection was greater than the

Figure 2. Growth analysis of different fadBA mutant combinations on medium (C₁₂:₀) and long chain-length fatty acid (C₁₄:₀, C₁₆:₀ and C₁₈:₁ω₉). Along with the wildtype PAO1 strain, mutants were grown in 1×M9 minimal medium supplemented with 0.4% different test FAs (A to D) or 1% control casamino acids (CAA, E) as sole carbon sources. Although fadBA mutants showed various defects when grown with FAs of different chain-lengths, no growth defects were observed for any of the mutants when grown with CAA as a control.

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initial inoculum (Fig. 4B), indicating that all these _P. aeruginosa_ strains maintained the ability to replicate within the mouse lung. The _ΔfadBA145_ mutant still replicated significantly _in vivo_ compared to its complement. Surprisingly, the _ΔfadBA145_ CI is quite high compared to other FAD mutants (i.e., _fadD_ mutants) we have previously published where CI is approximately 0.5 [17,18]. The _ΔfadBA145_ΔbetAB, _ΔfadBA145_ΔglpD _in vivo_ CI is lower than the _ΔfadBA145_ when compared to their respective complements (Fig. 4B), showing the importance of glycerol and choline degradation as potential nutrient sources _in vivo_. Most significantly, the mean CI value for the triple pathway mutant (i.e., _ΔfadBA145_ΔetABΔglpD) showed that the triple pathway mutant had a significantly reduced ability to survive and multiply in the lungs of mice compared to its complement. We monitored all strains tested _in vivo_ for different virulence expression, including proteases, rhamnolipid, hemolysins and lipases (Fig. S4). With similar level of these common secreted virulence factors observed between strains (Fig. S2), the low CI is most likely due to its inability to metabolize PC and the three components of PC (LCFAs, glycerol, and phosphorylcholine) as a nutrient source, rather than resulting from altered virulence expression. Overall, the altered ability for the pathway mutants to metabolize PC as nutrient _in vitro_ was clearly mirrored by their competitive fitness within the lung.

