A cryptic long-chain 3-ketoacyl-ACP synthase in the *Pseudomonas putida* F1 unsaturated fatty acid synthesis pathway

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Huijuan Dong,1,2 Jincheng Ma,1 Qunyi Chen,1 Bo Chen,1 Lujie Liang,1 Yuling Liao,1 Yulu Song,1 Haihong Wang1,4, and John E. Cronan2,3,*

From the 1Guangdong Provincial Key Laboratory of Protein Function and Regulation in Agricultural Organisms, College of Life Sciences, South China Agricultural University, Guangzhou, Guangdong, China; 2Department of Microbiology, 3Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

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The *Pseudomonas putida* F1 genome contains five genes annotated as encoding 3-ketoacyl-acyl carrier protein (ACP) synthases. Four are annotated as encoding FabF (3-ketoacyl-ACP synthase II) proteins, and the fifth is annotated as encoding a FabB (3-ketoacyl-ACP synthase I) protein. Expression of one of the FabF proteins, FabF2, is cryptic in the native host and becomes physiologically important only when the repressor controlling fabF2 transcription is inactivated. When derepressed, FabF2 can functionally replace FabF1 (3-ketoacyl-ACP synthase I) protein. Expression of the FabF proteins, FabF2, is cryptic in the native host and becomes physiologically important only when the repressor controlling fabF2 transcription is inactivated. When derepressed, FabF2 can functionally replace FabF, and when expressed from a foreign promoter, had weak FabF activity. Complementation of *Escherichia coli* fabB and fabF mutant strains with high expression showed that *P. putida* fabF1 restored *E. coli* fabF function, whereas fabB restored *E. coli* fabB function and fabF2 restored the functions of both *E. coli* fabF and fabB. The *P. putida* ΔfabF1 deletion strain was almost entirely defective in synthesis of cis-vaccenic acid, whereas the ΔfabB strain is an unsaturated fatty acid (UFA) auxotroph that accumulated high levels of spontaneous suppressors in the absence of UFA supplementation. This was due to increased expression of fabF2 that bypasses loss of fabB because of the inactive mutant strain still accumulated suppressors at low UFA concentrations.

*Pseudomonas putida* is a saprophytic soil γ-proteobacterium (1) that is readily distinguished from *Pseudomonas aeruginosa* by the lack of pyocyanin production and an inability to grow at 42 °C (2, 3). Although *P. putida* and *P. aeruginosa* have a high level of genomic conservation (85% of the predicted coding regions are shared), *P. putida* genomes lack key virulence factors including exotoxin A and type III secretion systems (4). Therefore, *P. putida* is considered a safe bacterium for cloning and expression of foreign genes and is a major *Pseudomonas* research organism (1, 5).

Fatty acids are the major components of membrane phospholipids (6). Bacteria use the type II fatty acid synthesis system to produce long-chain fatty acids through a cycle of elongation, reduction, dehydration, and reduction reactions catalyzed by a series of discrete enzymes (7) (Fig. S1).

The 3-ketoacyl-acyl carrier protein (ACP) synthases catalyze the elongation reactions of fatty acid synthesis (Scheme 1).

![Scheme 1](image)

*For correspondence: John E. Cronan, jecronan@illinois.edu; Haihong Wang, wanghh36@scau.edu.cn.

Escherichia coli has two long-chain 3-ketoacyl ACP synthases: 3-ketoacyl ACP synthase I (FabB) and 3-ketoacyl ACP synthase II (FabF). *E. coli* FabB is required to elongate cis-3-decenoyl ACP to long-chain unsaturated acyl-ACPs and together with FabA is a key enzyme in unsaturated fatty acid (UFA) synthesis (8). *E. coli* FabF is responsible for extending saturated acyl ACPs and for conversion of cis-9-hexadecenoyl-ACP (palmitoleoyl-ACP) to cis-11-octadecenoyl-ACP (cis-vaccenoyl-ACP), a reaction regulated by growth temperature (9). Although many diverse bacteria encode long-chain 3-ketoacyl ACP synthases similar to *E. coli* FabB and FabF, there are significant differences. The *Haemophilus influenzae* genome...
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codes only fabB and lacks fabF (10). Clostridium acetobutylicum and Ralstonia solanacearum each have only a single 3-ketoacyl ACP synthase, but these FabF enzymes have both 3-ketoacyl ACP synthetase I (FabB) and II (FabF) activities (11, 12). Lactococcus lactis FabB also has both FabB and FabF activities, but upon overexpression, the FabF can replace FabB, the short-chain 3-ketoacyl ACP synthase III (13). Shewanella oneidensis MR has one fabB and two fabF genes where fabF1 has the functions of both 3-ketoacyl ACP synthase I and II (14). Increased expression of fabF1 can restore the synthesis of UFAs when the pathway is blocked by inactivation of fabB (15).

