Pseudomonas aeruginosa Directly Shunts β-Oxidation Degradation Intermediates into De Novo Fatty Acid Biosynthesis

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We identified the fatty acid synthesis (FAS) initiation enzyme in Pseudomonas aeruginosa as FabY, a β-ketoacyl synthase KASI/II domain-containing enzyme that condenses acetyl coenzyme A (acetyl-CoA) with malonyl-acyl carrier protein (ACP) to make the FAS primer β-acetoacetyl-ACP in the accompanying article (Y. Yuan, M. Sachdeva, J. A. Leeds, and T. C. Meredith, J. Bacteriol. 194:5171-5184, 2012). Herein, we show that growth defects stemming from deletion of fabY can be suppressed by supplementation of the growth media with exogenous decanoate fatty acid, suggesting a compensatory mechanism. Fatty acids eight carbons or longer rescue growth by generating acyl coenzyme A (acyl-CoA) thioester β-oxidation degradation intermediates that are shunted into FAS downstream of FabY. Using a set of perdeuterated fatty acid feeding experiments, we show that the open reading frame PA3286 in P. aeruginosa PAO1 intercepts C8-CoA by condensation with malonyl-ACP to make the FAS intermediate β-keto decanoyl-ACP. This key intermediate can then be extended to supply all of the cellular fatty acid needs, including both unsaturated and saturated fatty acids, along with the 3-hydroxyl fatty acid acyl groups of lipopolysaccharide. Heterologous PA3286 expression in Escherichia coli likewise established the fatty acid shunt, and characterization of recombinant β-keto acyl synthase enzyme activity confirmed in vitro substrate specificity for medium-chain-length acyl CoA thioester acceptors. The potential for the PA3286 shunt in P. aeruginosa to curtail the efficacy of inhibitors targeting FabY, an enzyme required for FAS initiation in the absence of exogenous fatty acids, is discussed.

Pseudomonas aeruginosa is a versatile Gram-negative pathogen, being the causative agent of a wide range of both community-associated (folliculitis and otitis externa) and health care-associated (pneumonia, urinary tract, and bacteremia) bacterial infections (15, 34, 39, 55). While generally not considered a normal member of the human flora, the near ubiquitous environmental distribution of P. aeruginosa provides a ready reservoir for exposure and ensuing opportunistic infection. Infections due to P. aeruginosa are particularly prevalent among immunodeficient individuals in whom cutaneous or mucosal barriers have been breached by ventilators, catheters, or through trauma, as seen in burn units. In >28,000 cases of all health care-associated infections reported to the U.S. National Healthcare Safety Network during a 22-month period beginning in 2006, 7.9% were attributed to P. aeruginosa (24). Chronic Pseudomonas infections are especially problematic in the lungs of cystic fibrosis patients, in part due to a genetic defect that facilitates bacterial colonization through diminished mucociliary clearance (6). The breadth of difficult-to-treat P. aeruginosa-related infections, coupled with an impressive array of intrinsic and adaptive antibacterial resistance mechanisms (3, 38), makes developing new antipseudomonas drugs a challenging priority.