### Table 1. Bacterial strains used in this study.

| Strain          | Lab ID  | Genotype/Description                  | Reference                  |
|-----------------|---------|---------------------------------------|----------------------------|
| _E. coli_       | E1231   | F− λ− mcrA Δ(mrr-hsdRMS-mcrBC) rpsDlacZ ΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(xara, leu)7697 galU galK rpsL supG | BioRad                     |
| SM10            | E006    | thi thr leu tonA lacY supE recA1ΔR4-2Tc:Mu Km′ | [48]                       |
| _P. aeruginosa_ |         |                                        |                            |
| PAO1            | P007    | Prototroph                            | [49]                       |
| PAO1- _mucA_    | P447    | Cb′, PAO1 with pUC18 inserted in _mucA_ gene | This study                  |
| _ΔfadBA125_     | P122    | Gm′, PAO1-ΔfadBA1-FRT, ΔfadBA2:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA135_     | P124    | Gm′, PAO1-ΔfadBA1-FRT, ΔfadBA3:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA145_     | P319    | Gm′, PAO1-ΔfadBA1-FRT, ΔfadBA4:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA235_     | P317    | Gm′, PAO1-ΔfadBA2:FRT, ΔfadBA3:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA245_     | P130    | Gm′, PAO1-ΔfadBA1:FRT, ΔfadBA3:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA345_     | P126    | Gm′, PAO1-ΔfadBA3:FRT, ΔfadBA4:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA1-5_     | P102    | Gm′, PAO1-ΔfadBA1:FRT, ΔfadBA2:FRT, ΔfadBA3:FRT, ΔfadBA4:FRT, ΔfadBA5:Gm | This study                  |
| _ΔfadBA145_ΔglpD_ | P539    | Gm′, PAO1-ΔfadBA1:FRT, ΔfadBA4:FRT, ΔfadBA5:FRT, ΔglpD::Gm-FRT | This study                  |
| _ΔfadBA145_ΔbetAB_ | P555   | Gm′, PAO1-ΔfadBA1:FRT, ΔfadBA4:FRT, ΔfadBA5:FRT, ΔbetAB::Gm-FRT3 | This study                  |
| _ΔfadBA145_ΔbetAB_ΔglpD_ | P561   | Gm′, PAO1-ΔfadBA1:FRT, ΔfadBA4:FRT, ΔfadBA5:FRT, ΔbetAB::Gm-FRT3, ΔglpD::Gm-FRT1 | This study                  |
| _ΔfadBA145_ΔglpD_Δcomplement_ | P965   | Gm′, Tet′, ΔfadBA145 complemented with miniCTX2-fadBAS | This study                  |
| _ΔfadBA145_ΔglpD_Δcomplement_ | P1015  | Gm′, Tet′, ΔfadBA145 ΔglpD complemented with miniCTX2-fadBAS::glpD | This study                  |
| _ΔfadBA145_ΔbetAB_ΔglpD_Δcomplement_ | P1017  | Gm′, Tet′, ΔfadBA145 ΔbetAB complemented with miniCTX2-fadBAS::betAB | This study                  |
| _ΔfadBA145_ΔglpD_ΔmucA_ | P576   | Gm′, Cb′, ΔfadBA145 with pUC18 inserted in _mucA_ gene | This study                  |
| _ΔfadBA145_ΔglpD_ΔmucA_ | P570   | Gm′, Cb′, ΔfadBA145 ΔglpD with pUC18 inserted in _mucA_ gene | This study                  |
| _ΔfadBA145_ΔbetAB_ΔmucA_ | P572   | Gm′, Cb′, ΔfadBA145 ΔbetAB with pUC18 inserted in _mucA_ gene | This study                  |
| _ΔfadBA145_ΔbetAB_ΔmucA_ | P574   | Gm′, Cb′, ΔfadBA145 ΔbetAB ΔglpD with pUC18 inserted in _mucA_ gene | This study                  |
| _ΔfadBA145_ΔmucA_ | P584   | Gm′, Cb′, Tet′, ΔfadBA145_ΔmucA− complemented with miniCTX2-fadBAS | This study                  |
| _ΔfadBA145_ΔmucA_ | P578   | Gm′, Cb′, Tet′, ΔfadBA145 ΔglpD::mucA− complemented with miniCTX2-fadBAS::glpD | This study                  |
| _ΔfadBA145_ΔmucA_ | P580   | Gm′, Cb′, Tet′, ΔfadBA145 ΔbetAB::mucA− complemented with miniCTX2-fadBAS::betAB | This study                  |
| _ΔfadBA145_ΔmucA_ | P582   | Gm′, Cb′, Tet′, ΔfadBA145 ΔbetAB ΔglpD::mucA− complemented with miniCTX2-fadBAS::betAB::glpD | 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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In summary, *P. aeruginosa* possesses an impressive repertoire of virulence factors, and the expression of most of these only occurs during the HCD replication and their timely expression is regulated by QS [42], which occurs at HCD. *P. aeruginosa* requires large amount of readily available energy to reach and maintain HCD and produce the high-energy dependent virulence structure like biofilm. Thus, exploration of the nutrient sources supporting such an energy intensive processes is of importance, especially for chronic *P. aeruginosa* lung infections in CF patients. In addition, the identification of the genes and pathways for *P. aeruginosa* HCD replication in CF lungs provides fundamental knowledge for possibly developing new therapeutic strategies targeting bacterial nutrient metabolism in the lung, thereby preventing bacterial HCD. The expression of genes involved in *P. aeruginosa* PC degradation within the lungs of CF patients has been previously demonstrated [15]. Our study focused on providing further evidence to determine whether PC serves as a significant nutrient source during *P. aeruginosa* lung infection. In order to decipher the role of PC *in vivo*, we first characterized PC degradation pathways *in vitro*. Of the three components released by the enzymatic cleavage of PC by bacterial phospholipase C and lipases (phosphorylcholine, LCFAs, and glycerol), LCFAs are highly reduced and yield the most carbon and energy. In our study, five potential *fadBA*-operons were investigated and three of them (i.e., *fadBA1,4,5*-operons) proved to be significantly involved in Fad. The *in vitro* growth analysis of different pathway mutants (*ΔfadBA145*, *ΔfadBA145ΔglpD*, *ΔfadBA145ΔbetBA*, *ΔfadBA145ΔbetBAΔglpD*, *ΔfadBA145/ complement*, *ΔfadBA145ΔglpD/complement*, *ΔfadBA145ΔbetBA/complement*, *ΔfadBA145ΔbetBAΔglpD/complement*) on PC provided direct evidence to support that *P. aeruginosa* utilizes the FA, glycerol and choline degradation pathways to degrade individual components of PC.