The genome of P. putida F1 shows complexity in that four fabF genes are annotated: together with a single fabB, only the fabF1 and fabB genes are located with other fatty acid synthesis genes (Fig. S2). Complementation studies using E. coli fabB and fabB(Ts) fabF mutant strains showed that fabF2 has the functions of both E. coli enzymes when highly expressed. In the pseudomonads, fabF2 is ubiquitous and homologous genes are found in other bacteria (see below). In this report, the function of fabF2 and fabB in P. putida F1 was explored. Deletion mutants of fabB were unable to synthesize UFAs, although suppressors accumulated and accumulation required a functional fabB gene. We report that ΔfabB suppression required increased expression of fabF2 and this resulted from mutational activation of a repressor gene encoded in the same operon.

Results

P. putida F1 FabF proteins and FabB protein functionally replace E. coli FabB and FabF in vivo

The P. putida F1 genome contains one gene annotated as fabB, Pput_1693, and four annotated as fabF genes: Pput_3798 (fabF1), Pput_2422 (fabF2), Pput_2974 (fabF3), and Pput_2975 (fabF4) (Fig. S2). Alignments of the protein sequences showed that the residue identities between FabB and FabF2 with E. coli FabB are 66.3% and 34.2%, respectively, whereas the residue identities between FabF1 and FabF2 with E. coli FabF are 66.9% and 45.7%, respectively. The FabF3 and FabF4 identities with E. coli FabB are essentially the same methods to purify the FabB and FabF2 proteins had monomeric molecular masses of 46 kDa consistent with the values calculated from the sequences of the tagged proteins (Fig. S5). Tryptic peptide mass spectral analyses confirmed the identities of the purified proteins (Fig. S5). To assay the activities of P. putida F1 FabB and FabF2, we used essentially the same methods to purify the E. coli fatty acid biosynthetic proteins, FabB, FabD, FabA, FabG, and FabI plus Vibrio harveyi acyl-ACP synthetase. E. coli holo-ACP was also purified. To test the functions of FabB and FabF2 in vitro, the elongation steps of the fatty acid synthesis reaction were reconstituted, followed by analysis by conformationally sensitive gel electrophoresis. At both 37 °C and 42 °C, P. putida F1 FabB and FabF2 elongate octanoyl-ACP using malonyl-ACP to

Analysis of the P. putida F1 FabB and FabF2 activities in vitro

The P. putida F1 FabB and FabF2 proteins were expressed in E. coli strain BL21(DE3) as described in Experimental procedures. The histidine-tagged fusion proteins were purified by nickel chelate chromatography. As measured by denaturing gel electrophoresis, the purified FabB and FabF2 proteins had monomeric molecular masses of 46 kDa consistent with the values calculated from the sequences of the tagged proteins (Fig. S5). Tryptic peptide mass spectral analyses confirmed the identities of the purified proteins (Fig. S5). To test the functions of FabB and FabF2 in vitro, the elongation steps of the fatty acid synthesis reaction were reconstituted, followed by analysis by conformationally sensitive gel electrophoresis. At both 37 °C and 42 °C, P. putida F1 FabB and FabF2 elongate octanoyl-ACP using malonyl-ACP to
generate low levels of long-chain acyl-ACP species (Fig. 2A). We also assayed the UFA synthetic abilities of purified PpFabB and PpFabF2 because this is their primary role in fatty acid synthesis. E. coli FabA converted 3-hydroxydecanoyl-ACP to a mixture of trans-2- and cis-3-decenoyl-ACPs. In the presence of E. coli FabB, cis-3-decenoyl-ACP is elongated with malonyl-ACP to 3-ketodecanoyl-ACP, which is reduced by E. coli FabG to give a new band corresponding to 3-hydroxy-cis-5-dodecenoyl-ACP. PpFabB and PpFabF2 both produce the same products as E. coli FabB (Fig. 2B) and hence are active in UFA synthesis in vitro.

Construction of P. putida F1 fabF deletions and analysis of their phenotypes

To determine the physiological functions of the P. putida F1 fabF proteins in fatty acid biosynthesis, strains having each fabF gene deleted were constructed by allelic replacement. The fatty acid compositions of the ΔfabF1 and ΔfabF2 strains were determined by [1-14C]acetate labeling (Fig. 3) and GC-MS (Table 2). Relative to the WT strain, the ΔfabF1 strain showed a large decrease in cis-vaccenic acid (C18:1), with a concomitant increase in palmitoleic acid (C16:1). The fatty acid compositions of the fabF2, fabF3, and fabF4 deletion strains were indistinguishable from that of the WT strain. The fatty acid composition of the ΔfabF1 ΔfabF2 double mutant was essentially the same as that of the ΔfabF1 strain (Table S3).

The ΔfabF1 and ΔfabF2 strains plus the ΔfabF1 ΔfabF2 double-mutant strain were complemented with plasmids carrying the WT copy of a gene, and the fatty acid compositions of the strains were determined by GC-MS. In the fabF1 complemented strain, the levels of C18:1 sharply increased and the C16:1 level sharply decreased (Table S3). Hence, P. putida F1 FabF1 is responsible for saturated fatty acid (SFA) synthesis.