We initiated a target evaluation program focused on fatty acid synthesis (FAS) in P. aeruginosa. FAS plays a multifaceted role in both maintaining bacterial viability and virulence in P. aeruginosa, suggesting that inhibition of FAS in vivo may have added benefit beyond simply blocking division through depleting fatty acid pools available for phospholipid biosynthesis. Aside from being compulsory components of membrane phospholipids (12), fatty acids are utilized by multiple primary and secondary metabolic pathways in P. aeruginosa. Lipopolysaccharides (LPS), which are lipoglycans located in the external leaflet of the outer membrane, are dependent on 3-hydroxy (3-OH) acyl-ACP (acyl carrier protein) FAS intermediates for complete acylation and in turn the establishment of the permeability barrier (43). Lipoproteins, a large class of lipiddated membrane proteins that includes components of the LPS transport machinery and of the resistance-nodulation cell division (RND)-type drug efflux pumps (44), depend on phospholipid donors for acylation (40). Even partial FAS inhibition could therefore induce LPS hypoacylation, decrease LPS transport, and/or cripple efflux pumps, raising the potential for synergistic combinations between FAS inhibitors and membrane-impermeable or efflux-susceptible antibiotics. Fatty acids are also used in the assembly of two important metabolic enzyme cofactors, lipoate and biotin. Lipoate is an essential cofactor of α-keto acid dehydrogenases (49), including pyruvate dehydrogenase. Pyruvate dehydrogenase connects glycolytic flux to the tricarboxylic acid (TCA) cycle by forming acetyl coenzyme A (acyetyl-CoA) from pyruvate. P. aeruginosa lacking pyruvate dehydrogenase does not express the type III secretion system, critical machinery involved in delivering cytotoxic effectors, due to the metabolic defect (45). More recently, the essential cofactor biotin has also been proposed to hijack FAS biosynthetic enzymes for its own synthesis (11, 37). Biotin is required by enzymes involved in amino acid metabolism, gluconeogenesis, and FAS itself. The assembly of the high-affinity ferric iron siderophore pyoverdine is dependent on the fatty acid myristate carrier in P. aeruginosa (22). Finally, orchestrating the complex intercellular signaling and hierarchical gene regulation that is a hallmark of the P. aeruginosa social lifestyle are the three acylated quorum-sensing signal molecules [Pseudomonas quinolone signal (PQS), N-(3-oxodo-
decanoyl-1-homoserine lactone, and N-butanoyl-1-homoserine lactone (30) and the cis-2-decenoic fatty acid diffusible signal factor (DSF) (14). Collectively, these systems coordinate the expression of hundreds of genes (54), including pertinent virulence factors such as rhamnolipids, pyocyanin, and extracellular proteases, as well as the transition from planktonic to antibiotic-recalcitrant growth within biofilms. In addition to being an essential target in and of itself, the central roles of fatty acids in intrinsic antimicrobial resistance, in supporting diverse intermediary metabolism, in sensing environmental cues, and in mobilizing a suite of virulence factors all make FAS a promising antibacterial target in P. aeruginosa. The initiating step of FAS in Escherichia coli is catalyzed by the enzyme β-acetoacyl-ACP synthase encoded by fabH (52), which condenses malonyl-ACP with acetyl-CoA to form β-acetoacyl-ACP. The enzyme is defined by the signature β-ketoacyl synthase III (KASIII) domain, which is present in multiple, highly similar genes within the genome of P. aeruginosa PAO1 (56, 57). Our initial attempts in the accompanying article (56) to assign the FabH-type activity of P. aeruginosa to a single KASIII ortholog were unsuccessful; rather, it was determined that the predominant β-acetoacyl-ACP synthase is encoded by a new class of highly divergent KASI-II/type synthases named fabY (formerly PA5174). Deletion of fabY confirmed a pleiotropic phenotypic consistent with decreased FAS flux, as siderophore production, swarming motility, rhamnolipids, and fatty acid-dependent quorum-sensing signals (PQS and homoserine lactones) in tandem with the expression of their cognate regulatory targets were attenuated (56). Although the doubling time of the ΔfabY deletion mutant was three times longer in liquid media, the viability of the ΔfabY deletion mutant indicated that another P. aeruginosa gene(s) is capable of fatty acid initiation. In addition, we observed that the growth defect could be partially complemented by inclusion of exogenous free fatty acids in the growth media. We thus set out to address the fabY-independent route of FAS in P. aeruginosa and to understand how fatty acids are incorporated into de novo phospholipids. We herein show that the KASIII domain-containing enzyme from the previously unannotated open reading frame PA3286 shunts fatty acid degradation intermediates from the β-oxidation pathway by condensing octanoyl-CoA (C8-CoA) with malonyl-ACP to make β-keto-decanoyl-ACP, a key building block common to saturated fatty acids (SFA), unsaturated fatty acids (UFA), and LPS.

**Materials and Methods**

**Bacterial strains and growth conditions.** All P. aeruginosa strains were derived from the wild-type PAO1 strain (51), while Escherichia coli strains were derived from the reference strain BW25113 (CGSC7636). Strains were grown in LB-Miller medium at 37°C unless otherwise noted. Antibiotic markers were selected with gentamicin (Gm) (100 μg/ml in P. aeruginosa and 10 μg/ml in E. coli), carbenicillin (Carb) (150 μg/ml in P. aeruginosa and 100 μg/ml in E. coli), tetracycline (Tet) (125 μg/ml in P. aeruginosa and 15 μg/ml in E. coli), chloramphenicol (Cam) (20 μg/ml in E. coli), and kanamycin (Kan) (50 μg/ml in E. coli). Bacterial strains and plasmids used in this study are listed in Table 1.

**Growth analysis.** For P. aeruginosa and E. coli strains, starter cultures were made by scraping cells off LB agar plates with appropriate selection antibiotics. Cultures were typically resuspended to ~2 × 10^6 CFU/ml in LB with or without fatty acid supplement (1 to 100 μg/ml) and inducer (1 mM isopropyl-β-D-1-thiogalactopyranoside [IPTG]). Cultures were incubated according to the Sherlock microbial identification system (Microbial ID, Inc., Newark, DE). Fatty acid methyl ester (FAME) composition was determined by gas chromatography with flame ionization detection (GC-FID) or with mass spectrometry (GC-MS). An HP 6890 gas chromatograph with an HP-5MS 30 m column was connected to an HP 5973 mass selective detector. Results were analyzed on the HP MSD ChemStation (version D.01.02). Structural assignments were made by comparison of retention times to authentic FAME standards, as well as from the mass spectra.