**Figure 3. Growth analysis on phosphatidylcholine.** (A) Some mutants exhibited growth defects on PC as a sole carbon source. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wildtype PAO1 strain. (B) No growth defects in control LB medium were observed.

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in vitro. Our in vivo competition study was performed utilizing a mouse lung infection model [40] to evaluate the fitness of the pathway mutants within the lung environment. The triple pathway mutant $\Delta fadBA145\Delta betAB\Delta glpD$ exhibited the greatest growth defect on relevant carbon sources in vitro and was outcompeted by its complement in vivo. Since no altered expression of virulence factors was observed for all the pathway mutants and their complement pairs compared to wildtype PAO1, it is highly likely that the decreased ability to utilize PC resulted in lower replication fitness in the lung environment. This study strongly supports the hypothesis that *P. aeruginosa* utilizes lung surfactant PC as one of the nutrient sources for chronic lung infection.
Materials and Methods

Ethic Statement
All animal experiments were performed 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 conditions
Bacterial strains and plasmids utilized in this study are listed in Table 1 and 2. E. coli EP-Max10B was used as cloning strains and cultured in Luria-Bertani (LB) medium (Difco). Pseudomonas Isolation Agar or Broth (PIA or PIB; Difco) or LB medium were used to culture P. aeruginosa strain PAO1 and derivatives. All fatty acids (FAs) stocks were made as previously described [17]. Strains for growth analyses were cultured in 1x M9 minimal medium supplemented with each of C12:0 (medium-chain fatty acid), C16:0, C18:0, and C18:1 medium supplied with each of C12:0 (medium-chain fatty acid), C16:0, C18:0, or C18:1 (Sigma, Fig. 2) and 1x M9 minimal medium supplemented with each of C12:0, C16:0, C18:0, C18:1, and C18:2 constituting the rest [43], the growth analysis was performed in 1x M9 minimal medium supplemented with each of C12:0 (medium-chain fatty acid), C16:0, C18:0, and C18:1 (LCFAs) as a sole carbon source. Accordingly, the PC utilized in this in vitro experiments contains mostly LCFAs, approximately 33% C16:0, 13% C18:0, 31% C18:1, and 13% C18:2. The in vitro competition studies (Fig. 4A) were performed under the growth condition mentioned above as previously described [17].

General molecular methods
Oligonucleotides were synthesized through Integrated DNA Technology and are listed in Table 3. All molecular methods and their components utilized were employed as previously described [44].

Construction of mutants and complementation strains
All mutants were constructed as described previously [45]. Briefly, the fadBA (fadBA1, fadBA2, fadBA3, fadBA4, fadBA5) operons, betAB operon, and glpD gene were amplified by PCR using respective upstream and downstream primer pair listed in Table 3. The PCR products were purified from the gel, digested with appropriate restriction enzymes, and cloned into the gene replacement vector pEX18T, digested with the same restriction enzymes, to yield each of the pEX18T-target gene constructs. After deletions were made on plasmid in each of the fadBA- operons, the glpD gene, and the betAB-operon through restriction digestion [fadBA1: PsII, BamHI; fadBA2: StuI, BamHI; fadBA3: NotI, SmaI; fadBA4: EcoRV; fadBA5: SphI, PsII] and blunted-ended (except for glpD, which was blunt-ended using SmaI), the 1.1 kb FRT-Gmr^-FRT cassette obtained from pPS856 digested with SmaI was inserted into each gene. These newly constructed pEX18T vectors were transformed into E.coli SM10 or ER2566-mob, and conjugated into PAO1 to engineer the unmarked mutations as previously described [45]. To obtain all triple mutants, we invested an enormous amount of work to first create all single and double mutants with the proper confirmed FIP/FRT-excision of the gentamycin antibiotic resistance cassette to recycle this resistance marker for subsequent mutagenesis (data not shown).