Table 1
Fatty acid compositions of CL28 complemented strains

| Fatty acid % | CL28/vector | CL28/pfabB | CL28/pfabF1 | CL28/pfabF2 | CL28/pfabF3 | CL28/pfabF4 |
|-------------|-------------|------------|-------------|-------------|-------------|-------------|
| C16:0       | 4.0 ± 0.1   | 5.0 ± 0.1  | 3.5 ± 0     | 4.3 ± 0.1   | 4.8 ± 0.5   | 44.3 ± 0.2  |
| C18:0       | 27.0 ± 0.6  | 24.7 ± 0.2 | 29.2 ± 1.0  | 27.4 ± 1.1  | 25.7 ± 1.9  | 27.8 ± 0.7  |
| C18:1 cyclo | 64.8 ± 0.4  | 67.0 ± 0.3 | 48.9 ± 2.1  | 61.7 ± 1.3  | 67.4 ± 2.0  | 65.0 ± 0.9  |
| C18:0 cyclo| 3.0 ± 0.1   | 1.4 ± 0.1  | 1.2 ± 0.1   | 2.1 ± 0.1   | 1.7 ± 0.4   | 2.0 ± 0.2   |
| C16:0 cyclo| 0.3 ± 0.1   | 0.5 ± 0    | 2.0 ± 0.6   | 0.5 ± 0.1   | 0.3 ± 0.1   | 0.4 ± 0.1   |
| C18:1 cyclo| 0.9 ± 0.3   | 1.3 ± 0.4  | 15.3 ± 1.7  | 4.1 ± 0.1   | 0.1 ± 0     | 0.3 ± 0.2   |

These experiments were done using a high copy number plasmid with transcription from the strong arabinose promoter and translation from a strong ribosome-binding site. The increase in C18:1 detected in the CL28/pfabF2 strain is the product of high expression.
and for conversion of C16:1-ACP to C18:1-ACP. Expression of fabF3 or fabF4 failed to remedy defects in C18:1 synthesis because of loss of fabF1, whereas fabF2 had only weak activity (Table S3).

Construction and properties of the P. putida ΔfabB strain

A P. putida ΔfabB mutant strain was constructed by replacing the gene with a kanamycin resistance marker. The resulting ΔfabB mutant strain was a UFA (oleic acid) auxotroph demonstrating the importance of the gene in P. putida F1 UFA synthesis (Fig. 4A). However, the ΔfabB strain was unstable and spontaneously accumulated suppressors even at high oleate concentrations. This argued that a mutation that allowed the ΔfabB strain to synthesize UFA gave faster growth than that obtained by oleate supplementation. The dependence on oleate concentration argued that most of the oleate was consumed by β-oxidation, thereby limiting the amount available for phospholipid synthesis. Endogenous synthesis avoids β-oxidation because ACP rather than CoA thioesters are used and the 3-hydroxy thioester intermediates use opposite stereoisomers. The ΔfabB suppressor mutations restored UFA synthesis and allowed growth without oleate (Fig. 4B). The above data indicated that FabF2 has the functions of both E. coli FabB and FabF, so it seemed likely that some alteration of fabF2 could explain the accumulation of suppressors. To test this possibility, a ΔfabB ΔfabF2 double-mutant strain was constructed. The ΔfabB ΔfabF2 strain was a UFA auxotroph that failed to form suppressors (Fig. 4C). This indicated that a mechanism requiring fabF2 could replace fabB function.
Plasmids encoding fabF2 or fabB complemented the ΔfabB ΔfabF2 strain (Fig. 5). The complemented strains restored growth and UFA synthesis and no longer required oleic acid. The growth phenotypes of the complemented strains indicated that FabB was more active than FabF2 (Fig. 5A). We also tested plasmids encoding fabF1, fabF3, and fabF4 for the ability to restore UFA synthesis in the ΔfabB ΔfabF2 strain by [1-14C]acetate labeling (Fig. 5B) and found no role for these genes in *P. putida* UFA synthesis per se although FabF1 can elongate 16:1 to 18:1 (Table 1). The failure of fabF2 to complement the *P. putida* ΔfabF1 strain, although it complements the *E. coli* ΔfabF strain, seems likely to be poor expression from the pSRK vector promoter in *P. putida* plus the high levels of expression in *E. coli*. Note that upon FabB overexpression, significant levels of cis-vaccenolate are synthesized in a fabF strain (17).

*Aeromonas* PAO1 has three pathways for UFA synthesis (18), whereas *P. putida* F1 has only two pathways: the fabA-fabB pathway and the desA desaturase. Although the *P. putida* desaturase (Pput_0232) has 84% identity to the *Aeromonas* DesA protein, it is unable to support growth of a ΔfabB strain, although it may be responsible for the traces of UFAs seen in the ΔfabB ΔfabF2 strain. To further test the ability of *P. putida* FabF2 to convert C16:1-ACP to C18:1-ACP, a ΔfabF1 ΔfabF2 ΔdesA triple deletion strain was constructed by homologous recombination. In this triple mutant, the chromosomal fabF gene was replaced by fabF2 (Fig. S6). Therefore, FabF2 was the sole long-chain 3-ketoacyl-ACP synthase in this strain. Fatty acid compositions obtained by [1-14C]acetate labeling (Fig. S6) and GC-MS (Table S4) showed that the level of C18:1 increased only slightly (4.4%–6.4%) accompanied by a moderate decrease in the C16:1 level (69.3%–63.4%) relative to the ΔfabF1 ΔfabF2 ΔdesA triple mutant. Hence, in the native host, FabF2 has only a weak ability to convert of C16:1-ACP to C18:1-ACP.