**Synthetic lethal scoring between fabY and KASIII domain orthologs.** The conjugative vector pEX18ApGW (9) carrying genes encoding carbencillin resistance (Carb'), oriT transfer origin, and sucrose counterselection (sacB) was used to deliver an accA1 (Gm) resistance cassette with either a transcriptionally coupled and functional E. coli fabH gene (pTMT123) or an inactive fabH fragment (pTMT124) (56) (Table 1). The cassettes were flanked by ~1 kb of homologous DNA flanking the fabY gene in P. aeruginosa PAO1. Vectors were mobilized in trans using E. coli Stellar cells (Clontech) transformed with the helper plasmid pRK2013 (16) as the conjugation-proficient donor strain along with different recipient P. aeruginosa strains as has been described previously (56). Merodiploid colonies were confirmed by colony PCR and outgrown for 4 h in 1 ml of LB only at 37°C. Diluted aliquots were then spread on LB agar containing 7% sucrose (LB-7% sucrose agar) (without NaCl) to counter-select colonies harboring unresolved plasmid. In parallel, aliquots were plated on LB-sucrose agar with Gm or Gm plus decanoate (100 μg/ml) to select colonies from which fabY was replaced with fabH (pTMT123) or deleted (pTMT124). Colonies were counted after incubation at 37°C for 24 h (all pTMT123 constructs) or 48 h for slow-growing mutants (pTMT124 selected on Gm). In cases where less than 10 colonies arose and spontaneous sucrose tolerance was suspected, colonies were patched onto LB agar with Carb to confirm loss of plasmid backbone. The entire experiment was repeated three separate times in each P. aeruginosa recipient strain background.

**Complementation of ΔPA3286.** The pBAD-containing pRC9 plasmid derivative of the site-specific integration vector pMini-CTX1 (26) was used to complement the ΔPA3286 deletion strain. The PA3286 gene, along with 483 bp upstream encompassing the putative native promoter region, was amplified from P. aeruginosa PAO1 genomic DNA template using the primer pair Ctx-PA3286 EcoRV/CTX-PA3286 XhoI (Table 1). The fragment was introduced into pRC9 using the In-fusion system (Clontech), removing the pBAD regulatory element in the process. The resulting vector (pTMT131) was transferred into P. aeruginosa ΔPA3286 by conjugation, after which the integrated vector backbone encoding the tetracycline resistance (Tet') gene was removed by FLP-catalyzed recombination using pFLP2 (25) to generate the complemented strain TMT44 (Table 1).

**PA3286 complementation in E. coli.** The PA3286 open reading frame was cloned by restriction and ligation into the IPTG-inducible expression vector PET24b (+) (Novagen) using the PCR product of primers PA3286 for Ndel/PA3286 rev HindIII (rev stands for reverse) (Table 1). The expression plasmid pET-PA3286 was transformed into E. coli BW25113, in
which the fabH gene was deleted using the fabHEc::camR (fabHEc is the fabH gene from E. coli) cassette and the Red recombination system as described previously (56). The fabH74 strain was verified by flanking PCR, and maintained on 1 mM IPTG for PA3286 induction.

**Recombinant PA3286 protein expression and purification.** Recombinant PA3286 was obtained according to the protocol described for PA174 (56). Briefly, E. coli BL21(DE3) Rosetta 2 (Novagen) cells were transformed with the His tag expression vector pET-PA3286 (Table 1). Colonies were inoculated into 1 liter of LB medium and grown at 37°C to mid-exponential growth (OD600 of 0.6) before induction (see Table 1).

**TABLE 1** Bacterial strains, plasmids, and primers used in this study

<table>
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<th>Bacterial strain, plasmid, or primer</th>
<th>Relevant genotype or phenotypea or primer sequence</th>
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a Carb‡, carbenicillin resistant; Cam‡, chloramphenicol resistant; Kan‡, kanamycin resistant; Gmr‡, gentamycin resistant; Tet‡, tetracycline resistant; FRT, Flp recombinase target; MCS, multicloning site.
b Strain CGSC7636 at the Coli Genetic Stock Center (CGSC).
c Cloned using the In-fusion system (Clontech).
d Cloned into vectors by DNA digestion and T4 DNA-catalyzed ligation.

RESULTS

Exogenous fatty acids C8 and longer rescue growth of P. aeruginosa ΔfabY. The P. aeruginosa ΔfabY strain has a pronounced growth defect both in LB liquid medium and on agar plates. The results of initial experiments suggested that growth on agar could at least partially be rescued by inclusion of the fatty acid decanoate

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FabY or fatty acids must be efficiently utilized by the β-oxidation cycle in order to be degraded to acetyl-CoA. In the latter case, high concentrations of intracellular acetyl-CoA pools would then ultimately be responsible for rescuing growth by improving turnover rates of a putative low-affinity FabH/FabY-type enzyme. To differentiate between these two possibilities, we fed the wild-type and ΔfabY P. aeruginosa strains perdeuterated C_{10} and analyzed the fatty acid composition as fatty acid methyl ester (FAME) derivatives by gas chromatography with flame ionization detection (GC-FID) and mass spectrometry (Fig. 2 and 3). If the decanoate (C_{10}) substrate is being completely degraded to acetyl-CoA, the label should become dispersed with random reincorporation into fatty acids, while a shunt would retain the label and generate unique deuterium-containing fatty acid peaks. Indeed, the FAME GC trace of the wild type revealed additional peaks specific to samples fed perdeuterated C_{10} (in comparison with unlabeled C_{10}), eluting slightly ahead of every major constituent fatty acid peak (Fig. 2). Since the amount of deuterium incorporated into FAMEs is inversely correlated with retention time (42), the shortened retention time along with peak symmetry and relative abundance (≈40% of unlabeled FAME peak area) suggested a shunt mechanism. We then fed perdeuterated C_{10} to the P. aeruginosa ΔfabY strain, which has a rate of de novo FAS flux of less than 5% of the wild type as determined by phospholipid macromolecular labeling with radiolabeled acetate (56). In this case, only the labeled peak was observed, as would be expected in the ΔfabY genetic background, since the incorporation rate of the shunt pathway greatly exceeds de novo FAS initiation.