The single copy integration vector, miniCTX2, was used to engineer the complemented strains for each triple-pathway mutant

Table 2. Plasmids used in this study.

| Plasmids | Lab ID | Relevant properties | Reference |
|----------|--------|---------------------|-----------|
| pFlp2    | E0067  | Ap^r, sacB^r; Flp-containing plasmid | [45]       |
| pPS856   | E0050  | Ap^r, Gm; plasmid with Gm^-FRT-cassette | [45]       |
| pUC18    | E0135  | Ap^r, cloning vector | [50]       |
| pUC18-mucA | E1907 | Ap^r, mucA internal region cloned into pUC18 | This study |
| pUC19    | E0014  | Ap^r, cloning vector with Puc | [50]       |
| pUC19-glPD | E1843 | Ap^r, pUC19 with glpD gene cloned in downstream of Puc | This study |
| pEX18T   | E0055  | Ap^r, oriT, sacB^r; gene replacement vector | [45]       |
| pEX18TfadBA1::Gm | E0202 | Ap^r, Gm; pEX18T with fadBA1 operon with Gm^-FRT-cassette insertion | This study |
| pEX18TfadBA2::Gm | E0224 | Ap^r, Gm; pEX18T with fadBA2 operon with Gm^-FRT-cassette insertion | This study |
| pEX18TfadBA3::Gm | E0225 | Ap^r, Gm; pEX18T with fadBA3 operon with Gm^-FRT-cassette insertion | This study |
| pEX18TfadBA4::Gm | E0226 | Ap^r, Gm; pEX18T with fadBA4 operon with Gm^-FRT-cassette insertion | This study |
| pEX18TfadBA5::Gm | E0461 | Ap^r, Gm; pEX18T with fadBA5 operon with Gm^-FRT-cassette insertion | This study |
| pEX18TglPD::Gm | E1066 | Ap^r, Gm; pEX18T with glPD operon with Gm^-FRT-cassette insertion | This study |
| pEX18TbetAB::Gm | E1070 | Ap^r, Gm; pEX18T with betAB operon with Gm^-FRT-cassette insertion | This study |
| miniCTX2 | E0076  | Tet^r; site-specific integration vector | [46]       |
| miniCTX2-fadBA5 | E1765 | Tet^r; miniCTX2 with cloned fadBA5 | This study |
| miniCTX2-fadBA5::glpD | E2035 | Tet^r; miniCTX2 with cloned fadBA5::glpD | This study |
| miniCTX2-fadBA5::betAB | E1953 | Tet^r; miniCTX2 with cloned fadBA5::betAB | This study |
| miniCTX2-fadBA5::betAB::glpD | E1992 | Tet^r; miniCTX2 with cloned fadBA5::betAB::glpD | This study |

^aFor plasmids constructed in this study, please see text for further details.

^bPlease use Lab ID for requesting plasmids.

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Table 3. Primers used in this study.

| Primer number and name   | Sequence*                                      |
|--------------------------|------------------------------------------------|
| 186; fadBA1-upstream     | 5′-CGAAGCCTGGATGTTATCTTCTTCC-3′               |
| 187; fadBA1-downstream   | 5′-GGCGAGATCGCTCTATTGCCGCAAG-3′               |
| 218; fadBA2-upstream     | 5′-CGTGAAGCCTTCTCGCACC-3′                     |
| 219; fadBA2-downstream   | 5′-GGCGAGATCGCTCTATTGCCGCAAG-3′               |
| 220; fadBA3-upstream     | 5′-GGCAAGTCACTACGAGAAGAAGGACACGACC-3′        |
| 221; fadBA3-downstream   | 5′-TGCGAATCCGACGTTAGTGGCGGAGGCACGTCAG-3′      |
| 211; fadBA4-upstream     | 5′-CGTAAAGTGGGAGTGGGAGGCGGC-3′               |
| 212; fadBA4-downstream   | 5′-CCCGAACTTGCGAGAGCAGCGAAGGACCGAAG-3′       |
| 272; fadBA5-HindIII      | 5′-AGTCAAGCTCTATTGCTACGC-3′                   |
| 273; fadBA5-EcoRI        | 5′-CCCGAATCTTTCTGAGAAGCCTGTAAG-3′            |
| 518; glpK-BamHI          | 5′-AGCTGAAGTCCATCGAACAA-3′                    |
| 519; glpK-SacI           | 5′-CTGCGAATCTCAGAAGCCGCAAGCGCAGCC-3′         |
| 522; beta-SacI           | 5′-CAAGCAGCTCAGGGATACAGGAGCGGC-3′            |
| 523; beta-HindIII        | 5′-GCCAAAGCTTGAGACGAAACAGCCAT-3′             |
| 888; Xho-fadB5           | 5′-CTGGCGAGGAGGGCTCGAGAGAGGC-3′              |
| 889; fadB5-Bam           | 5′-GGCCCAAGTAGCATGCTGCTG-3′                   |
| 895; Spe-betB            | 5′-CGTAACTCGAGCTGTCGCTG-3′                   |
| 896; Hind-glpD           | 5′-GCTGTGCTAGCTGTCGCTG-3′                    |
| 927; SacI-Pvu-glpD       | 5′-CGCTCGCGAATGCTGCTG-3′                     |

*Restriction enzyme sites utilized in this study are underlined.