### Analysis of suppressor phenotypes: Interaction with the β-oxidation pathway and inhibition by octanoic acid

A high concentration of oleic acid (5 mM) generally decreased the accumulation of ΔfabB suppressors relative to lower concentrations, suggesting that the suppressor accumulation is related to the concentration of oleic acid (Fig. 6).
After entry into the cell, oleic acid becomes activated by an acyl-CoA synthetase (FadD), and most of the acyl-CoA will enter the \( \beta \)-oxidation cycle for degradation to acetyl-CoA. Thus, only a small fraction of the UFA is available for synthesis of membrane phospholipids.

FadD1 and FadD2, which are homologs of the \( P. \) aeruginosa \( \text{fadD1} \) and \( \text{fadD2} \) acyl-CoA synthetase genes, have been identified as the major acyl-CoA synthetases in \( P. \) putida (18, 19). However, the \( \Delta \text{fadD1} \) \( \Delta \text{fadD2} \) double mutant remains able to grow with oleic acid as the sole carbon source in minimal medium (20, 21), indicating the presence of other fatty acid activation pathways. We inactivated both \( \text{fadD} \) genes in a \( \Delta \text{fabB} \) strain and found that oleic acid supplementation remained effective indicating that, as in \( P. \) aeruginosa (20), activation systems other than FadD1 and FadD2 are present in \( P. \) putida F1. Alignments with \( E. \) coli FadB and FadA indicated that Pput_3606 and Pput_3605 encoded \( \beta \)-oxidation cycle enzymes: enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (FadB) and 3-ketoacyl-CoA thiolase (FadA), respectively. Inactivation of the \( \beta \)-oxidation cycle enzymes in the

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**Figure 4. Characterization of \( \Delta \text{fabB} \) and \( \Delta \text{fabB} \) \( \Delta \text{fabF2} \) mutants of \( Pseudomonas putida \).**

A, the \( \Delta \text{fabB} \) strain was a UFA auxotroph and suppressors (1–3) (denoted as Supp) accumulated on LB medium plates containing oleic acid (see inset). B, upon restreaking, the suppressors restored growth on LB plates lacking oleic acid. C, the \( \Delta \text{fabB} \) \( \Delta \text{fabF2} \) strain was stably auxotrophic and did not accumulate suppressors.

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**Figure 5. Either the \( \text{fabB} \) or \( \text{fabF2} \) genes of \( Pseudomonas putida \) F1 complement the \( \Delta \text{fabB} \) \( \Delta \text{fabF2} \) double mutant.**

A, both \( \text{fabB} \) and \( \text{fabF2} \) restore the growth of \( \Delta \text{fabB} \) \( \Delta \text{fabF2} \) mutant in the absence of oleic acid. The growth of the \( \text{fabB} \) complemented strain is significantly better than that of the \( \text{fabF} \) complemented strain upon induction with 1 mM IPTG. B, the phospholipid fatty acids of \( P. \) putida F1 WT, \( \Delta \text{fabB} \), \( \Delta \text{fabB} \) \( \Delta \text{fabF2} \), and the complemented strains labeled with [1-\(^{14}\)C]acetate (\( \Delta \text{fabB} \) (\( \Delta \text{B} \)), \( \Delta \text{fabB} \) \( \Delta \text{fabF2} \) (\( \Delta \text{BF2} \))) lack UFA synthesis. These are scans of autoradiograms of two different thin layer chromatographic separations. In the right-hand autoradiogram, an erroneously loaded lane was to the right of the first lane. This lane was deleted, and the remaining lanes spliced together. The splice junction is depicted by the vertical line. The right-hand three lanes are from a separate thin layer.
ΔfabB strain blocked accumulation of suppressors as did inactivation of FadD1 and FadD2 (Fig. 6), indicating that accumulation of ΔfabB suppressors is closely related to the fatty acid β-oxidation pathway. Degradation of most of the oleate supplements limits the supply of oleoyl-CoA available for incorporation into phospholipids and provides a strong selection for accumulation of suppressors. The phenotype of the ΔfabB ΔfadA ΔfadB triple mutant was as expected, whereas the ΔfabB ΔfadD1 ΔfadD2 triple mutant argues that the product of the unknown pathway that activates oleate in this strain somehow avoids β-oxidation even when grown in a rich medium (Fig. 6). We first suspected that mutations in the fabF2 coding sequence or the upstream promoter region were responsible for suppression of the ΔfabB mutant strain. However, sequencing showed that this was not the case. We then considered Pput_2425, a gene annotated as encoding a putative regulatory protein of the TetR family, which is the first gene in the fabF2 operon (fabF2 is the last gene, the two intervening genes encode an unrelated efflux pump) (Fig. S2). Pput_2425 seemed a good candidate because it appeared to be cotranscribed with fabF2 and several members of the TetR family participate in regulation of fatty acid synthesis (22, 23). To test if the protein encoded by Pput_2425 is involved in ΔfabB suppression, we constructed a ΔfabB ΔPput_2425 double-mutant strain. The double mutant grew stably in LB medium containing 5 mM oleic acid and weakly in the absence of oleic acid with accumulation of suppressors. Given these data, we selected several independent suppressor strains and sequenced their Pput_2425 genes plus the upstream regions and found that all suppressors had mutations within the coding sequence. Five had deletion mutations (two 299(A), 265(C), 50–51(AT), 159(C)) and one had a missense mutation 130(G-C). The frameshifts caused by the 50 to 51(AT), 299(A), and 265(C), respectively, gave extended out-of-frame translation products of 18, 19, and 5 residues before a termination codon was encountered, whereas the 159(C) mutation directly created a TAA termination codon. These data together with the deletion allele indicate that the regulatory protein encoded by Pput_2425 is a repressor that negatively regulates fabF2 expression. Note that deletion of the putative exporter genes, Pput_2424 and Pput_2423, in the ΔfabB strain failed to alter the phenotype of the ΔfabB strain. These strains remained auxotrophic and accumulated suppressors (data not available).