The structure of each deuterium-labeled FAME peak was assigned using mass spectrometry (Fig. 3). In comparison to the corresponding unlabeled FAME peak (see Fig. S1 in the supplemental material), the deuterated FAME molecular ions were 15 Da heavier and had odd mass numbers for all of the saturated and hydroxylated fatty acids. The odd masses suggested that the terminal ω-carbon was deuterated (~CD_{3}). The McLafferty rearrangement ion, which is a characteristic fragmentation ion for a

**FIG 1** Fatty acid rescue of the P. aeruginosa ΔfabY mutant. (A) Growth curves in LB medium alone (P. aeruginosa PA01 [●] and ΔfabY mutant [▲]) or in LB medium supplemented with decanoate (1 μg/ml [■], 10 μg/ml [▲], or 100 μg/ml [▲]) for the ΔfabY mutant. Growth was measured at 37°C by recording the optical density at 600 nm. (B) The ΔfabY strain was streaked onto LB agar supplemented with the indicated fatty acid (C_{2} to C_{12}) at 100 μg/ml. The plates were incubated at 37°C for 16 h before imaging.

**FIG 2** Fatty acid composition analysis of P. aeruginosa. (A) Fatty acid methyl esters (FAMEs) prepared from strains grown overnight on LB agar supplemented with either 100 μg/ml of decanoate (C_{10}) or perdeuterated decanoate (d_{15}-C_{10}) were analyzed by gas chromatography with flame ionization detection. FAME peaks that contain deuterated fatty acids analyzed by mass spectrometry in Fig. 3 are indicated with an asterisk. Wt, wild type.
FIG 3  Mass spectra of deuterated FAMEs. Unique FAME peaks appearing after supplementation with perdeuterated decanoate (indicated by asterisks in Fig. 2) were analyzed by mass spectrometry. The parent molecular ions ([M]+) and select fragment ions are indicated, including for the McLafferty ion (m/z = 74). The corresponding unlabeled FAME mass spectra are included for comparison in the supplemental information (see Fig. S1 in the supplemental material).
cultures had been fed perdeuterated C10 fatty acid, deuterium labels of each fatty acid were fully substituted with deuterium. Since the spectra supports the conclusion that the 7 terminal carbon atoms of each fatty acid were shunted in the absence of fatty acid (UFA) biosynthesis (33). Similar analysis of the other cis-3-decenoyl-ACP during anaerobic unsaturated fatty acid (UFA) biosynthesis (33). Analysis of the other spectra supports the conclusion that the 7 terminal carbon atoms of each fatty acid were fully substituted with deuterium. Since the cultures had been fed perdeuterated C10 fatty acid, deuterium labels were uniformly lost at C-2 and C-3. The data are consistent with a fatty acid metabolism model whereby P. aeruginosa degrades C10 via one round of the β-oxidation cycle to make C8-CoA. This intermediate is then shunted into FAS, at which point the C9 fatty acid is rebuilt by using unsubstituted malonyl-ACP as the substrate. The terminally labeled β-keto-decanoyl-ACP ester can then be used to supply all the different types of fatty acids needed by the cell, including saturated (SFA), unsaturated (UFA), and 3-hydroxyl fatty acids.