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as previously described [46]. Briefly, fadBA5 and betAB were PCR amplified with primers 888/889 and 522/895, respectively. The miniCTX2-fadBA5 was derived by inserting fadBA5 fragment into miniCTX2, both digested with XhoI and BamHI. The betAB gene was sub-cloned in using SacI and SpeI, yielding miniCTX2-fadBA5-betAB. glpD was first cloned into pUC19 by digesting the PCR product with HindIII and SacI, which was amplified using primers 896/519. The pathway mutants and complement strains were purified on LB medium. At each time point, aliquots of the diluted cultures were transferred to a sterile, polystyrene 96-well assay plate (Falcon Microtest flat bottom plate, catalog no. 35–1172; Becton-Dickinson Labware). Growth was recorded using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT) under the following conditions: temperature 37°C, and shaking at a low speed. The plate was read at 630 nm every 30 min for 40 h. All of the data was transferred and plotted using Prism.

Virulence factors detection

Strains used for virulence factors detection were grown in LB medium. At each time point, aliquots of individual culture were used for OD640 measurement (Fig. 3B). The detection of proteases, hemolysins, lipases, and rhamnolipid was performed as described elsewhere [17]. All assays were conducted in triplicate, and the data were analyzed as previously described [17].

Growth Phenotype Confirmation of Mucoid and Non-mucoid Strains

To confirm that mutations in mucA do not have additional effects on nutrient metabolism of the pathway mutant strains, all of the pathway mutants and complement strains were purified on LB plate or LB plate supplemented with 250 μl/ml carbenicillin (Cb250) for mucA− strains. After 24 h incubation at 37°C, single colony of each strain was patched on 1× M9 solid medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) C18:1^ω9, 40 mM glycerol or 30 mM choline as sole carbon source. They were also patched on LB plate, which served as a control. The growth pattern was observed after 24–36 h incubation at 37°C (Fig. S3).
In vitro and in vivo competition studies

In vitro and in vivo competition studies were performed as previously described [17]. Briefly, seven growth media with different carbon sources, including Luria-Bertani (LB) medium, casamino acids (CAA), glucose, PC, C11:0-A9, choline, and glycerol, were used in this study. The bacterial CFU were determined after inoculation into each of the medium for 24–48 h. The CI was calculated as the CFU ratio of mutant/wildtype recovered at each time point divided by the CFU ratio of mutant/wildtype in the input inoculum [47]. The smaller the CI value, the more the growth defects were observed for the rest of single fadBA mutants. All of the mutants grew to the same level as wildtype when grown in LB.

Figure S3 Growth Phenotype Confirmation of Mucoid and Non-mucoid Strains. Along with the wildtype PAO1 and PAO1-mucA- strains, all of the pathway mutants and their corresponding complement strains were patched on 1 x M9 solid medium +1 % (w/v) Brij-58 supplemented with 0.2% (w/v) C11:0-A9 (B), 40 mM glycerol (C), or 30 mM choline (D). (A) Growth on LB was performed as a control. Alginate over-producing strains show a light sheen surface indicated by white arrow in panel A. Similar growth defects were shown between mucoid and non-mucoid strains on different plates. A detailed plate layout is shown in panel E with strains identification of Table 1 in parentheses.

Figure S4 Analyses of proteases, hemolysins, lipases, and rhamnolipid productions by P. aeruginosa various pathway mutant. No mutants displayed significant (P≤0.05, based on student t-test) decrease in productions of proteases (A), rhamnolipid (B), hemolysins (C), and lipases (D).

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Author Contributions

Conceived and designed the experiments: ZS YK MHN RMT HPS SWD. Performed the experiments: ZS YK MHN RMT. Analyzed the data: ZS YK MHN. Contributed reagents/materials/analysis tools: TTH HPS YK. Contributed to the writing of the manuscript: ZS TTH.

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