Figure 6. Analysis of the growth phenotypes of fabB mutants in different concentrations of oleic acid and dependence on β-oxidation. The ΔfabB ΔPput_2425 strain (denoted as ΔΔPput_2425) grew stably with 5 mM oleic acid, but as the concentration of oleic acid decreased, suppressors accumulated. The ΔfabB ΔPput_4737 strain denoted by ΔΔPput_4737 and the ΔfabB strain denoted by ΔB both accumulated suppressors at the lower concentrations of oleic acid. The ΔfabB ΔfadD1 ΔfadD2 and ΔfabB ΔfadB ΔfadA strains (denoted as ΔfabB ΔfadD1 ΔfadD2 and ΔB, respectively) grew stably at the high oleic acid concentration.
shown). To test the possible influence of transcriptional polarity, an in-frame deletion allele of Pput_2425 was constructed in the ΔfabB strain. This strain accumulated suppressors in the absence of oleic acid as previously seen for the original ΔfabB strain that contained the prior Pput_2425 deletion allele (data not shown).

Suppression of the ΔfabB mutation is due to increased fabF2 expression resulting from inactivation of Pput_2425 (Fig. 6). Another putative regulatory gene is Pput_4737, which encodes a protein that is 72.6% identical to P. aeruginosa PAO1 DesT, a negative regulator of UFA synthesis (24). To test the possible involvement of this regulatory protein in ΔfabB suppression, ΔPput_4737 and ΔPput_4737 ΔfabB strains were constructed. The ΔPput_4737 mutation failed to alter the phenotype of the ΔfabB strain. The ΔPput_4737 ΔfabB strain grew stably in LB medium containing 5 mM oleic acid and grew weakly with suppressor accumulation in the absence of oleic acid (Fig. 7A). Moreover, the ΔPput_4737 mutation failed to affect the phenotype of the ΔfabB strain (Fig. 7A). Labeling with [1-14C]acetate demonstrated that only in the ΔfabB ΔPput_2425 strain was UFA synthesis restored (Fig. 7B).

An interesting phenotype of the ΔfabB suppressor strains is that they are variably sensitive to octanoic acid (Fig. S7). A concentration of 5 mM octanoate completely inhibits growth of some suppressor strains but only partially inhibits growth of others, whereas the WT strain grows normally (Fig. S7). A possible scenario to explain growth inhibition is discussed below.

Expression of the Pput_2425, Pput_2424, fabF2, and fabA genes in P. putida F1

The upstream 200-bp regions of Pput_2425 and fabF2 were fused to a promoter-less lacZ of the pSRK-lacZ expression vector (25), to allow detection of possible promoters. The Ppput_2425 fusion plasmids were transferred into the WT strain, the ΔPput_2425 strain plus the ΔfabB suppressor strains, and β-galactosidase activity was measured. The β-galactosidase activity of the Pput_2425 mutant and suppressor strains was much higher than that of the WT strain (Fig. 8, A and C). The Ppput_2424 and PfabF2 lacZ fusion plasmids were then transformed into the WT and Δpput_2425 strains. However, for PfabF2, the β-galactosidase activity of the Δpput_2425 strain was only slightly higher than that of the WT strain (Fig. S8A), indicating that the fabF2 upstream 200-bp segment lacked a promoter, whereas the β-galactosidase activity of Ppput_2424 was greatly increased in the Δpput_2425 strain (Fig. 8B). These results indicated that Pput_2425 controls expression of itself, the fabF2 gene cluster, and thus fabF2 expression. Reverse transcription PCR confirmed that fabF2 is cotranscribed with Pput_2425 (Fig. S9).