**PA3286 is required for de novo FAS initiation in the absence of fabY and for fatty acid shunting.** On the basis of the pattern of labeled fatty acid incorporation (Fig. 3), we hypothesized a fatty acid shunt consisting of a single enzyme that condenses C5-CoA with malonyl-ACP to make β-keto-decanoyl-ACP. This hypothetical catalytic activity is reminiscent of FabY/FabH, which condense acetyl-CoA with malonyl-ACP; the only difference being the acyl chain length of the CoA thioester acceptor. Since fatty acids were shunted in the absence of fabY and recombinant FabY displays an absolute substrate preference for short-chain acyl-CoA acceptors (56), we focused our search among the previously identified four KASIII domain-containing proteins sharing similarity to FabH of E. coli (56). If one of the KASIII domain-containing proteins were responsible for fatty acid shunting, then C9 supplementation should not rescue growth in a ΔfabY::Gmr exchange allele in the wild-type (Wt) strain. Data are representative of 3 separate experiments and for fatty acid rescue. (A) Vectors conferring aacC1-mediated gentamicin (Gm) resistance that targeted the P. aeruginosa fabH gene were designed to either exchange fabH of E. coli (pTMT123 fabH) or to delete fabY (pTMT124). Methidiploid intermediates (after step 1) were confirmed by PCR analysis and passively resolved by outgrowth in LB medium (step 2). Aliquots were plated on LB-sucrose agar to counterselect unresolved clones and to enumerate the sum of A- and B-type recombination events. In parallel, aliquots were plated on LB-sucrose agar plus Gm and on LB-sucrose agar plus Gm with 100 μg/ml of the fatty acid supplement decanoate (FA) to select only the recombinants arising through A-type recombination. (B) The pTMT123/ΔfabH vectors were individually introduced into the wild-type (Wt) P. aeruginosa, TMT16 (ΔP0998 ΔP0999 ΔP3333 ΔP3286), ΔP3286, and TMT44 (ΔP3286 attB::P3286). CFU resulting from resolving either pTMT123 (black bars) or pTMT124 (hatched bars) were determined by counting colonies that appeared after 24 h or 48 h (#) of incubation at 37°C. The selection media and strain background are indicated below the x axis. Data are representative of 3 separate experiments. The results for the ΔP0998, ΔP0999, and ΔP3333 mutant strains are shown in Fig. S2 in the supplemental material. (C) Gas chromatograms with FID detection of FAME extracts obtained from P. aeruginosa strains grown on LB agar with either 100 μg/ml of decanoate (C10:0) or perdeuterated decanoate (d15-C10:0). Asterisks indicate FAME peaks unique to d15-C10:0-fed samples with structures assigned as in Fig. 3. **FIG 4** Synthetic lethal analysis for fabH with KASIII domain fabH orthologs and fatty acid rescue. (A) Vectors conferring aacC1-mediated gentamicin (Gm) resistance that targeted the P. aeruginosa fabH gene were designed to either exchange fabH of E. coli (pTMT123 fabH) or to delete fabY (pTMT124). Methidiploid intermediates (after step 1) were confirmed by PCR analysis and passively resolved by outgrowth in LB medium (step 2). Aliquots were plated on LB-sucrose agar to counterselect unresolved clones and to enumerate the sum of A- and B-type recombination events. In parallel, aliquots were plated on LB-sucrose agar plus Gm and on LB-sucrose agar plus Gm with 100 μg/ml of the fatty acid supplement decanoate (FA) to select only the recombinants arising through A-type recombination. (B) The pTMT123/ΔfabH vectors were individually introduced into the wild-type (Wt) P. aeruginosa, TMT16 (ΔP0998 ΔP0999 ΔP3333 ΔP3286), ΔP3286, and TMT44 (ΔP3286 attB::P3286). CFU resulting from resolving either pTMT123 (black bars) or pTMT124 (hatched bars) were determined by counting colonies that appeared after 24 h or 48 h (#) of incubation at 37°C. The selection media and strain background are indicated below the x axis. Data are representative of 3 separate experiments. The results for the ΔP0998, ΔP0999, and ΔP3333 mutant strains are shown in Fig. S2 in the supplemental material. (C) Gas chromatograms with FID detection of FAME extracts obtained from P. aeruginosa strains grown on LB agar with either 100 μg/ml of decanoate (C10:0) or perdeuterated decanoate (d15-C10:0). Asterisks indicate FAME peaks unique to d15-C10:0-fed samples with structures assigned as in Fig. 3.
established. The fabY deletion is thus synthetic lethal when introduced into the KASIII domain knockout strain TMT16. We then repeated this experiment in each of the single deletion strains to decipher whether a lone KASIII domain gene was the synthetic lethal partner of fabY or if the relationship was more complex. The merodiplod ΔPA3286 deletion strain resolved with efficiencies that closely mirrored that of TMT16 (Fig. 4B), whereas the other three single deletion strains (ΔPA3333, ΔPA0998, and ΔPA0999) behaved akin to the wild-type P. aeruginosa genetic background (see Fig. S2 in the supplemental material). Reintroduction of a functional copy of the PA3286 gene into the chromosome of the ΔPA3286 mutant at the phage CTX attB site (TMT44) allowed the fabY deletion to now be established, indicating no confounding polarity issues and confirming a true synthetic lethal pairing between fabY and PA3286.

The synthetic lethal relationship between PA3286 and fabY indicates that deacyl-CoA:malonyl-ACP condensing activity. Unlike the P. aeruginosa ΔfabY strain, the ΔPA3286 deletion strain is afeoptoty with respect to growth (56). This suggests that the presumptive FAS initiating activity of PA3286 that allows ΔfabY to grow, albeit slowly, is secondary to another main cellular role utilizing similar substrates. To determine whether PA3286 was indeed the hypothesized C₈-CoA:malonyl-ACP condensing enzyme responsible for fatty acid shunting, we fed perdeuterated C₁₀ to the ΔPA3286 mutant. Whereas perdeuterated C₁₀ fatty acids noticeably had a negative impact on the growth of the wild type and in particular the ΔfabY mutant on LB agar, no inhibitory isotope effect was observed for the ΔPA3286 strain (data not shown). Since the primary isotope effect arising through breaking of C-D bonds in shunted fatty acids to make UFA by the anaerobic FAS pathway was likely responsible, this hinted at a lack of shunting in the ΔPA3286 mutant. We next analyzed the FAME profile by GC-MS as we had been done previously (Fig. 4C). The GC trace for the ΔPA3286 mutant fed perdeuterated C₁₀ showed no en bloc incorporation of deuterated fatty acids and was nearly superimposable with traces derived from samples that had been grown with unlabeled C₁₀. In the complemented PA3286 strain TMT44, deuterated FAMEs having retention times and mass spectra identical to those of deuterated FAMES assigned for the wild type reappeared (Fig. 2). The data support the identification of the PA3286 enzyme as the enzyme responsible for shunting fatty acids in vivo, as well as implicating PA3286 secondary enzymatic activity as the source of FAS initiation in the ΔfabY background.