Figure 7. The phenotypes of the ΔfabB ΔPput_2425 and ΔfabB ΔPput_4737 strains. A, the growth phenotype of the ΔfabB strain (ΔB), the ΔfabB ΔPput_2425 strain (ΔBΔPput_2425), and the ΔfabB ΔPput_4737 (ΔBΔPput_4737) strain on LB plates in the presence or absence of oleic acid. The left side of the middle plate contains ΔPput_2425Δ fabB suppressors (denoted as Supp), whereas the right side is the parental ΔPput_2425Δ fabB strain. B, the phospholipid fatty acids of Pseudomonas putida F1 WT, ΔfabB, ΔfabB ΔfabF2, ΔfabB ΔPput_2425, and ΔfabB ΔPput_4737 strains labeled with [1-14C]acetate. The two mutations of the ΔfabB ΔPput_2425 strain restored UFA synthesis. Panel B is a scan of the autoradiogram of the thin layer plate used to separate the radioactive methyl ester species. UFA, unsaturated fatty acid.
Although fabF2 is involved in the synthesis of UFA, the ∆Pput_2425 ∆fabB double mutant grew weakly and suppressors appeared on plates lacking oleic acid. Sequencing the fabF1, fabF2, and fabA genes of the suppressors plus their promoter regions gave only WT sequences. The fatty acid composition of a ∆fabB suppressor strain was similar to that of the WT strain although the UFA content was somewhat decreased (Table S5).

The anaerobic FabA-FabB pathway is the major pathway of bacterial UFA synthesis (26). Increased expression of FabF2 improved growth and replaced fabB function and it seemed possible that increased expression of fabA might also aid UFA synthesis in the ∆fabB suppressor strains. The transcription start site of the fabA was identified by rapid amplification of 5’ complementary DNA ends and was located 120 bp upstream of the fabA coding sequence (Fig. S10).

The upstream 200 bp of the fabA promoter was fused to the promoter-less lacZ of the expression vector. The fusion vector was transferred into WT, ∆Pput_2425, and ∆fabB suppressor strains, and β-galactosidase activities were determined (Fig. 8C). The β-galactosidase activity in the ΔfabB suppressor strains (Fig. 8C) and the ΔPput_2425 strain was much higher than that of the WT strain (Fig. 8C). This showed that the accumulation of suppressors might also be related to the fabA expression level. Note that deletion of Pput_4737 had only a very modest effect on fabA expression (Fig. S8B).

Deletion of P. aeruginosa PAO1 fabF1 has been reported to affect swimming mobility (27). Hence, the swimming motility phenotypes of the P. putida F1 ΔfabF1 and ΔfabF2 strains were tested. The ΔfabF1 strain had significantly decreased swimming motility, whereas the ΔfabF1 ΔfabF2 strain was basically immobile, indicating that fabF1 affected swimming, whereas the ΔfabF2 mutation aggravated the effects of fabF1 on swimming (Fig. S11A). Because the genes downstream of fabF2 are related to drug transport, we tested the resistance of the fabF2 mutant strain to three antibiotics and found that after the ΔfabF2 mutation, the resistance of P. putida F1 to ampicillin, erythromycin, and carbenicillin increased (Fig. S11B).

**Discussion**

P. putida is a soil and water bacterium that can utilize a very wide variety of organic compounds as carbon and energy sources, and thus, P. putida strains are often utilized in bioremediation (1, 5). This lifestyle may provide a rationale for the complexity of UFA synthesis and regulation in P. putida F1 (Fig. 9) and other pseudomonads compared with the paradigm E. coli pathway. Degradation of a number of the utilized compounds proceeds through acyl-CoA intermediates, which could enter the fatty acid synthesis pathway and alter the UFA:SFA ratio. Intermediates in β-oxidation have been shown to enter the fatty acid synthesis pathway of P. aeruginosa via a 3-ketoacyl-ACP synthase encoded by the PA3286 ORF (28). P. putida F1 encodes a protein, Pput_1345, with 74% identity to Pa3286 and...
Figure 9. Scheme of the Pseudomonas putida F1 UFA synthesis pathway. Upon inactivation of fabB, greatly increased expression (large arrow) of fabF2 resulting from inactivation of the Pput_2425 repressor replaced FabB function. Expression of fabA also was modestly increased (small arrow). FabB and FabF2 are unable to elongate palmitoyl-ACP (C16:1) to cis-vaccenoyl-ACP (C18:1), whereas FabF1 elongates palmitoyl-ACP (C16:1) to cis-vaccenoyl-ACP (C18:1) but is unable to perform the first elongation in UFA synthesis (cis-3-decenoyl-ACP to 3-keto, cis-5-dodecenoyl-ACP). ACP, acyl carrier protein; UFA, unsaturated fatty acid.

Like Pa3286 elongates octanoyl-CoA with malonyl-ACP in vitro (data not shown). Hence, a possible scenario is that the complexity of P. putida UFA synthesis and regulation may be a means to cope with the diversity of acyl-CoA intermediates generated in consumption of diverse organic compounds.

The fatty acid components of P. putida are all straight-chain fatty acids as found in E. coli. At present, there are two routes for UFA synthesis in bacteria, anaerobic and aerobic pathways (although the anaerobic pathway also functions aerobically). Thus far, the anaerobic FabA-FabB is the major pathway, whereas aerobic desaturation is in general a supplementary pathway (29). Although the aerobic desaturation pathway of P. aeruginosa PAO1 can partially compensate for loss of the anaerobic pathway (30), this is not the case in P. putida F1. The P. putida ΔfabB strain is a UFA auxotroph, and hence, the aerobic pathway is unable to support growth. Indeed, deletion of the desA gene had little or no effect on UFA synthesis, although DesA may account for traces of UFA synthesis in the ΔfabB strain.

The ΔfabF2 strain spontaneously accumulates suppressors in which fabF2 restores UFA synthesis. Suppressors accumulate because of inactivating mutations in a regulatory protein encoded upstream in the fabF2 gene cluster, which determines whether the otherwise cryptic fabF2 gene has gained enough expression to significantly participate in UFA synthesis. This regulatory protein, Pput_2425, belongs to the TetR family of transcriptional repressors, which are often involved in the regulation of fatty acid synthesis (22, 23). The P. putida mechanism is different from that of the ΔfabB suppressors of Shewanella. In Shewanella, fabF1 is located in the fatty acid synthesis gene cluster, and mutations that eliminate an upstream transcriptional terminator allow increased transcription of fabF1 that compensates for loss of fabB (15).

Analysis of growth phenotype of suppressors found that the growth of suppressors was significantly inhibited by exogenous octanoic acid (Fig. 57). This phenotype may result because the activity of FabB2 is lower than the activities of FabB and FabF1. As noted above, P. putida F1 encodes a protein that condenses octanoyl-CoA and malonyl-ACP in vitro (data not shown). A possible scenario that could explain the octanoic acid inhibition is as follows. Upon entry of octanoic acid into the cytosol, it becomes activated to octanoyl-CoA, which can then either enter the fatty acid synthesis pathway or be degraded by β-oxidation. The branch point for the synthesis of SFA and UFA takes place after the formation of 3-hydroxydecanoyl-ACP. The trans double bond is introduced by FabA or FabZ, but only FabA is capable of the isomerization of trans-2 to cis-3-decenoyl-ACP (28) (Fig. S1). Addition of octanoic acid may increase the levels of cis-3-decenoyl-ACP but because elongation by FabB2 is weak, excess cis-3-decenoyl-ACP would be isomerized back to trans-2-decenoyl-ACP where it would enter the SFA synthetic pathway. This may lead to an imbalance in the ratio of SFA and UFA causing growth inhibition.

DesT is a regulator of UFA synthesis in P. aeruginosa PAO1, where it controls expression of fabA (24). A P. putida homolog of DesT is encoded by Pput_4737. Deletion of Pput_4737 resulted in modestly increased expression of the fabA fabB operon as seen in P. aeruginosa PAO1. Upon loss of P. putida F1 FabB activity, there is a strong selection for inactivation of the Pput_2425 regulatory protein, which results in increased expression of fabF2 to bypass loss of fabB. From the properties of P. aeruginosa DesT, we expected that fabA expression would be altered by the DesT homologous gene Pput_4737 (Fig. S8B). However, sequencing the upstream 200-bp regions of DesT homologous gene Pput_4737 and the ΔfabB suppressor strains showed no differences from the WT sequences. Although the expression of fabA slightly increased upon deletion of Pput_4737 (Fig. S8B), the properties of the ΔPput_4737 ΔfabB and ΔfabB strains were the same, indicating that Pput_4737 was not involved in suppressor accumulation. Note that, although the ΔPput_2425 mutation restored UFA synthesis of the ΔfabB strain, suppressors still accumulated. This may be related to fabA expression, but the mechanism will require further study.

Phylogenetic tree analysis showed that the homologs of P. putida fabF2 not only exist in Pseudomonas, but highly homologous genes are found in other species. These genes are often located in gene clusters resembling that of fabF2 and all such clusters contain a regulatory factor of the TetR family (Fig. S12). Perhaps, these bacteria may also compensate for the defects in UFA synthesis by activating fabF2 expression.

**Experimental procedures**

**Bacterial strains, plasmids, and growth conditions**

The strains and plasmids are given in Table S1. E. coli and P. putida F1 strains were grown at 37 °C and 30 °C in LB medium containing (in g/l) tryptone, 10; yeast extract, 5; NaCl, 10; pH 7.0. When required, antibiotics and inducers were added as follows (in μg/ml): sodium ampicillin, 100; kanamycin sulfate, 30; gentamicin, 30; tetracycline hydrochloride, 90; L-arabinose, 200; IPTG, 240; and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 20. Olate was used at a final concentration of 5 mM. Bacterial growth was determined by growing on solid media.

**Expression and purification of His<sub>6</sub>-tagged FabB, FabF1, and FabF2 proteins**

Vector pET28b constructs carrying with fabF1, fabF2, and fabB were transformed into strain BL21(DE3). The
transformants were incubated in LB medium at 37 °C with
50 μg/ml kanamycin to an absorbance at 600 nm of 0.6 and
then were induced by 1 mM IPTG for another 4-h incubation.
The cells were harvested and lysed in the lysis buffer (50 mM
sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM
imidazole). The supernatant was loaded onto the Ni-NTA
column. The column was eluted with a wash buffer (50 mM
sodium phosphate (pH 8.0), 300 mM NaCl, and 40 mM
imidazole), and then the tagged proteins were eluted with the
same buffer containing 250 mM imidazole. The eluted pro-
teins were dialyzed against 50 mM sodium phosphate (pH 8.0)
and 300 mM NaCl, glycerol was added after dialysis to 15%,
and the proteins were stored at −80 °C.