**PA3286 specifically shunts C₈-CoA in vivo.** While decanoate is likely intercated as a C₈-CoA β-oxidation cycle intermediate by PA3286 (Fig. 3 and 4C), we wanted to determine whether this was specific to C₁₀ or for all fatty acids in general. Perdeuterated long-chain fatty acids (C₁₄ and C₁₆) were therefore fed to the wild-type and ΔPA3286 P. aeruginosa strains and analyzed by GC-MS (Fig. 5A). FAME peaks with retention times and mass spectra consistent with the terminal 7 carbon atoms being fully substituted with deuterium atoms were once again observed only in the wild-type strain upon either C₁₄ or C₁₆ supplementation. An additional FAME peak was present in the C₁₆-fed samples for both the wild-type and ΔPA3286 strains (marked by ○). Analysis of the mass spectrum identified the fatty acid as perdeuterated Δ₁₁₀-C₁₆ (Fig. 5B), based on the parent molecular ion mass (m/z = 301) and a fully deuterated McLafferty ion [CD₂ = C(OD)⁺⁻OCH₃ m/z = 77]. Long-chain fatty acid-CoA esters can be recycled en bloc and incorporated into de novo phospholipids by the glycerol-phosphate and acylglycerol-phosphate acyltransferases PlsB/PlsC in P. aeruginosa (60). Under these growth conditions, the PA3286 shunt clearly occurs in tandem with the direct en bloc PlsB/PlsC-catalyzed incorporation into phospholipids. Collectively, the data suggest that PA3286 has high in vivo substrate specificity for C₈-CoA intermediates and that the shunt is relevant during the catabolism of both long- and medium-chain exogenous fatty acids.

**PA3286 cross complements fabH and confers a fatty acid shunt in E. coli.** Fatty acid metabolism is especially complex in pseudomonads, with an unusually large genetic allocation to both the catabolism and anabolism of fatty acids (32, 50, 51). To further confirm the two activities of PA3286 observed in P. aeruginosa PAO1, to probe the substrate specificity and to determine whether PA3286 requires partner proteins for intercepting β-oxidation cycle intermediates, we introduced the PA3286 gene into the model E. coli strain BW25113. In the presence of a high-copy-number...
plasmid harboring PA3286, we were able to delete the otherwise essential fabH gene that encodes the FAS initiating enzyme β-acetoacyl synthase III in enterobacteria. Unlike the fabY gene that achieved full restoration of the growth rate (56), the PA3286 gene only partially complemented fabH and indicates weak intrinsic β-acetoacyl-ACP synthase catalytic activity (Fig. 6A). This is consistent with our inability to identify PA3286 among a P. aeruginosa genomic cosmid library, which is introduced at low copy number without any strong endogenous E. coli promoters (56).

We next fed the recombinant E. coli strain TMT47 [fabH::catR(pET-PA3286)] a mixture of perdeuterated C10 and unlabeled C16 fatty acid (10:1 [wt/wt]) in order to induce the fad genes involved in β-oxidation (Fig. 6B). The wild-type E. coli strain did not incorporate the deuterium label for any of the peaks, indicating that a medium-chain fatty acid shunt in order to extend exogenous fatty acids is not native to E. coli, in agreement with previous reports (47). Likewise, replacement of fabH with fabY of P. aeruginosa did not result in fatty acid labeling. Only when fabH was replaced with PA3286 did deuterium-substituted FAME peaks become evident. Structure assignment based on the mass spectra of all fatty acids common to P. aeruginosa and E. coli (labeled with asterisks; spectra similar to those in Fig. 3), as well as those specific to E. coli (see Fig. S3 in the supplemental material), were entirely consistent with complete labeling of the terminal 7 carbons. Hence, PA3286 is intrinsically specific for C8-CoA and can intercept β-oxidation intermediates without the need for a partner protein. The second aspect specific to the FAME profile of TMT47 is the near absence of odd-numbered carbon fatty acids (indicated by ◊ in Fig. 6B) and of terminally labeled d7-C16:0 (indicated by + in Fig. 6B; mass spectrum is included in Fig. S3E in the supplemental material). In E. coli, odd-numbered fatty acids arise through utilization of propionyl primers as alternative substrates to acetyl-CoA for FAS by FabH (23, 27). The low-abundance d7-C16:0 peak that is present only in perdeuterated C16-fed E. coli wild-type or TMY32 [fabH::catR(pET-FabY)] may arise through utilization of d7-butyryl-CoA produced by the β-oxidation cycle as a FAS primer by either FabH or FabY, respectively. In line with the previously observed in vivo substrate specificity in P. aeruginosa, PA3286 did not efficiently utilize either short-chain acyl-CoA primer in E. coli. These short-chain acyl-CoA substrates are