**Assay of long-chain 3-ketoacyl-ACP synthase activities in vitro**

The PCR products containing *E. coli* acpP and acpS were cloned into pET-28b or pBAD33 to yield plasmids pET-ACP
and pBAD33-AcpS, respectively. These two plasmids were introduced into *E. coli* BL21(DE3) cells, and holo-ACP was
expressed at high levels and purified. The abilities of FabF2 and
FabB to function in the cycle of fatty acid synthesis were
assessed with reaction mixtures containing 0.1 M sodium
phosphate (pH 8.0); 0.1 μg each of EcFabD, EcFabG, EcFabA,
and EcFabI; 50 μM NADH; 50 μM NADPH; 1 mM β-mer-
captoethanol; 100 μM acyl-ACP; 100 μM malonyl-CoA; and
50 μM holo-ACP in a final volume of 40 μl. The reactions were
initiated by the addition of FabF2 or FabB to the mixture,
followed by incubation for 1 h. The reaction products were
resolved by conformationally sensitive gel electrophoresis on
13.5% polyacrylamide gels containing a concentration of urea
optimized for separation.

**Thin layer chromatography analysis of phospholipid fatty
acids**

The complemented K1060 derivatives and the *P. putida* F1
strains and their complemented derivatives were cultured in LB
medium with or without oleic acid and labeled with radioactive
[1-14C]acetate as follows. The strains were grown to an absorbance at 600 nm 0.5 with 5 mM oleic acid, and the cells
were washed with 0.5% Brij 58 three times to remove oleate, resuspended in media lacing oleate, and incubated for
another 3 h at 37 °C (K1060) or 30 °C (*P. putida* F1) in the
presence of [1-14C]acetate (final concentration of 1 μCi/ml)
followed by cell lysis with methanol–chloroform (2:1). The
phospholipids were further extracted with chloroform and
dried under nitrogen. The fatty acyl groups on phospholipids
were then converted to their methyl esters by tran-
sesterification with 25% sodium methoxide, extracted into
petroleum ether, taken to dryness under nitrogen, resuspended
in hexanes, and loaded onto the silver nitrate thin layer
chromatography plates (Analtech), which were developed in
toluene at −20 °C (inclusion of silver allows separation of
saturated and unsaturated esters with different double bond
positions). The plates containing the [1-14C]-labeled esters
were analyzed by phosphorimaging using a GE Typhoon FLA
7000 Scanner and analyzed by the ImageQuant TL program.

**GC-MS analysis of phospholipid fatty acids**

The strains were cultured in LB medium to an absorbance at
600 nm 0.5. Cultures were standardized by absorbance at 600
nm, and fatty acid methyl esters were generated as above and
then analyzed by GC-MS using a highly polar chiral CP-Si 88
column (Agilent Technologies). The CP-Si88 column allows
baseline separation of the methyl esters of acids based on their
double-bond positions.

**Extraction of total RNA, cDNA synthesis, and reverse
transcription PCR**

Total RNA was purified using the RNeasy Mini Kit (Qiagen).
RNAs were nonspecifically converted to single-stranded
cDNAs using the ProtoScript First Strand cDNA Synthesis
Kit (NEB). The control samples were made during cDNA
synthesis from the total RNA using the ProtoScript First
Strand cDNA Synthesis Kit without the addition of reverse
transcriptase. The resulting cDNA served as the template for
PCR amplification of the P.putida_2425 gene cluster, using
specific primers (Table S2) and an Eppendorf thermal cycler.

**RLM-RACE**

The 5’ends of fabA mRNA in *P. putida* F1 were mapped
using RLM-RACE using the First-Choice RLM-RACE kit
(ThermoFisher) according to the manufacturer’s instructions.
To identify the 5’ends of the fabA mRNA, the PCR products
were cloned into vector PCR 2.1 and sequenced.

**Swimming and antibiotic resistance assays**

The swimming assays were performed on a semi-solid
plate containing 0.3% agarose, and the plate was allowed to
air-dry on a clean bench for 5 to 10 min before use. The
bacteria were first cultured in liquid LB medium to the sta-
tionary phase and transferred to 5-ml fresh liquid LB me-
dium at a ratio of 1:100 to allow the culture to achieve the
log phase, and the absorbance at 600 nm value was deter-
mined. The test culture was diluted to an absorbance at
600 nm 0.5, and 1 μl of the bacteria solution for each sample
was placed on the swimming plate or plates containing the
antibiotics to be tested. Growth of the lawn was observed
after placing them at 25 °C for 12 h to 16 h. Three repeti-
tions were performed.

**Data availability**

All data are contained within the article.

**Supporting information**—This article contains supporting
information.

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Abbreviations—The abbreviations used are: ACP, acyl carrier protein; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

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