FIG 6

Complementation of E. coli fabH by the P. aeruginosa PA3286 gene. (A) The E. coli strain TMT47 [fabH::catR(pET-PA3286)] was grown at 37°C in LB medium alone (●) or with 1 mM IPTG (○) and compared to the parent Wt strain (□). Growth was monitored by measuring the optical density at 600 nm. (B) The gas chromatogram-FID trace for FAME samples prepared from E. coli strains grown on LB agar with perdeuterated decanoate (d19-C10:0 100 µg/ml) and palmitate (C16:0 10 µg/ml) for induction of fatty acid degradation genes. The parent wild-type E. coli BW25113 and TMY32 [fabH::catR(pET-PA3174)] strains were included as controls. Asterisks indicate deuterated FAMEs of identical structures to those already assigned in Fig. 3 based on time of elution and mass spectra analysis. Mass spectra and assignment of deuterated FAME peaks unique to E. coli expressing PA3286 (C14:0 3-OH, C17:0 cyclo α7c, and C19:0 cyclo α7c) along with the terminally labeled d7-C16:0 (+) peak present only in the absence of PA3286 are shown in Fig. S3 in the supplemental material. Odd-numbered acyl chain FAMEs with reduced abundance in strain TMT47 are indicated (○).
present at much lower intracellular concentrations in comparison to the glycolytic product/tricarboxylic acid cycle (TCA) intermediate acetyl-CoA. Thus, while PA3286 can condense enough of the abundant acetyl-CoA substrate to maintain FAS initiation in the absence of fabH, other low-affinity short-chain acyl-CoA substrates present in trace amounts are not appreciably utilized.

**Characterization of recombinant PA3286 β-ketoacyl ACP synthase activity.** The PA3286 open reading frame is predicted to encode a 350-amino-acid protein (http://www.ncbi.nlm.nih.gov/). However, repeated attempts to express recombinant PA3286 failed to produce soluble protein. We thus chose an upstream start codon and cloned the gene (encoding a 373-amino-acid protein) into a PET-24b(+) expression vector so as to append a C-terminal His tag for purification. The longer protein was readily soluble and amenable to characterization. The substrate specificity of the PA3286 enzyme was explored using various straight-chain saturated acyl-CoAs (C₂ to C₁₆) as acceptors along with malonyl-CoA, as has previously been described for FabY (56). Reaction products were separated by conformation-sensitive urea-PAGE (46), and the gels were stained with Coomassie blue. PA3286 only weakly accepted short-chain acyl-CoA substrates, including acetyl-CoA, butyryl-CoA, and hexanoyl-CoA (Fig. 7). The substrate specificity suggests why shorter exogenous fatty acid did not rescue growth in the *P. aeruginosa ΔfabY* mutant (Fig. 1B), even though they can be utilized as the sole carbon source (31). The two best substrates were clearly C₈-CoA and C₁₀-CoA. When the experiment was repeated with C₈-CoA and C₁₀-CoA acceptors using radiolabeled [2-¹⁴C]malonyl-ACP, radioactivity was incorporated into the faster-migrating bands (data not shown). This confirmed PA3286 is a malonyl-ACP condensing KAS as opposed to an acyl-CoA:ACP transacylase. CoA thioester acceptors with longer acyl chains (C₁₂, C₁₄, and C₁₆) were all excluded as substrates. The in vitro substrate specificity is consistent with the *in vivo* characteristics of PA3286, and together, these results support our assignment of PA3286 as a β-keto-decanoyl-ACP synthase that condenses malonyl-ACP with C₈-CoA originating from the fatty acid β-oxidation degradation cycle.

**DISCUSSION**

While it is clear that FAS in *P. aeruginosa* is a central metabolic pathway upon which the expression of multiple virulence and regulatory factors depend, exogenous C₁₀ fatty acid noticeably muted the growth-defective phenotype in the *ΔfabY* strain (Fig. 1). It might be expected, therefore, that the efficacy of a small-molecule inhibitor of FabY would likewise be subject to the fatty acid content of the environment where it is administered. This is a particularly pressing concern with any intended anti-pseudomonal agent, as an important segment of the intended patient population has cystic fibrosis. *P. aeruginosa* can be found in the respiratory tracts of 80% of all cystic fibrosis patients by the time they are 18 years old (20), an environment where there is an already abundant lipid nutrient source in the form of pulmonary surfactant. Pulmonary surfactant, being composed of 90% lipids (mostly dipalmitoyl phosphatidylcholine) along with 10% surfactant proteins (1, 19), is an important *in vivo* nutrient source for *P. aeruginosa* (31, 48). Transcription of *fad* β-oxidation cycle genes is induced *in vivo*, and mutants deficient in fatty acid degradation exhibited decreased fitness in a mouse lung infection model (32). Our goal in the present work was to address how the Δ*fabY* strain is rescued by exogenous fatty acids and whether the mechanism(s) might potentially compromise an inhibitor targeting FabY in *P. aeruginosa*.

Based on the perdeuterated feeding experiments with *P. aeruginosa* and the defined isogenic mutants (Fig. 2 to 5), the synthetic lethal relationship between PA3286 and *fabY* (Fig. 4B), the PA3286 cross complementation of *fabH* activity and fatty acid shunting in *E. coli* (Fig. 6), and *in vitro* characterization of recombinant PA3286 (Fig. 7), we propose that fatty acids rescue the *P. aeruginosa ΔfabY* mutant through incorporation via the PA3286-mediated β-oxidation cycle to the FAS shunt (Fig. 8). In this pathway, C₈-CoA intermediates originating from the oxidation of exogenous fatty acids C₆ and longer are intercepted by PA3286 and condensed with malonyl-ACP to make the FAS intermediate β-keto-decanoyl-ACP. PA3286 belongs to the β-keto-acyl carrier protein synthase III family (KASIII), with *E. coli* FabH being the prototypical member (52). As in *E. coli* FabH and most other characterized orthologs (18), PA3286 contains the class-defining Cys-His-Asn catalytic triad common to KASIII enzymes (56), and so the catalytic function of condensing malonyl-ACP with an acyl-CoA is not altogether unexpected. However, KASIII domain-containing enzymes generally exhibit high preference for short-chain acyl-CoA substrates (18). The use of longer-chain acyl-CoA primers by KASIII domain synthases does have precedent, as the *Mycobacterium tuberculosis* FabH prefers long-chain acyl-CoA substrates *in vitro* and *in vivo* during the synthesis of mycolic acids (5, 8). With an overall sequence identity between *E. coli* FabH and PA3286 of 27%, there is significant difference between the two proteins that may contribute to its substrate specificity. Structural information of PA3286 is needed to shed more light on how the KASIII fold has been co-opted by PA3286 into accommodating longer acyl chains and whether there are common themes with FabH from *M. tuberculosis*.

The β-keto-decanoyl ACP thioester shunt product generated by PA3286 is a versatile precursor that is uncommitted to the biosynthesis of any specific essential fatty acid (Fig. 8). Pathways for the biosynthesis of LPS (3-OH decanoyl-ACP/3-OH dodecanoyl-ACP), UFA (via trans-2-decenoyl-ACP), and SFA can all utilize nutrient-acquired fatty acids for extension without having to degrade them down to C₂-CoA before reincorporation via de novo FAS. This is critical, as FAS is bioenergetically the most costly synthetic process for any membrane component (59); for instance, building an n-acyl-CoA thioester (where n = C₁₀⁻) from C₂-CoA consumes [(n/2) − 1] ATP molecules along with [(n/2) − 1] × 2 reducing equivalents. In comparison, degradation of n-acyl-CoA β-oxidation intermediates down to C₂-CoA before reassembly into n-acyl-ACP thioesters by FAS consumes a net [(n/2) − 1] ATP molecules. However, the PA3286 C₈ shunt uses...
only \((n/2 - 4)\) ATP molecules to accomplish the same feat, yielding a further net savings of 3 ATP molecules per metabolized \(n\)-acyl fatty acid molecule (Fig. 8). The use of a \(C_n\) fatty acid chain length for shunting by \(P. aeruginosa\) now joins certain \(Vibrio\) species which are also capable of extending exogenous fatty acids en bloc using the FAS pathway (7, 21, 28). In the case of \(Vibrio\), exogenous medium-chain fatty acids are directly ligated to free ACP to form acyl-ACP thioesters. While mechanistically distinct and independent of the \(\beta\)-oxidation pathway, the end result is equivalent to the \(PA3286\) shunt in that the acyl-ACP produced can be utilized for incorporation into LPS, SFA, and UFA (29).

It has been reported that the entire FAS can be bypassed by exogenous fatty acid uptake from serum in certain Gram-positive pathogens, including \(Streptococcus\) (4). The essentiality of the FAS pathway has not been questioned in Gram-negative bacteria, in part due to the dependence of LPS biogenesis on FAS supplied \(3\)-hydroxyacyl-ACP precursors (41). The LPS of \(P. aeruginosa\) contains both \(3\)-hydroxydecanoyl and \(3\)-hydroxydodecanoyl acyl chains (35). Although the \(PA3286\) shunt can supply \(3\)-hydroxydecanoyl-ACP (Fig. 8), it should be noted that FabG is still needed to introduce the \(\beta\)-hydroxyl group. Further, even if a \(C_{16}/C_{18}\) fatty acid source were available for direct incorporation into UFA/SFA membrane phospholipids by the combined activities of the acyl-glycerol-phosphate acyltransferases PslB/PslC and the aerobic desaturases DesB/DesC/DesA (60), at least one more turn from the FAS cycle enzymes would still be necessary to form \(3\)-hydroxydodecanoyl-ACP. Hence, it seems unlikely that \(PA3286\) would impact the efficacy of FAS inhibitors beyond FabY. In the absence of having access to a FabY-specific small-molecule inhibitor, whether FabY can be entirely bypassed by uptake of exogenous fatty acids still must remain an open question. However, if one assumes that treatment with a FabY inhibitor will phenocopy the \(fabY\) deletion strain, then it certainly seems likely that a FabY inhibitor could be compromised by exogenous fatty acids. More than 95% of the total extracted fatty acids were terminally labeled with deuterium in the \(fabY\) sample (Fig. 2). This suggests that the shunt can satisfy the majority of the cellular needs for acyl-ACP primers, in effect making \(\beta\)-acetoyactyl synthase (FabY/FabH) ac-
tivity superfluous. Indeed, there may even be intrinsic cellular regulatory mechanisms to repress de novo FAS initiation by FabY, as synthesis and degradation in most bacteria are tightly regulated in order to maintain homeostasis and to avoid a futile metabolic cycle (17, 58). We are currently studying these putative regulatory mechanisms in order to understand how the PA3286 shunt is integrated into the global fatty acid metabolism network of P. aeruginosa.

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